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Turing Revisited: Decoding the microRNA Messages in Brain Extracellular Vesicles for Early Detection of Neurodevelopmental Disorders

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Abstract

The prevention of neurodevelopmental disorders (NDD) of prenatal origin suffers from the lack of objective tools for early detection of susceptible individuals and the long time lag, usually in years, between the neurotoxic exposure and the diagnosis of mental dysfunction. Human data on the effects of alcohol, lead and mercury and experimental data from animals on developmental neurotoxins and their long term behavioral effects have achieved a critical mass, leading to the concept of the Developmental Origin of Health and Disease (DOHaD). However, there is currently no way to evaluate the degree of brain damage early after birth. We propose that Extracellular Vesicles (EVs) and particularly exosomes, released by brain cells into the fetal blood may offer us a non-invasive means of assessing brain damage by neurotoxins. We are inspired by the strategy applied by Alan Turing (a cryptanalyst working for the British government) who created a first computer to decrypt German intelligence communications during World War II. Given the growing evidence that miRNAs, which are among the molecules carried by EVs, are involved in cell-cell communication, we propose that decrypting messages from EVs can allow us to detect damage thus offering an opportunity to cure, reverse or prevent the development of NDD. This review summarizes recent findings on miRNAs associated with selected environmental toxicants known to be involved in the pathophysiology of NDD.

*The corresponding author is Larissa Takser.

Compliance with Ethics Guidelines

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of Interest

Virginie Gillet, Darel Hunting, and Larissa Takser declare that they have no conflict of interest.

Keywords

Neurodevelopmental disorders; microRNAs; Extracellular vesicles; environmental exposure; biomarkers

Introduction

The development of specific non-invasive biomarkers of neurotoxicity in humans is a high priority for scientists, industry and governments, but accurate and efficient tools are lacking. Well characterized environmental toxicants such as lead or mercury, as well as chemical exposures such as alcohol, affect the developing brain [1–3] and predispose to future mental disorders [4–7]. The underlying mechanisms of developmental neurotoxicity are not well characterized but recent evidence implicates miRNAs. Indeed, miRNAs are key regulators of gene expression and environmental toxicants affect their expression [3, 8, 9]. NDD may have several causes but those caused by environmental exposures are challenging to demonstrate in humans mostly due to the time lag between exposure and the first symptoms but also because of the lack of objective markers. Currently, virtually all diagnoses of NDD are based on clinical observations which are usually made several years after the moment of initial brain damage. Growing evidence supports the potential of miRNAs as biomarkers of brain health because of their tissue-specific properties and their accessibility in the blood through their presence in Extracellular Vesicles (EVs) [10]. MiRNAs are a class of single stranded, small noncoding RNA molecules of ~ 22 nucleotides (nt) in length, evolutionarily well conserved and derived from genome-encoded stem-loop precursors [11]. MiRNAs induce gene silencing at the post-transcriptional level by recognizing target messenger RNA (mRNA) through complementary binding of 3' or 5' untranslated regions (3' or 5' UTR), and represent an important mechanism for regulating the expression of the mammalian genome. This recognition of mRNAs leads to inhibition of the translation by sequestration or degradation of the target mRNAs. The biogenesis of miRNAs and their mechanisms of action have been explained in excellent detailed reviews [12].

MiRNAs influence and reflect almost every cellular and developmental process investigated so far such as embryonic development, cell functions, immune reactions and adaptation to stress, as well as disease etiology [8], [13]. MiRNAs are implicated in several pathologies such as: immune disorders [14–17], cardiovascular disease [18–20], endocrine dysfunction [21–23], biogenesis pathways in cancer [24, 25], nephropathy [26], as well as in drug addiction [27], gynecological / reproductive diseases [28] and CNS disorders [29–33]. Since the discovery of miRNAs in *Caenorhabditis elegans*, approximately 2600 mature miRNAs have been identified in humans and, based on computational analysis, it is believed that all mRNAs are potential targets of miRNAs [34–36]. Interestingly, miRNAs are released from virtually all cell types and most are encapsulated in EVs (Extracellular Vesicles) which are found in all biological fluids [37].

EVs are spherical-structures limited by a lipid bilayer, secreted by cells into extracellular spaces and are commonly classified into 3 categories, according to the International Society of Extracellular Vesicles [37, 38]. First, exosomes, nanovesicles of 40 – 100nm diameter,

formed within the endosomal network and released by cells after the fusion of multivesicular bodies with the plasma membrane [38]. The second group includes microvesicles, microparticles, and ectosomes, larger than 100nm that are shed from the plasma membrane [37]. And finally, the third vesicle group comprises apoptotic bodies released when plasma membrane shedding occurs during apoptosis. This latter group is eliminated during EV isolation procedures due to their size (> 800nm). Currently, there is a need for additional specific markers for each EV type since some features are shared by two group (lipid composition; certain membrane markers). The biogenesis and secretions of EVs have been recently reviewed in detail [39]. EVs are released by cells in a constitutive manner but also as a consequence of cellular reactions to external signals or pathological states such as hypoxia, oxidative stress, cytokines, cancer and infection [37, 40, 41]. EVs and particularly exosomes are now recognized as an important mode of cell-cell communication and could be one of the means by which cells adapt to stimuli. Indeed, exosomes contain cytosolic cellular components such as proteins, RNAs (miRNAs, mRNAs) and lipids and are able to transfer their contents to targeted cells but can also induce cascade signaling in recipient cells by binding to the plasma membrane [37, 42]. Moreover, it appears that miRNAs are not passively loaded into EVs since their miRNA repertoire differs from that of the parent cells and certain miRNAs are usually excluded from EVs, indicating an active selection of miRNA [43–45]. Currently three online databases, EVpedia, Exocarta and Vesiclepedia provide an inventory of all proteins, lipids and RNAs found associated with EVs [46–50]. Interestingly, EVs have been found in virtually all biological fluids including urine, blood, breast milk, saliva, amniotic fluid, cerebrospinal fluids, which supports their potential utilization as biomarkers of the status of their cell of origin [37]. EVs released by brain cells were reported to be involved in CNS development as well as in the pathogenesis of certain mental disorders such as Alzheimer disease, fronto-temporal dementia, schizophrenia [51–56]. Currently, there are no studies on miRNA expression in EVs linked with environmental exposures, particularly neurotoxicants, relevant for neurological functions. Here, we summarize the rationale for testing the potential of EVs and their miRNAs as markers to identify damaged brains in newborns, who are destined to suffer from NDDs. We highlight the fact that environmental exposures affect miRNAs with relevant functions in brain physiology, as demonstrated both *in-vitro* and *in-vivo* where changes in cellular or blood miRNA levels were reported after neurotoxicant exposure. Moreover, several animal models of NDD, and human studies showed differential expression of certain miRNAs in patients compared to neurotypical subjects. EVs are secreted by brain cells, are found in biological fluids and contain cellular miRNAs. Thus, we suggest it should be possible to use a few well studied developmental neurotoxins which give rise to specific NDD, to decode the meaning of the messages present in the miRNAs within EVs released by brain cells (Fig. 1). i.e, the intended target cells and biological effects. Thus, EVs with their miRNAs represent a potential tool which should be urgently explored in molecular epidemiology focused on NDD.

1. Known neurotoxic xenobiotics perturb miRNAs in the brain

It is clear that exposure to neurotoxic chemicals (metals, polychlorinated biphenyls, endocrine disruptors, volatile organic compounds) during early fetal development can cause

brain injury predisposing to NDD as well as subclinical brain dysfunction[1, 57–59]. Sanders et al. reviewed epidemiological studies on perinatal and childhood exposure to metals (cadmium, lead, manganese, or their combination) and their effects on neurodevelopment with a focus on cognitive function and behavior [2]. Here we summarize the results of observational studies and in-vitro/in-vivo experiments determining the effects of a few recognized environmental neurotoxins on miRNA expression, with a focus on NDD (Table 1).

• Arsenic

Arsenic is one of the most common environmental pollutants and a worldwide health concern. Arsenic, a known risk factor for NDD, induces oxidative stress which affects *in-vitro* EV-secretion by astrocytes [60]. Moreover, arsenic, even at low concentrations, impairs CNS functions and induces cognitive dysfunction, including learning and memory deficits and mood disorders, and children are particularly vulnerable [60]. Rager et al. examined human cord blood samples (n=40) for miRNA expression changes associated with in utero arsenic exposure and reported an increased expression of 12 miRNAs [61]. This suggests that the target miRNAs in the recipient cells should generate fewer protein molecules, which should in turn alter the function of the recipient cells.

• Lead

Lead is a well-studied developmental neurotoxin which induces adverse effects on neuronal differentiation, synaptic plasticity, learning and memory, neurogenesis and neuroregeneration, essential functions which are linked to the development of several NDD [62–63]. In addition, early life exposure to lead is related to increased risk of Schizophrenia [64]. Epidemiological studies provide sound evidence that even relatively low blood levels of Pb have adverse effects on the developing brain, especially during fetal life and childhood [63, 65, 66]. Recently, An et al. have reported that chronic exposure to lead alters the miRNA expression profile in the adult rat hippocampus and bioinformatics analysis as well as mRNA profiling analysis showed that the target genes of these affected miRNAs are involved in neural injury, neurodegeneration, axon and synapse function, neural development, and regeneration and apoptosis regulation [67].

• Mercury

Mercury is a well-recognized developmental neurotoxin, which induces learning disabilities, reduced cognitive function, and several neurological disorders, especially when the exposure occurs in utero even at very low doses [68–70]. Using differentiating human pluripotent cells repeatedly treated with non-cytotoxic doses of methylmercury chloride (MeHgCl), Nerini-Molteni et al. reported significant changes in the expression of 12 miRNAs associated with neural development [71]. In a mixed neuronal/glia culture, derived from NT2 cells, exposure to methyl mercury chloride during neuronal differentiation induced overexpression of 5 miRNAs (miR-302b, miR-367, miR-372, miR-196b and miR-141) [72]. These miRNAs were associated with developmental processes and cellular responses to stress, and pathway analysis revealed possible links to CNS development including axon guidance, learning and memory processes [72]. The data suggest that miRNA profiling could provide a functional evaluation of the toxicity pathways involved in developmental neurotoxicity [72]

• Other neurotoxic metals

Pogue et al. provided evidence that in primary cultures of human neural (HN) cells, exposure to iron- and aluminum-sulfate (ROS-generating neurotoxic metal sulfates) induced up-regulation of miR-9, miR-125b, miR-128 and miRNA-146a. MiRNA-146a down-regulates the expression of complement factor H, a repressor of inflammation, leading to increased neuroinflammation [73,74]. These results suggest that miRNA-146a may aggravate the inflammatory response in stressed HN cells [73].

Silver nanoparticles (NPs) are commonly found in medical devices, pharmaceuticals and cosmetics and may cause adverse effects on the CNS [75]. Oh et al. performed the first comprehensive profiling of genes as well as miRNAs indicating epigenetic regulation in silver NP-treated neural stem/progenitor cells (NPCs) derived from embryonic stem cells (hESC). This cell model showed neural cell differentiation capacity allowing them to determine the 'global' effects of silver NPs on the entire neural cell system. In the NPCs, 6 miRNAs were deregulated (miR-297, miR-132, miR-22, miR-27b, miR-196b, miR-1226) and neuronal function, such as synaptic long-term potentiation, and axonal guidance signaling, were strongly regulated by the miRNA targeted genes in response to silver NPs [18]

In a zebrafish model, copper induced deregulation of miRNAs (from olfactory system tissues) involved in neurogenesis (e.g., let-7, miR-7a, miR-128, and miR-138), which suggests a role for miRNAs in copper-mediated toxicity via interference with neurogenesis processes [76].

• Ethanol

Ethanol is highly toxic for the developing brain. Prenatal exposure to ethanol leads to a myriad of developmental disorders known as fetal alcohol spectrum disorder, often characterized by intellectual disability, central nervous system damage, and specific craniofacial dysmorphic features. Studies in both animals and cultured cells reported that exposure to ethanol during the in-utero period, or during brain cell differentiation affected the expression of brain miRNAs involved in functions which are often altered in NDD (Table 1). Briefly, Wang et al. reported that ethanol caused both reduced cognitive function in offspring mice and deregulation of several miRNA including the miR-10 family which was significantly up-regulated in the fetal mouse brain but could be restored to normal with folic acid treatment [77]. In rodent cultured neurons [78–80] and fetal neural stem cells [79], ethanol treatment altered the expression of miR-9 (targeted calcium- and voltage-activated potassium channel in the brain) and miR-153, an miRNA that protects neurons from cell death. A recent study also revealed that miR-29b (a miRNA that prevents cell death by regulating Bcl-2 expression) was decreased in cerebrum and cerebellum granule neurons isolated from mice exposed to ethanol, which may explain the ethanol-induced neuronal apoptosis in the developing cerebellum [81]. Ethanol increased the expression of pro-inflammatory cytokines in the brain of mice due to miR-155 overexpression [82]. In humans, comprehensive analysis of miRNA levels in the post-mortem frontal cortex of alcoholic patients showed deregulation of 35 miRNAs involved in cell cycle regulation,

differentiation and CNS development. Thus, miRNA deregulation may either be part of the mechanism of brain injury or may be a reflection of a damaged brain.

- **Valproic acid**

Valproic acid (VPA) is a widely prescribed drug to control epilepsy and pain. VPA is a recognized teratogen which increases the risk of malformations but also of neurodevelopmental disorders such as ASD [83–85] and ADHD [86]. For example, the risk of ASD is increased 5-fold in mothers who took VPA prior to conception [85]. Animal models suggest that VPA induces apoptosis, altered neurotransmitter profiles, and impaired synaptogenesis which are some of the mechanisms responsible for its cognitive and behavioral effects [85]. Aluru et al, reported that exposure to VPA altered the miRNA expression profiles in the developing embryo of zebrafish [87]. The targets of the miRNAs included several genes which are involved in the normal functioning of the CNS. In murine embryonic stem cells, VPA changes the miRNA expression profile during neural differentiation of neurons and astrocytes, in addition to the shifts in the lineage specification from neural to myogenic differentiation [88]. Concomitantly, the expression of myogenic regulatory factors as well as muscle-specific genes were elevated, while genes involved in neurogenesis were repressed [88]. Considering that prenatal exposure to VPA induces autistic-like features in rodents [83, 89] as well as deregulation of miRNAs involved in CNS development, it is possible that this deregulation contributes to the development of ASD-like features.

Investigating the role of miRNAs in NDD disorders may provide molecular diagnostic tools for psychiatric disorders and further studies focusing on brain miRNA deregulation following *in utero* exposure to environmental toxicants will help to identify mechanisms underlying the neurotoxic effects. Excitingly, miRNAs and brain-EVs show great potential as biomarkers and studies in neuro-oncology and neurodegenerative diseases support the potential use of EVs as biomarkers of NDD.

Apoptosis, calcium intracellular regulation, neurotransmitter secretion and neuro-inflammation, which have been reported to be affected by neurotoxins, are critical for the secretion of EVs by brain cells which led us to hypothesize that such neurotoxic xenobiotics will affected both miRNA content and EV secretion within the developing brain.

2. Release of extracellular vesicles (EV) by brain cells is mediated by neurotransmitters and the activation of inflammatory/oxidative stress pathways

EVs are now recognized as key actors in neuron-glia communication and their release by neurons and glial cells appears to be controlled by neurotransmitters (e.g. serotonin [90], glutamate [91]), intracellular calcium, but also, oxidative stress, microglia activation and neuro-inflammation [91–93]. The secretion of EVs by neurons, oligodendrocytes (ODC), astrocytes, microglia, and Schwann cells has been reported *in-vitro* and *in-vivo* [94–100]. Astrocytes, essential for trophic support of neurons but also very involved in cellular communications, release EVs [96, 101]. Under hyperthermia and oxidative stress, cultured

astrocytes released EVs carrying the heat shock protein Hsp70 and synapsin I conferring pro-survival effects on neurons [102]. ODC treated with a calcium ionophore increase the release of exosomes containing myelin components [95].

The secretion of exosomes by ODC also depends on the electrical activity of neurons and appears to be controlled by the neurotransmitter glutamate [103]. Indeed, exosomes produced by ODC regulate, in an autocrine fashion, the membrane expansion, axonal myelination and myelin formation in response to trophic factors of neuronal origin but do not influence the differentiation of their precursors [104]. Finally, exosomes derived from ODC augmented neuron integrity and survival, axonal maintenance and immune surveillance by transferring myelin proteins, lipids and miRNAs to neurons and microglia [103, 105].

Microglia, the resident macrophages of the brain, were reported to secrete exosomes exhibiting similar protein content as B cell-derived exosomes and this *in-vitro* secretion is up-regulated following microglia activation with interferon treatment [97]. Serotonin released by neurons induced exosome secretion by microglia (both in murine microglial BV-2 cells and mouse primary microglia), suggesting that a neurotransmitter can control exosome release [90]. Microglia activation is normally neuroprotective, allowing the pruning of inactive synapses through phagocytosis of ODC-derived exosomes. However, their activation can also cause neurodegeneration, and propagation of inflammation by a mechanism which involves the uptake of microglia-derived exosomes [92].

3. EVs released by brain cells contain miRNAs which are involved in brain development and function, and the pathogenesis of mental disorders

Since the discovery of the first miRNA, *lin-4*, in *C.elegans* [36] and the identification of miRNAs conserved across species [106], our understanding of the role of miRNAs in brain development and function has been steadily growing. In mammals, more than 600 miRNA species have been discovered in the brain with specific temporo-spatial expression patterns [107, 108]. In the developing human brain, Moreau et al. uncovered distinct temporal expression patterns of miRNAs in post-mortem brain tissues from the fetus, early postnatal and adult brain tissue and constructed a miRNA expression atlas of the developing human brain [108]. Patterns of expression of miRNAs were studied during brain development in neurons, oligodendrocytes, astrocytes and microglia [109]. For example, in rodents, miR-124 and miR-128 are primarily expressed in neurons, whereas miR-23, miR-26, and miR-29 are present in large amounts in astrocytes [110]. Ziats et al. discovered differentially expressed miRNAs across both temporal and spatial dimensions, and between the male and female prefrontal cortex. The targets of the specific miRNAs were highly enriched for gene sets related to ASD, schizophrenia, bipolar disorder but not for adult-onset psychiatric diseases [107]. It is clear that miRNAs contribute to the maintenance of normal neuronal function and homeostasis which are often affected in NDD [111].

miRNAs and EVs in brain physiology

• **miRNAs as key regulators of brain development**—In one of the first studies using mice to examine the role of miRNAs in neuron integrity, it was reported that the depletion of all mature miRNAs (by interfering within the miRNA biogenesis pathway using a knock out strategy to target a key protein for miRNAs biogenesis) resulted in a progressive neurodegenerative phenotype characterized by loss of motor control [112]. For example, results using *Drosophila* showed that disruption of the Argonaute protein (the ribonuclease needed for miRNA biogenesis) results in miRNAs depletion and caused developmental defects with malformation of the CNS in embryos [113]

Subsequent studies in developmental models in several species (i.e. zebrafish, mice, rat, *C.elegans*) and in-vitro (human neural stem cells) reported that the depletion of miRNAs reduces the number of neurons and glial cells, impairs axon guidance and neuronal differentiation, and induces cerebellum and midbrain malformations [112, 114–117]. MiRNAs regulate the transition of pluripotent stem cells to the neural lineage [50]. For example in mice, miR-9 and miR-124a, which were reported to be altered by neurotoxins such as aluminium, methyl-mercury chloride or ethanol, have been implicated in the decision of neural precursors to differentiate into neurons or glial cells [117]. Moreover, when miR-124a (expressed in neurons but not in astrocytes) is experimentally expressed in non-neuronal cells it promotes a neuronal-like mRNA profile [117] and additional studies showed that miRNAs can regulated the shift from neuronal to glial fate and promote the generation of astrocytes and oligodendrocytes [50, 118, 119]. Evidence for the implication of EVs in neuroembryology comes from the discovery of exosomes in rodent and human embryonic CSF (eCSF) that contain evolutionarily conserved molecules important for coordinating intracellular pathways [120]. Feliciano et al. showed that eCSF served as a medium for molecules that regulate embryonic neural stem cell amplification during corticogenesis through the distribution of exosomes [120]. Recently, Lopez- Verrilli et al. showed that EVs from Schwann cells were selectively captured by neurons (*in vitro*) which resulted in a substantial neurite outgrowth, a sharp increase in axonal regeneration and a change in the RNA profile of neurons. Analysis of the contents of EVs showed that miRNAs and mRNAs were selectively packaged into EVs and some mRNAs coded for neuron specific proteins [98]. Thus, these results, confirmed by other studies, revealed the roles of miRNAs in developmental processes of the CNS such as tissue morphogenesis, neural patterning, neuronal specification and differentiation, and axonal path finding [111, 121].

• **Role of EVs and miRNAs in synaptic plasticity**—Synaptic plasticity is a critical process in learning and memory and its disruption triggers NDD such as ASD or ADHD [122, 123]. *In vitro* studies in rodent cells reported that release of EVs by neurons at somato-dendritic (post-synaptic) sites is modulated by glutaminergic synaptic activity. The EVs contained subunits of neurotransmitter receptor suggesting roles for EVs in homeostatic synaptic-scaling. Moreover, neuronal EVs contained synaptic-plasticity-associated protein and miRNAs and preferentially interacted with targeted neurons at the pre-synaptic terminal. This suggests that EVs act as vehicles for both anterograde and retrograde information transfer, consistent with a role in synaptic plasticity and memory [51, 91, 124].

Considering the vital role of miRNAs in post-transcriptional regulation, their widespread expression in different brain regions and their regulation by neuronal activity, miRNAs are increasingly considered as central players in synaptic plasticity. Bak et al showed that several miRNAs are expressed specifically in the hippocampus and cortex, and Kye et al, reported that a small number of miRNAs including miR-26a are highly enriched in dendrites compared to the cell body in mouse brain [125, 126]. For example, miR-132, is required for memory acquisition, spatial memory formation, and it regulates the visual cortex plasticity in a developmental and experience-dependent manner [127, 128]. Karpova et al. in a review focused on the epigenetic control of the brain-derived neurotrophic factor (BDNF), a central player in neuronal plasticity, reported that miRNAs, such as miR-206, targeted BDNF transcripts resulting in memory impairment and were up-regulated in neurodegenerative disorders [129]. Several reviews summarize the regulatory mechanisms of miRNAs in synaptic plasticity and learning memory and the picture that emerges is that miRNAs are ideally suited to contribute to the regulation of long-term potentiation-related gene expression, that miRNAs are pleiotropic, synaptically located, tightly regulated, and function in response to synaptic activity [130]–[133]. The first direct links between miRNA dysfunction and synaptic pathologies are emerging, raising the interest in these molecules as potential biomarkers and therapeutic targets in NDD.

• **miRNA, EVs and neuroprotection**—In rodent models as well as in humans, changes in miRNA expression after ischemic stroke have been reported [134]. Ischemia in rodents induced miRNA changes (192 miRNAs up-regulated and 95 miRNAs down-regulated) in cortical tissue [135]. These results suggest that certain miRNAs (those with altered expression following neurotoxic xenobiotic exposure) may act in a neuroprotective manner. This is further supported by studies in which individual miRNAs (e.g. neuron-enriched miR-29, Mir-132, and miR-124; astrocyte-enriched miR-146a and miR181) were shown to reduce neuronal death and to improve neurological outcomes after ischemia [134]. In addition, an *in vitro* study showed that EVs derived from ODC maintained neuron and axon integrity and exhibited pro-survival effects on neurons [93].

miRNAs and EVs in CNS disorders: focus on NDD disorders

Recent evidence supports the essential roles of EVs and miRNAs in the pathogenesis of neurodegenerative disorders such as prion disease, Alzheimer disease, multiple sclerosis, CNS infections and brain tumorigenesis. Deregulation of miR-124, the most abundant brain miRNA, has been linked to neurodegeneration, neuroimmune disorders and CNS stress among others [136]. In the context of mental disorders, it appears that EVs and miRNAs could represent the underlying mechanisms of disorders such as bipolar affective disorder, schizophrenia, major depression and anxiety disorders [137–140]. Whether miRNA deregulation is a cause or a consequence of such disorders is not clear and the majority of studies which examined the expression of miRNAs in mental disorders are case-control studies. In addition, one must control for the influence of psychotropic drugs on miRNA expression since several studies showed that lithium, haloperidol or valproate induced changes of the miRNA profiles in brain [141, 142]. For NDDs such as ASD, ADHD and intellectual deficiency, little is known about the role of miRNAs in the pathogenesis of these disorders and surprisingly no publication reports the analysis of EVs for these disorders.

Autism spectrum disorder (ASD)—Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders caused by the interaction between genetic vulnerability and environmental factors [143]. Direct evidence for miRNA involvement in ASDs is currently lacking and the existing human studies were case-control studies which reported altered cellular or serum miRNA (i.e. not in EVs) levels in tissues from patients with ASD. The miRNA profile of olfactory cells of autistic patients, revealed differential expression of several miRNAs relevant for the pathophysiology of autism such as: miR-146a, miR-221, miR-654-5p, and miR-656 [143]. In post-mortem cerebellar cortex and temporal cortex, two studies showed deregulated miRNAs and among their predicted targets there were genes that are known genetic causes of autism, such Neurexin and SHANK3 [144, 145]. Three other studies reported that in lymphoblastoid cells, miRNAs were differentially expressed in ASD [146–148]. Finally, more recently, two studies reported differentially expressed miRNAs in the blood of children and adults with ASD compared to controls [149,150]. Unfortunately, no studies have compared the miRNAs levels in different tissues of the same patients; however, changes in the levels of certain miRNAs (miR-106b, miR-663 and miR-23a) have been reported in both post-mortem cortex, lymphoblastoid cells and serum of ASD patients. Some of these miRNAs (miR-23a, miR-132, miR-134 and miR-146b) have been shown to regulate dendritic spine structure and morphology, synaptic connectivity and maturation (two process altered in ASD), were related to anxiety behavior, and were affected by alcohol, metals and VPA exposure [151, 152].

Further evidence for the role of miRNAs in the pathogenesis of ASD comes from the VPA rat model of ASD where elevated miR-181c levels in the amygdala were observed. Experimental suppression of miR-181c attenuated neurite outgrowth and branching and reduced synaptic density in primary amygdalar neurons to levels which were similar to controls [153].

Attention deficit/hyperactivity disorders (ADHD)—ADHD is one of the most prevalent NDD, often associated with cognitive handicap and learning deficit [154]. Although the etiology and pathogenesis remain unknown, several theories suggest that it results both from genetic and environmental factors [154]. The blood of ADHD patients has been reported to contain altered levels of miRNAs involved in differentiation from pluripotency to neurogenesis and in the maintenance of pluripotency, somatic cell reprogramming, tissue regeneration, and neural stem cell plasticity [154, 155].

Numerous studies support a role for miRNAs in environmentally-related disease since neurotoxins affect miRNA expression. In parallel, is it clear that miRNAs/EVs play a crucial role in CNS development and particularly in processes such as synaptogenesis, synaptic plasticity, neural differentiation and fate which are often impaired in NDDs. On the other hand, in NDDs like ASD and ADHD where there is a crucial lack of human studies, a reasonable hypothesis is that exposure to environmental toxins during prenatal life leads to NDD through deregulation of the release of EVs or alterations in their miRNA content.

4. miRNA profiles of brain EVs as potential biomarkers for neurodevelopmental disorders?

The miRNA content of EVs is recognized to be a fingerprint of the releasing cell type and its physiological state [37]. Studies focused on miRNA changes in the context of brain physiopathology support the possibility of their use as biomarkers of CNS disorders. Most of the studies have been dedicated to neurodegenerative diseases and neuro-oncology [156–158].

Indeed, while the Blood Brain Barrier (BBB) as well the blood-CSF barrier are generally considered to have a highly selective permeability to blood compounds or drugs [159, 160], recent studies suggest that exosomes have the ability to cross the BBB in both directions, although the route of transfer remains unclear [161–164]. Moreover, exosomes were shown to be able to enter the brain parenchyma at the choroid plexus and were internalized both in neurons and glial cells (in-vitro and in-vivo rodent studies) [99]. The most promising finding comes from Kapogiannis et al. who successfully isolated neuronal-EVs in plasma using a specific marker of neuronal origin, NCAM/ L1-CAM [165]. Moreover, accumulating experimental and clinical evidence indicates that a number of brain diseases (i.e glioblastoma) are associated with BBB dysfunctions resulting in elevated barrier permeability that may enable leakage of EVs from the brain. Indeed, Shao et al. successfully detected and quantified glioma markers in glioma-derived exosomes in the blood circulation, with a detection rate of > 90%, suggesting the possibility of using EVs derived from brain tumors as an effective diagnosis platform [166]. In ASD, often associated with immune dysfunction, the detection of autoantibodies against neural cells of the cerebellum in the plasma suggests the existence of BBB disruption [167]. Moreover, environmental neurotoxicant induced neuroinflammation may participate both in BBB disruption and EV release [92–168].

EVs have been found in cord blood and taking into account the BBB immaturity during the first year of life [169], it is possible that EVs arising from brain cells can be detected in cord blood.

Conclusion

There is an urgent need to develop a molecular tool to evaluate brain damage at birth, whether it results from exposure to neurodevelopmental toxins or others causes. On the basis of the information compiled in this review, we propose that the miRNA profiles of EVs released by brain cells and present in cord blood may reveal the degree of brain damage (Fig. 1). EVs are a messenger for intercellular communications among the community of cells within the brain. The miRNA profiles of EVs play important roles in virtually all aspects of brain development and functioning; however, we are not yet able to decode the information contained in the miRNA profiles. Yes, we know which target mRNAs in the recipient cells will be degraded or sequestered and thus not be translated into proteins, but what will be the resulting effects on the brain? This is a far more complex situation than analyzing the effects of a given neurotransmitter and this is why we use the term “decoding” and make reference to the remarkable achievement of Alan Turing. We may be able to use

well studied neurotoxins, such as lead, mercury and ethanol, which predispose to NDD, as “Rosetta stones”, to allow us to decode EV messages and thus to identify damaged brains at birth, thus opening the possibility of preventive / therapeutic interventions to reduce the incidence of NDD. Finally, there are an increasing number of environmental chemicals to which fetuses are exposed and for which there are no neurodevelopmental toxicity data. A molecular tool to evaluate brain damage at birth would be a major advance for the field of molecular epidemiology by permitting timely, non-invasive detection of neurodevelopmental toxins in humans.

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Figure 1. Conceptual model

(1) Exposure to environmental neurotoxins during prenatal period provokes oxidative stress / neuron inflammation / excitotoxicity through neurotransmitter and Ca^{2+} perturbation. (2) pathological states perturb both the secretion of EVs by brain cells and their miRNAs profiles; (3) MiRNAs and EVs are key players in brain development, homeostasis and function (i.e synaptic plasticity) and the miRNA profiles of EVs secreted by specific brain cells may affect brain development and reflect brain damage. (4) In NDD, synaptic plasticity, synaptogenesis, cell differentiation, and the balance between proliferation versus apoptosis are impaired which lead to NDD (4). (5) Since EVs and miRNAs are tissue-specific and reflect brain responses and phenotype, decoding of the information in EVs in cord blood could help us identify early brain damage to prevent the development NDD(6).

Table 1

Environmental exposures and microRNA expression relevant for CNS functions

In-vitro and animals studies				
Environmental exposures	miRNAs	Functions / miRNAs targets	Species or cells lines or tissues	References
Metals				
Mercury	(miR-302b, miR-367, miR-372, miR-196b, miR-141) ↑	axon guidance, learning and memory processes	neuronal/glia culture derived from NT2 cell line (carcinoma pluripotent stem cells)	[72]
Methyl-mercury chloride	12 miRNA deregulated	neuronal development	differentiating human pluripotent cells	[71]
Copper	let-7, miR-7a, miR-128, and miR-138	neurological process, neurotoxicity	zebrafish	[170]
Lead	↑(miR-204, miR-211, miR-448, miR-449a, miR-34b, and miR-34c) ↓miR-494	Neural injury and neurodegeneration, axon and synapse function, neural development and regeneration.	rat (hippocampus)	[67]
Silver nanoparticles	(miR-297, miR-132, miR-22, miR-27b, miR-196*, miR-1226) deregulated	oxidative stress, neuronal function, inflammatory response	human embryonic stem cell(HES)-derived neural stem/ progenitor cells	[75]
Aluminium	↑ (miR-9, miR-125b, miR-128)	neuronal differentiation, inflammation	human neural (HN) cells	[74]
Aluminium and iron sulfate	↑(miR-146a)	Down-regulation of inflammation repressor and neurotoxicity	human neural (HN) cells	[73]
Chemicals				
Ethanol	↑ (miR-10a, miR-10b)	Early embryo development.	mouse fetal brain	[77]
	↓ miR-21, miR-335, miR-9, and miR-153	anti-apoptotic factor, pro-apoptotic, anti-mitogenic factor,	ex-vivo model of fetal cerebral cortical neuroepithelium	[78]
	↓miR-153	preventing premature Neural Stem Cells differentiation	mouse fetal cortical Neural Stem Cells	[79]
	↓miR-9, miR-29a, miR-29b and miR-133, ↑miR-34b	inflammation and neuronal apoptosis pro-apoptotic miRNA	mice cerebellum granule neurons	[81]
	↑miR-9	neuronal plasticity	adult mammalian brain	[81]
	↑miR-155	neuroinflammation	mice cerebellum	[82]
	↑miR-10a-5p, ↓miR-26a and miR-495	target brain-derived neurotrophic factor, neurodevelopment as well as in the fine-tuning of synaptic plasticity	rat hippocampus	[172]
	↓miR-9	neural-enriched miRNA, neural progenitor cell migration and neurogenesis	Zebrafish embryos, murine NSCs	[174]
	↑ (let 7 family members, miR-34c, miR-146a, miR-194, miR-203, and miR-369)	neuronal plasticity effectors targeted	Post mortem human prefrontal cortex (alcohol patients vs normal)	[175]

In-vitro and animals studies				
Environmental exposures	miRNAs	Functions / miRNAs targets	Species or cells lines or tissues	References
	↑ (miR-497, miR-302b, miR-34b)	neuronal cell death, mitochondria-dependent neuronal apoptosis	human neuroblastoma cell line	[176]
Valproic acid	↑(mir-206, mir-133a and mir-10a) ↓ (mir-124a, mir-128 and mir-137)	muscle-abundant miRNA neural specific miRNA, neural differentiation	murine embryonic stem cells (mESCs)	[88]
	(miR-16a, miR-18c, miR-122, miR-132, miR-457b, and miR-724)	neurodevelopment	zebrafish	[87]
Cocaine	↓ (miR-133b, miR-144, miR-191) ↑ (miR-134, miR-135, miR-181c, miR-22, miR-26b, miR-382, miR-409-3p and miR-50, miR-30a)	dopamine receptors, brain disorders, drug abused	Rat hippocampus	[177]
Endocrine Disruptors				
Bisphenol A	↑miR-146	inflammation	HTR-8 and 3A Human placental cells	[178]
Trimethyltin	↑miR-9* and miR-384-5p	Neurotoxicity	Rat serum and brain	[180]
Air pollution				
Cigarette smoking	↓ (miR-16, miR-21, miR-93, miR-146a)	growth and developmental processes	Human placenta + TCL-1 cells	[181]
Ambient PM	↓ (miR-1, miR-126, miR-135a, miR-146a, miR-155, miR-21, miR-222, miR-9)	neuronal differentiation	leukocyte	[183]
Carbon tetrachloride	↑(miR-122, miR-133a, and miR-124)	liver-, muscle- and brain-specific miRNAs	rat plasma	[185]
Others				
RDX	13 miRNAs deregulated	neurotoxicity, carcinogenesis and toxicant-metabolizing enzymes	Mice brain and liver	[186]
	↑ (miR-71, miR-27ab, miR-27b, miR-98, miR-135a, miR-674-5p), ↓ (miR-320, miR-129*, miR-342-3p)	neurotoxicity	Rat brain	[187]
Ionizing radiation	↑miR-132, miR-212, miR-134	synaptic actin remodeling, neurogenesis, neuroinflammation	brain of neonatal mice	[188]
Human in-vivo studies				
Environmental exposures				
Arsenic	↑12 miRNAs	innate and adaptive immune signaling	Human cord blood	[67]
Bisphenol A	↑miR-146a	immune inflammatory and neural disease pathways	2 nd trimester human placentas	[179]
Cigarette smoking	↓ (miR-129, miR-634), ↑ (miR-340, miR-365)	embryo development, particularly cell death and apoptosis	Human spermatozoa	[182]

In-vitro and animals studies				
Environmental exposures	miRNAs	Functions / miRNAs targets	Species or cells lines or tissues	References
Benzene	↑ (miR-34a, miR-205, miR-10b, let-7d, miR-185 and miR-423-5p-2) ↓ (miR-133a, miR-543, hsa-miR-130a, miR-27b, miR-223, miR-142-5p and miR-320b)	Regulation of transcription, axon guidance, nervous system development, and regulation of actin cytoskeleton organization.	human peripheral blood mononuclear cells	[184]