Methylmercury Exposure and Developmental Neurotoxicity: New Insights from Neural Stem Cells

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Abstract

Methylmercury (MeHg) is a widespread neurotoxic environmental contaminant. The developing nervous system is particularly vulnerable to MeHg toxicity that may have adverse consequences on neurodevelopment depending on the timing and level of exposure. In our daily lives, we can be exposed to MeHg via consumption of contaminated seafood, which is the major MeHg source for humans. While the deleterious effects of exposure to high levels of MeHg are well known and characterized, the consequences and the mechanisms behind the developmental neurotoxic effects induced by low-level exposure are still unclear.

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Neural stem cells (NSCs) have been proposed as a powerful model to identify the adverse effects on the complex process of nervous system development. This chapter gives an overview of in vitro studies performed in NSCs of different origin with special emphasis on the effects induced by MeHg levels considered to be environmentally relevant to human exposure. Altogether, the data show that MeHg exerts harmful effects at very low concentrations by dysregulating critical neurodevelopmental steps such as proliferation, differentiation, migration, and neurite outgrowth. The use of NSC-based models has made possible the identification of relevant signaling pathways and molecular alterations that point to novel mechanisms of MeHg toxicity.

Keywords

Developmental neurotoxicity · Neural stem cells · Cellular mechanisms · Apoptosis · Neurogenesis · Oxidative stress · Epigenetics

Abbreviations

aNSCs	Adult neural stem cells
CpG	Cytosine dinucleotide
GD	Gestational day
GSH	Glutathione
hiPSC	Human-induced pluripotent stem cells
hNES	Human neuroepithelial-like cells
MeHg	Methylmercury
NSCs	Neural stem cells
PND	Postnatal day
ROS	Reactive oxygen species

1 Introduction

Methylmercury (MeHg), a widespread environmental and food contaminant, originates from inorganic mercury by the methylating activity of microorganisms in oceans, rivers, lakes, wetlands, sediments, and soils. In the aquatic food chain, MeHg is accumulated at the highest levels in sea mammals, predatory fish, and shellfish, which are considered the primary sources of MeHg exposure for humans (Hintelmann, 2010) (Fig. 1).

MeHg exerts its toxic effects via multiple mechanisms including mitochondrial function impairment, increased generation of reactive oxygen species (ROS), interaction with sulfhydryl groups of thiol-containing compounds targeting cysteine- and methionine-containing peptides and proteins, and perturbation of intracellular Ca^{2+} levels (Ke et al., 2019). Alterations of these intracellular processes pose a major risk to the nervous system, especially during development.

It has long been known that the nervous system is the main target organ for MeHg, particularly during prenatal life when it exerts adverse effects on

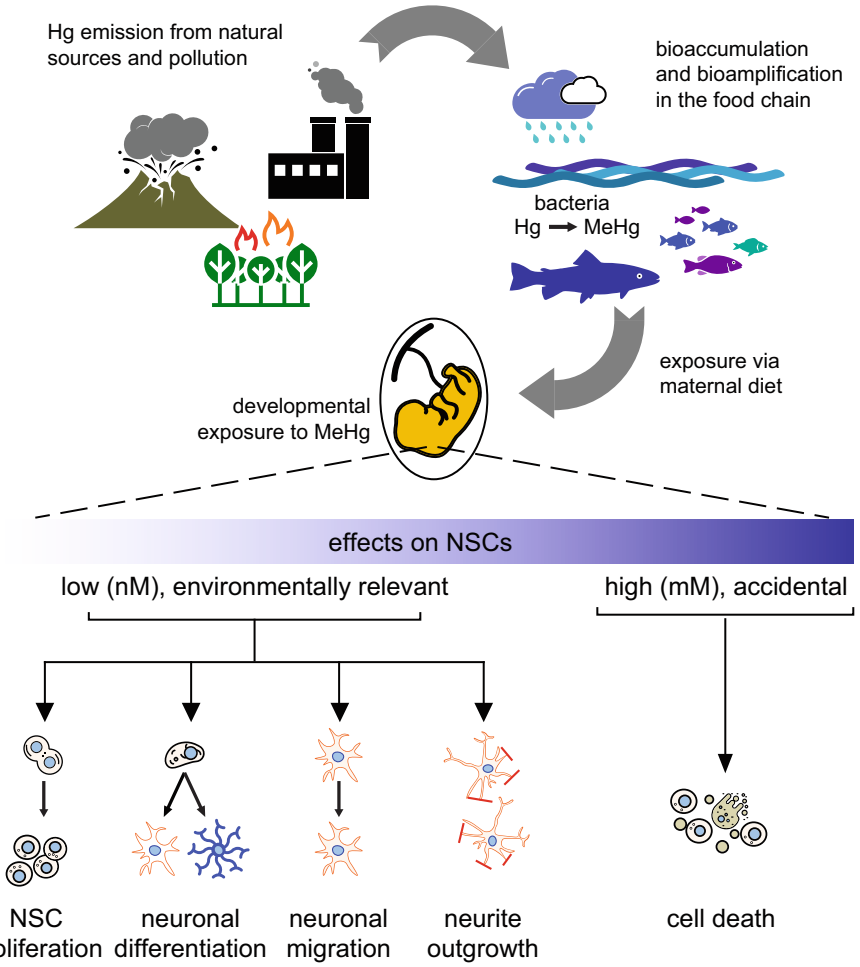

Fig. 1 An overview of MeHg-induced neurotoxicity. Natural and anthropogenic sources are the origin of inorganic mercury that is released into the environment. Once inorganic mercury falls in the water, it can be converted to MeHg by sulfate-reducing bacteria present in aquatic environments. MeHg then enters the aquatic food chain and the highest levels are found in predatory fish. Humans are mainly exposed to MeHg via consumption of contaminated seafood. MeHg is almost completely absorbed in the gastrointestinal tract. Pregnant and lactating women pass on MeHg to their fetus/child posing serious risks to the developing brain. Long-lasting effects on NSC impact fundamental neurodevelopmental processes

neurodevelopment in both humans and animals by passing through the blood-brain barrier as well as the placenta (Johansson et al., 2007). Notably, exposure during pregnancy results in MeHg levels in fetal blood that are about 1.7 times higher than those in the mother (Stern & Smith, 2003), which explains why fetuses can be affected in the absence of maternal toxicity.

The first well-documented cases of prenatal poisoning were reported after the MeHg outbreak occurred in Japan more than 60 years ago. Mercury-contaminated wastewater from a chemical factory had been discharged for some decades into the Minamata Bay. The accumulated mercury entered the food stream as MeHg-contaminated fish were consumed by the local population. The most severe MeHg effects on the human nervous system were observed in the offspring of women who had consumed contaminated fish during pregnancy. When not lethal, MeHg prenatal exposure led to a variety of clinical signs including ataxia, spasticity, blindness, impairment of motor skills, and different degrees of mental retardation depending on the level of exposure (Harada, 1995).

Over the years, man-made MeHg contamination has decreased substantially, thanks to the efforts to minimize the release of mercury in the environment. However, there is a major concern about the adverse long-lasting consequences on the nervous system induced by developmental exposure to low levels of MeHg via maternal diet (Castoldi et al., 2008). Epidemiological studies done in seafood-eating populations and experimental animal studies performed in rodents have shown that developmental exposure to MeHg can induce behavioral alterations and decrease cognitive abilities (Grandjean et al., 2010). Therefore, in many countries women who are pregnant or planning to become pregnant, nursing mothers, and young children are advised to avoid some types of fish that contain high levels of MeHg (American Academy of Pediatrics, 2019).

The development of the nervous system is a well-orchestrated process characterized by specific cellular and molecular events regulating proliferation, migration, differentiation, myelination, and synaptic pruning. Disruption of this complex series of sequential steps can result in structural and/or functional deficits. Different regions of the nervous system develop at different times, creating specific *windows of susceptibility* that determine the impact of exposure to neurotoxic stimuli, such as chemical contaminants. Also, the lack of an efficient blood-brain barrier makes the developing nervous system more susceptible to neurotoxicants even at levels that do not harm the adult brain. The damaging effects induced by neurodevelopmental insults may be unnoticeable for long time (*silent neurotoxicity*) until physiological events, such as aging, or harmful insults of different nature reveal or exacerbate the damage, which can be at structural, cellular, molecular, and/or biochemical level (Tamm & Ceccatelli, 2017). More recently, it has been shown that cellular alterations induced in NSCs by developmental stressful stimuli persist in the progeny never directly exposed (long-lasting effects), pointing to the occurrence of epigenetic modifications (Bose et al., 2010). As described in the next subchapter, a similar mechanism is brought into play by MeHg in NSCs (Bose et al., 2012).

NSCs have been proven to be a valid model to identify the effects and mechanisms behind neurodevelopment alterations induced by MeHg. This chapter gives an overview on studies performed in NSCs originating from mice, rats, and humans (see Tables 1 and 2), with special emphasis on the effects of MeHg concentrations considered to be environmentally relevant to human exposure.

Table 1 Studies reporting effects of exposure to MeHg in NSCs isolated from rats or mice

Species and model	Exposure doses (nM)	Duration of exposure	Alterations	Analyzed markers	Reference
Rat, NPCs (E13)	2.5–50 500–50,000	2 days	Decreased NSC proliferation Induced apoptosis	Cyclin E Cleaved caspase 3; cell viability (MTT assay)	(Xu et al., 2010)
Rat, NPCs (E14)	10 30, 100 >100	2 days	Decreased proliferation Induces cell death	Cyclin E BrdU, Cyclin D1, and CDK2 TUNEL staining	(Fujimura & Usuki, 2015)
Rat, NPCs (E15)	2.5–5	2 days	Reduced proliferation; senescence; reduced mitochondrial gene expression Inheritable effects associated with epigenetic alterations	*p16*, *p21; bmi*, *Hmga1*, Hp1ɣ; *Nd3*, *Cytb* *Dnmt3b*, decreased global DNA methylation	(Bose et al., 2012)
Rat, NPCs (E15) C17.2	2.5, 5 25, 50 250, 500	2 days 1 day 1 day	Decreased neuronal differentiation Induced cell death (blocked by pan-caspase and calpain inhibitors)	Tuj1 Trypan blue, nuclear condensation (Hoechst 33342, propidium iodide); Bax activation; cytochrome *c* release; caspase 3 activation calpain activation	(Tamm et al., 2006)
Rat, NPCs (E15)	2.5–10	12 h	Decreased neuronal differentiation (reversed by metalloprotease inhibitor)	Notch signaling Tuj1	(Tamm et al., 2008)
Rat, hippocampal NSCs (E16)	2.5–5.0	2 days	Decreased neuronal differentiation, increased astrocyte differentiation (prevented by *Lycium barbarum* polysaccharides)	MAP-2, GFAP	(Tian et al., 2016)
Mouse, NPCs (E12–14.5)	0.25 0.5–5	3 days	Decreased proliferation; increased differentiation Decreased differentiation	Ki67, Nestin, Sox2 Tuj1	(Yuan et al., 2018)
Mouse, ESCs (D3); EB	2.5, 25, 250 250 200	3–13 days	Decreased neurite outgrowth Increased % cells in undifferentiated state Induced cell death	% EB with >75% outgrowth *Nestin*, *SSEA1* Alamar blue cytotoxicity assay	(Theunissen et al., 2010)

(continued)

Table 1 (continued)

Species and model	Exposure doses (nM)	Duration of exposure	Alterations	Analyzed markers	Reference
Mouse, ESCs (D3); EB	25	8 days	Downregulation of transcription and development-related genes; upregulation of neurodevelopment- and cell motion-related gene sets Decreased neural outgrowth	Whole-genome transcriptomics analysis % EB with >75% outgrowth	(Theunissen et al., 2011)
Mouse, ESCs (D3)	15.13–1000 1000	8–14 days 16 h	Decreased neuronal differentiation Induced cell death	Nestin, Tuj1, MAP-2, GABAA-R, GFAP Cell viability (MTT assay)	(Kang et al., 2014)
Mouse, ESCs (D3); EB	0.1–100 >100	14 days	Altered gene expression-related neuronal differentiation Decreased cell viability	Nestin, Pax6, Tuj1, NCAM1, Nefm, MAP-2 *(Mtap2)* Resazurin reduction	(Stummann et al., 2007)
Mouse, adult NSCs	100, 500	16 h	Induced apoptosis (males more affected than females)	Condensed nuclei (Hoechst 33342)	(Ceccatelli et al., 2013)
Mouse, NPCs (E14.5); EB	100, 1000	48 h	Induced apoptosis (prevented by antioxidant treatment)	TUNNEL staining	(Watanabe et al., 2009)
Mouse, NPCs (E14.5); EB	100, 500, 1000	3–24 h	Induced apoptosis (prevented by caspase inhibitors)	Cleaved caspase 3, Bcl-2, Bax, DNA fragmentation (ladder)	(Watanabe et al., 2013)
Rat, NPCs (E14.5); EB	3000	6 h 18 h	Reduced proliferation Induced apoptosis	Cyclin E, BrdU Cleaved caspase 3	(Burke et al., 2006)

Abbreviations: CDK2, cyclin dependent kinase 2; MAP-2 (Mtap2), microtubule-associated protein 2; Nd3, mitochondrially encoded NADH dehydrogenase 3; Cytb, mitochondrially encoded cytochrome b; Bmi1, polycomb ring finger oncogene; Hmga1, high mobility group AT-hook 1; Hp1γ, heterochromatin protein 1; EB, embryoid body; Nefm, neurofilament medium polypeptide

Table 2 Studies reporting effects of exposure to MeHg in NSCs of human origin

Models	Exposure doses (nM)	Duration of exposure	Alterations	Analyzed markers	Reference
Human NPCs (NHNP, neurospheres)	500–1000	2 days	Decreased neuronal differentiation Decreased neuronal migration	Tuj1 Migration distance	(Moors et al., 2009)
Human NPCs (NHNP, neurospheres)	750	2 days	Decreased neuronal migration	Migration distance ERK 1/2 phosphorylation	(Moors et al., 2007)
hNSC (HB1.F3 cell line)	1000	2 days	Decreased RA-induced neuronal differentiation; increased astrocyte differentiation Induced autophagy Induced apoptosis	Nestin, GFAP LC3-I, II Akt1, mTOR, p53	(Chang et al., 2013)
hNSC (umbilical cord, HUCB-NSC)	50 (undifferentiated) 1000 (differentiated)	2 days	Decreased proliferation Induced apoptosis Decreased S100b expression in astrocytes		(Buzanska et al., 2009)
Human iPSC-derived neuroepithelial stem (NES)	10	2 days	Increased astrocyte differentiation (reversed by DAPT – Notch signaling blocker)	GFAP, MAP2, DCX	(Raciti et al., 2019)
Human fetal neural progenitor cells (hNPC)	10 25–100	4 days	Decreased neuronal differentiation Disruption of neurite extension and cell migration (males more affected than females) Increased cell death	Tuj1 BDNF, CDKL5 Neurite length, migration distance Condensed nuclei (Hoechst 33342)	(Edoff et al., 2017)
ihNPCs (ReNcell CX cell line)	10, 50	1 day	Decreased proliferation Mitochondrial biogenesis ROS production	EdU uptake; *p16*, *p21*, *p53* miR-25, 30d, 1285; mtDNA TFAM, PGC-1a, P53R2 ROS measurement	(Wang et al., 2016b)

(continued)

Table 2 (continued)

Models	Exposure doses (nM)	Duration of exposure	Alterations	Analyzed markers	Reference
ihNPCs (ReNcell CX cell line)	10, 50 50	1 day	Mitochondrial damage, ROS production Mitochondrial DNA mutations Induced apoptosis	ND1, Cytb, ATP, mitochondrial membrane potential (JC-1 aggregation) Point mutations in ND1, Cytb, ATP6 genes MTT assay, Annexin V, PI	(Wang et al., 2016a)
ESCs (H9-derived neural crest cells)	5–50	2 days	Decreased neuronal migration	Migration distance	(Zimmer et al., 2012)
Human, LUHMES (CRL-2927)	1	6 days	Decreased neurite extension DNA hypomethylation	Neurite length % 5-mC; DNMT3B	(Go et al., 2021)
hESC	25	12 days	Decreased neuronal differentiation	MAP2, NEUROD1, NCAM1	(Stummann et al., 2009)

Abbreviations: *ND1*, mitochondrially encoded NADH dehydrogenase 1; *ATP6*, mitochondrially encoded ATP synthase 6; MAP 2, microtubule-associated protein 2; Cyt *b*, mitochondrially encoded cytochrome b; NEUROD1, neurogenic differentiation 1 transcription factors; NCAM1, neural cell adhesion molecule 1; DNMT3B DNA, methyl transferase 3b; BDNF, brain-derived neurotrophic factor; CDKL5, cyclin-dependent kinase-like 5 (CDKL5); DCX, doublecortin; GFAP, glial fibrillary acidic protein; Tuj1, neuron-specific class II beta-tubulin; p16, p21 cyclin-dependent kinase inhibitors; p53, tumor suppressor phosphoprotein; TFAM; PGC-1a; p53R2, p53-inducible ribonucleotide reductase; ERK 1/2, extracellular signaling-regulated kinase 1/2

2 In Vitro Models for Developmental Neurotoxicity Studies

2.1 Neural Stem Cells

Among the cells that have been mostly used for in vitro neurodevelopmental toxicity studies are NSCs and neural progenitor cells (NPCs). The two definitions, often used interchangeably, refer to undifferentiated cells of the nervous system with different specific properties. NSCs are multipotent cells, whereas the potential of NPC is more restricted (Homem et al., 2015). Multipotency of NSCs is described as the ability to differentiate into neurons, astrocytes, and oligodendrocytes; and self-renewal is the ability to maintain multipotency through an indefinite number of cell divisions. Both are essential for the elaboration of a specific spatial organization and neuronal network during development and maturation. For experimental purposes, primary NSCs can be derived from various regions of the embryonic/fetal nervous system, such as the olfactory bulb, subventricular zone, hippocampus, cerebellum, cerebral cortex, and spinal cord. In the adult human brain, the persistence of NSCs and their ability to produce new neurons have not been fully clarified. In the adult rodent brain, NSCs are active and continuously produce new neurons, astrocytes, and oligodendrocytes throughout life in two specific brain regions: the subventricular zone of the lateral ventricle (basal ganglia) and the subgranular layer of the dentate gyrus (hippocampus). The integration of newly generated neurons into preexisting neuronal networks is essential for the function and plasticity of neural circuits of the adult brain. Thus, NSCs are highly relevant for neurotoxicity studies not only in the developing brain but also in the adult nervous system. Several NSC models can be used for investigating known and suspected neurotoxicants, as briefly discussed below.

2.2 Cell Lines and Primary Cultures of Mouse, Rat, and Human-Derived Neural Stem Cells

The C17.2 cell line is a murine neonatal cerebellum-derived immortalized NSC line, which has been widely used for understanding cell fate and differentiation of neural progenitors. These cells maintain the capacity to follow developmental cues. For example, C17.2 cells can generate fully functional neurons when transplanted into mid-embryonic mouse brain, but not at later developmental stages, when gliogenesis is predominant (Snyder et al., 1992).

Primary cultures of cortical NSCs are isolated from the telencephalon of rat embryo at embryonic day 15 (E15), and about 50% of these cells have been found to retain multipotential properties (Johe et al., 1996). These cells can be cultured as a monolayer on coated surfaces, or in suspension on non-coated surfaces, and the stemness is maintained by the addition of fibroblast growth factor (FGF). Upon removing FGF from the culture medium, NSCs can differentiate into the major cell types found in the telencephalon, including pyramidal neurons and interneurons, astrocytes, and oligodendrocytes.

Primary cultures of adult neural stem cells (aNSCs) are obtained from the anterior portion of the lateral walls of the lateral ventricles of adult rats (Johansson et al., 1999). These cells are cultured in suspension in the presence of epidermal growth factor (EGF) and propagate as neurospheres within a week. Thereafter, neurospheres can be subsequently trypsinized and either passaged for de novo neurosphere propagation or plated onto coated surfaces for monolayer culture. Similarly to other models, aNSCs can differentiate into neurons, astrocytes, and oligodendrocytes in the absence of EGF.

Human NPCs are collected from fetal forebrain at gestational weeks 8–12 (Åkesson et al., 2007), cultured in presence of FGF and EGF and maintained in suspension as proliferating neurospheres (Brannen & Sugaya, 2000; Piper et al., 2000). When NPCs are cultured on coated surfaces without growth factors, they give rise to the major lineages found in the adult brain.

Human NSCs (hNSCs) generated from stem cells isolated from umbilical cord blood (HUCB-NSC cell line) can differentiate into neurons, which express functional voltage- and ligand-gated ion channels, and can establish functional networks similar to immature neurons. In addition, HUCB-NSC cells differentiate to generate astrocytes and oligodendroglia (Buzanska et al., 2009).

Human iPSC-derived neuroepithelial-like stem cells (AF22 cell line, hNES cells) have long-term self-renewal capacity and display a rosette-like growth pattern after 8–12 days in culture with EGF and FGF. hNES cells give rise to neurons and glia when they are induced to differentiate by growth factor withdrawal. In addition, these cells retain neuro- and gliogenic potential even after long-term proliferation (Koch et al., 2009).

A recent advance in hiPSC technology is the generation of brain organoids, which recapitulate early stages of development and allows the direct investigation of processes associated with altered development. For the toxicology field, brain organoids may offer an unprecedented possibility for large-scale screening and molecular studies on compounds with developmental neurotoxic potential (Bose et al., 2021).

3 MeHg Exposure Affects NSCs by Dysregulation of Developmental Processes

3.1 Levels of MeHg Exposure Reported in Humans During Development

The level of MeHg exposure during development in human populations is usually inferred from the levels measured in the umbilical cord at birth. Peak levels detected in populations heavily exposed from industrial pollution have been reported to be in the range of 1.5–3.5 μM. Such massive exposure leading to so high concentrations is mostly due to man-made outbreaks that hopefully will not occur in the future. As previously described, the main source of exposure to MeHg in the general population is the intake of contaminated seafood, fish, and marine mammals (Fig. 1), and

there is a direct correlation between maternal fish intake and the concentration of MeHg in cord blood even at low maternal fish consumption (Ursinyova et al., 2019). Since MeHg readily crosses the placenta, there is a consistent correlation between the concentration of MeHg in fetal and maternal blood, with cord blood levels being 1.7–1.9 times higher than maternal levels (Stern & Smith, 2003). The concentration of MeHg in the cord blood ranges between 10 and 50 nM in most populations studied and reaches 250 nM in populations with diet based primarily on marine food. Therefore, the potential developmental neurotoxic effects of MeHg are of genuine concern.

3.2 Relevant Endpoints for Assessing Neurotoxic Effects in NSCs

The effects of MeHg exposure on NSCs have been studied in monolayer and suspension culture systems (e.g., neurospheres, embryoid bodies); the latter require considerably higher MeHg concentrations (in low μM range) to elicit similar effects. From the papers reviewed and summarized in Tables 1 and 2, it appears that primary NSCs are more sensitive to the toxic effects of MeHg than NSC cell lines and 3D culture systems.

3.2.1 Apoptosis

Exposure for >24 h to 25 nM or higher concentrations induces apoptosis in proliferating NSCs of mouse or rat origin by activating caspase- and calpain-dependent pathways (Fujimura & Usuki, 2015; Tamm et al., 2006; Xu et al., 2010). Similarly, exposure to 50 nM MeHg for 48 h induces apoptosis in HUCB-NSC cells (Buzanska et al., 2009). In cultures using cells of human origin, such as human fetal or embryonic NPCs, cell death is induced by concentrations of 25 nM and higher (Edoff et al., 2017; Wang et al., 2016a, b). Exposure to 50 nM for 24 h has been described to induce mitochondrial damage, altered mitochondrial biogenesis, and increased ROS production in immortalized human cortical NPCs (ReNcell® CX) (Wang et al., 2016a, b). In contrast, apoptosis is induced in C17.2 cells by exposure to 250 nM for 24 h (Tamm et al., 2006). In embryoid bodies, apoptosis is induced by exposure to >100 nM for 14 days (Stummann et al., 2007), 200 nM for 11 days (Theunissen et al., 2010), or 1000 nM for 16 h (Kang et al., 2014). Interestingly, exposure to 1000 nM MeHg for 48 h causes caspase-dependent apoptosis and autophagy via inhibition of Akt1/mTOR signaling (Chang et al., 2013). Of note, aNSCs derived from male mice are more sensitive than cells derived from female mice, as observed following exposure to either 100 or 500 nM MeHg for 16 h (Ceccatelli et al., 2013). Apoptosis induced by exposure to MeHg can be prevented by caspase (Tamm et al., 2006; Watanabe et al., 2013) and calpain (Tamm et al., 2006) inhibitors. In addition, inhibition of glutathione (GSH) synthesis promoted cell death, while antioxidant treatment (NAC or alpha-tocopherol) prevented apoptosis induced by 48-h exposure to 100 nM MeHg in mouse cortical neural progenitors (Watanabe et al., 2009).

3.2.2 Proliferation

Exposure regimens which do not decrease cell viability typically reduce the proliferation rate and alter differentiation in rodent primary NSCs. Cell proliferation has been shown to be reduced at doses ranging from 2.5 nM to 10 nM following 48-h exposure in NSCs derived from rat embryonic telencephalon (E14.5) (Bose et al., 2012; Fujimura & Usuki, 2015). In a recent study using rat embryonic NSCs derived from an earlier stage (E12), Yuan and colleagues describe a significant reduction in the proliferation rate at 0.25 nM, but not at higher doses (0.5–5 nM) (Yuan et al., 2018). Exposure to MeHg induces cellular senescence in rat NSCs, as shown by the expression of *Bmi1*, *Hmga1*, and *Hp1γ* (Bose et al., 2012). The cell cycle arrest can be explained by the observed upregulation of CDK inhibitors, such as p16 and p21, accelerated cyclin E degradation, GSK-3b upregulation, and altered cytoskeleton dynamics by direct interference with microtubule polymerization (Bose et al., 2012; Burke et al., 2006; Fujimura & Usuki, 2015; Tian et al., 2016; Xu et al., 2010). Similar effects have been described in immortalized cortical human NPCs (ReNcell® CX) exposed to 10 nM MeHg for 24 h, where the decrease in proliferation is associated with the upregulation of p16, p21, and p53 (Wang et al., 2016b).

3.2.3 Differentiation

Spontaneous differentiation of NSCs in culture is triggered by withdrawal of growth factors (FGF or EGF). In rat NSC, neuronal differentiation has been shown to be decreased at doses ranging from 0.5 to 10 nM following 48-h exposure (Tamm et al., 2006, 2008; Tian et al., 2016; Yuan et al., 2018). Oxidative stress appears to play a role also in the effects on NSC differentiation at subcytotoxic concentrations (2.5 or 5 nM for 48 h): Tian et al. have shown that antioxidant treatment with polysaccharides from *Lycium barbarum* reversed the decreased neuronal differentiation and increased astrocyte differentiation in hippocampal NSCs derived from rat embryos (Tian et al., 2016). Decreased neuronal differentiation has been described also in NSC of human origin following exposure to 10 or 25 nM MeHg for between 2 and 12 days (Edoff et al., 2017; Raciti et al., 2019; Stummann et al., 2009). Interestingly, Yuan et al. have found that exposure to very low concentrations (0.25 nM for 3 days) increased neuronal differentiation but decreased the proportion of precursor cells in NSCs isolated from mouse embryonal cortex (Yuan et al., 2018). Human fetal NPCs exposed to 10 nM MeHg for 4 days display decreased neuronal differentiation associated with BDNF downregulation (Edoff et al., 2017). ERK 1/2 dephosphorylation and Notch have been identified as relevant signaling pathways mediating the effects of MeHg on NSC differentiation. In rat cortical NSCs, neuronal differentiation is rescued by GM-6001, a potent metalloprotease inhibitor which reduces extracellular cleavage of Notch1 receptor (Tamm et al., 2008).

Astrocytic differentiation is also affected by MeHg as shown by the increase observed in human iPSC-derived neuroepithelial stem (NES) cells exposed to 10 nM for 2 days. The increase in astrocytes can be reversed by DAPT, a gamma-secretase inhibitor which blocks extracellular Notch cleavage (Raciti et al., 2019). Interestingly, the alterations induced by MeHg resemble the alterations observed in NES cell derived from a patient bearing a mutation in NRXN1 gene linked to autism spectrum disorder (Raciti et al., 2019).

3.2.4 Migration and Neurite Outgrowth

The occurrence of decreased neuronal migration and neurite outgrowth have been described at concentrations between 1 and 50 nM of MeHg in cells of human origin (Edoff et al., 2017; Go et al., 2021; Zimmer et al., 2012). Remarkably, the MeHg-induced reduction in cell migration and neurite extension is more pronounced in cells of male origin (Edoff et al., 2017). Decreased neuronal migration has been described in neurospheres grown from human NPCs exposed to 500–1000 nM MeHg for 48 h (Moors et al., 2007, 2009), and the effect was dependent on the reduction in ERK 1/2 phosphorylation. These findings are corroborated by the abnormal positioning of cerebrocortical neurons following in vivo administration of MeHg (0.1 or 1 mg/kg/day i.p. GD11-21) (Guo et al., 2013). In this experimental model, MeHg reportedly interfered specifically with neuronal migration, but not with the proliferation or differentiation of NSCs (Guo et al., 2013).

3.3 Inheritable Effects

An interesting observation made in primary cultures of NSCs is the persistence of the MeHg-induced alterations in the progeny of the exposed cells (Bose et al., 2012). Briefly, rat NSCs were exposed to 2.5 or 5 nM MeHg for 48 h (parent cells). Note that the exposure is not repeated after passaging the cells (daughter cells), which results in Hg levels similar to the control cells. Nevertheless, daughter cells exhibited the same alterations in proliferation and differentiation, cellular senescence, gene expression, and global DNA methylation as observed in parent cells. The latter points to the occurrence of epigenetic changes.

In summary, the reports available to date point to similar effects in NSC cultures of either rodent or human origin. Concentrations above 25 nM induce apoptosis by activating the caspase 3- and calpain-dependent pathways. Oxidative stress contributes to triggering apoptosis, as shown by the protection exerted by antioxidants. Proliferation is decreased without significant effects on cell viability at concentrations between 0.25 and 10 nM by cell cycle arrest. Neuronal differentiation, migration, and neurite outgrowth are altered by exposure to concentrations between 0.5 and 10 nM, and Notch signaling appears to mediate the effects on neuronal differentiation in NSC of either rodent or human origin. Oxidative stress plays a role also in the effects on neuronal differentiation. The effects of low concentration MeHg exposure persist in daughter cells never directly exposed and are associated with epigenetic changes.

4 Critical Mechanisms Behind NSC Dysregulation Induced by MeHg

4.1 Mitochondria Impairment and Oxidative Stress

Among the various mechanisms that have been shown to be involved in MeHg-induced neurodevelopmental toxicity, oxidative stress appears to be most critical in NSCs, as indicated by a number of studies performed in different types of NSC

models (Tian et al., 2016; Wang et al., 2016a, b). Compelling evidence links the overproduction of ROS to MeHg-dependent impaired mitochondrial function, as shown in in vivo and in vitro studies (Mori et al., 2007). Mitochondria are equipped with antioxidants, including GSH, thioredoxin (TRX), and the catalase system to quench ROS maintaining a steady-state concentration of oxidants at not toxic levels. The negative effects of MeHg on the antioxidant defenses further alter the delicate REDOX balance necessary for proper mitochondrial functions.

Based on these data, it appears that the extent of mitochondrial damage in NSCs depends on the level of MeHg exposure. While subtoxic concentrations induce transcriptional repression of mitochondrial respiratory chain enzymes of complexes I and III that do not affect cell survival (Bose et al., 2012), higher concentrations induce release of cytochrome *c* with subsequent activation of the mitochondrial caspase-dependent apoptotic cell death pathway (Tamm et al., 2006).

The importance of oxidative stress in MeHg-induced damage in NSC is strongly supported by the protective effects exerted by antioxidants, which can prevent both apoptosis (Watanabe et al., 2009) and the alterations in neuronal differentiation (Tian et al., 2016) in NSCs isolated from embryonal rodent brains exposed to low-dose MeHg.

There are several pathways potentially connecting oxidative stress with genetics as well as with epigenetics. Both free radicals and nonradical oxidants are generated during oxidative stress. While free radicals are reactive and lead to macromolecular damage, nonradical oxidants (e.g., H_2O_2, peroxynitrite, lipid hydroperoxide, and disulfides) disrupt REDOX signaling and physiological regulation pathways. For example, a free radical, hydroxyl (•OH), is the most reactive among ROS and has a relatively short half-life. The •OH reacts with guanosine directly, which is oxidized to produce 8-oxo-7,8-dihydro-2 deoxyguanosine (8-oxo-dG). While 8-oxo-dG can be repaired by a multistep process of base excision repair (BER) mechanisms, it can also mispair with adenine instead of cytosine and thereby generate G→T transversions. This hypothesis is supported by the mutations in mitochondrial DNA described in human NPCs (ReNcell® CX) exposed to 10 or 50 nM MeHg for 48 h (Wang et al., 2016a). The following subchapter will discuss the relevance of epigenetic modifications in NSC exposed to MeHg.

4.2 Epigenetic Changes

Epigenetics is defined as the regulation of gene expression through chromatin remodeling without changing the DNA sequence. The epigenetic mechanisms for regulating gene transcription include histone modifications, which control DNA availability for transcription factors to bind (by condensation or relaxation of chromatin wrapping in nucleosomes); DNA methylation, which modulates the efficiency of DNA transcription machinery (DNA methylation in the promoter region typically represses gene expression); and noncoding RNA, such as microRNA (miRNA) strands, which silence gene expression by binding to mRNA. Epigenetic changes are inherited across cell generations and have been demonstrated

to regulate a wide range of physiological and pathological processes, from cell cycle to the activity of non-diving cells – such as neurons. In addition, epigenetic marks are subject to influence from exogenous factors, such as methyl donors in the diet, environmental pollutants, and toxicants, such as MeHg (Culbreth & Aschner, 2019).

4.2.1 DNA Methylation

DNA methylation is regulated by DNA-methyl transferases (Dnmt), which add a methyl group to cytosine in position 5 and generate 5-methylcytosine (5-mC), and Ten-eleven translocation methylcytosine dioxygenases (Tet), which oxidize 5-mC to 5-hydroxymethylation (5-hmC), and the demethylated is completed by replication. In addition, 5-hmC can be further oxidized by Tet to produce 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-CaC) before demethylation by replication. Several mechanisms linking oxidative stress to DNA demethylation have been proposed. First, the affinity for sulfhydryl functional groups allows MeHg to bind to GSH and thereby disrupt antioxidant defense systems, and depletion of GSH per se is sufficient to lead to DNA hypomethylation. Second, methionine adenosyltransferase (MAT), which catalyzes the synthesis of *S*-adenosylmethionine (SAM; the main donor of methyl groups for DNA methylation by Dnmt), and methionine synthase (MS), which is required for methionine synthesis, are particularly sensitive to oxidative stress. Thus, the availability of methyl-group donors is decreased by oxidative stress, and DNA methyltransferases lack substrate for maintenance or de novo DNA methylation. A third potential mechanism is initiated by DNA oxidation by ROS. Thus, the presence 8-oxo-dG, the most commonly occurring oxidized nucleotide, activates DNA base repair enzyme OGG1, which binds to 8-oxo-dG, preventing DNMTs from methylating the DNA. In addition, OGG1 binding to 8-oxo-dG leads to CpG demethylation either directly by recruiting Tet1, which targets the adjacent 5-mC, or indirectly via deamination followed by DNA repair mechanisms during replication (see Fig. 2). Wang and colleagues show that ROS production increased significantly in hNPCs exposed to low MeHg dose (10 or 50 nM) (Wang et al., 2016a). Using primary embryonic rat NSCs, the effects of MeHg have been shown to be long-lasting (Bose et al., 2012). The decrease in proliferation (senescence) was associated with p16 and p21 upregulation (Bose et al., 2012). In addition, the global DNA hypomethylation is associated with Dnmt3b downregulation. This is in line with an earlier study showing that inhibition of DNMT in human umbilical cord blood-derived stem cells upregulates the expression of p16 and p21, thereby decreasing cell proliferation rate and inducing cellular senescence (So et al., 2011). Using a different model and exposure protocol, Go et al. report global DNA hypermethylation and *DNMT1*, *3A*, and *3B* upregulation in differentiating LUHMES (CRL-2927) cells exposed to 1 nM MeHg for 6 days, with similar results following in vivo exposure (3 mg/kg/day between GD12 and GD14) (Go et al., 2021). However, these experimental protocols lead to accumulation of Hg to levels relevant for massive, accidental exposure during development, which can explain the apparent contradiction in reported outcome. Further evidence linking oxidative stress and DNA demethylation comes from experimental models of aging (Guillaumet-Adkins et al., 2017) and brain tumors, where Barciszewska and

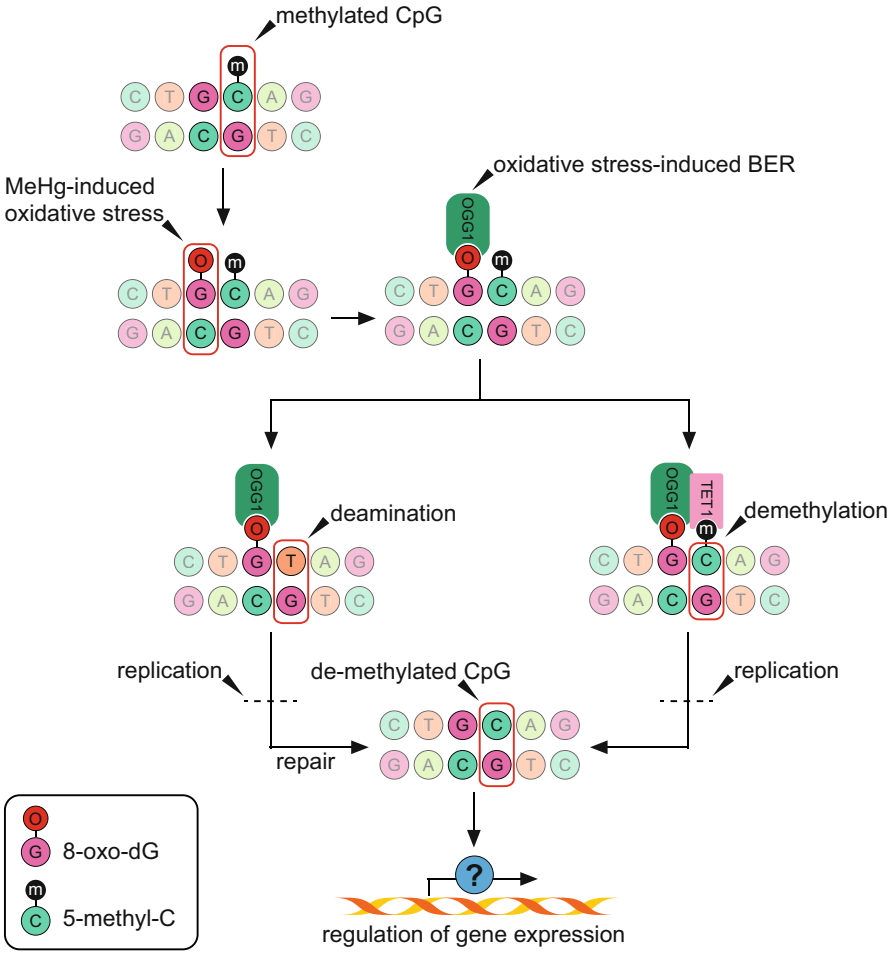

Fig. 2 MeHg causes both genetic mutation and epigenetic alterations in NSCs. MeHg-induced ROS (mostly hydroxyl group; •OH) oxidized guanosine that produced several oxidized products, including 8-oxoguanine glycosylase (OGG1), a DNA base repair enzyme involved in base excision repair (BER) arrives at the site of oxidation and repairs the base by a series of processes. For example, methylated cytosine (5-mC) is demethylated by recruiting TET1, which results in alteration in gene expression and functions. However, 5-mC adjacent to 8-oxoguanosine interferes with the repair process with the OGG1, which results in DNA lesions, including point mutations

colleagues describe an inverse correlation between the 8-oxo-dG and 5-mC content of DNA (Barciszewska et al., 2019).

In addition to global DNA methylation, the methylation status can be assessed at individual gene level. A DNA fragment which shifted methylation status (either methylated de novo or demethylated) after exposure is defined as differentially methylated region (DMR). The occurrence of DMRs potentially changes the rate

at which the gene is expressed. In a recent study, the methylation status of specific genes involved in brain development and neuronal signaling was assessed in 7-year-old children in relation to prenatal exposure to MeHg from maternal diet (Cediel Ulloa et al., 2021). The methylation of CpG sites in the promoter regions of NR3C1 (glucocorticoid receptor), GRIN2B (NMDA-receptor subunit), and BDNF (neurotrophic factor modulating neuronal development and function) was increasing with the developmental exposure level of exposure inferred from Hg levels in maternal hair. For NR3C1, prenatal MeHg exposure increased methylation in CpG3 and CpG5 sites. CpG3 is located in a transcription factor binding site for Hen-1 and together with CpG4 is part of the binding site for the transcription factor NGFI-A. Thus, MeHg-induced methylation of CpG3 site inhibited NGF1-A binding, leading to lower NR3C1 expression. MeHg-induced methylation of CpG4 did not have effects on NR3C1 expression regulation by NGF1-A. However, CpG4 is part of a conserved predicted binding site for nuclear respiratory factor 1 (Nrf1), and its methylation decreased GRIN2B expression. Alteration of GRIN2B function has been associated with neurodevelopmental disorders, such as attention deficit hyperactivity disorder, autism spectrum disorder, and schizophrenia. Similarly, CpG5 methylation induced by MeHg decreased expression of BDNF, which encodes for a neurotrophin with fundamental role in neural development, nerve cell survival, and synaptic plasticity (Castrén et al., 2007). BDNF is particularly relevant in relation to MeHg exposure because polymorphisms in the BDNF gene increase the susceptibility to neurotoxic effects, and the alterations in BDNF expression have been associated with depression as found following MeHg exposure in vivo (Onishchenko et al., 2007, 2008).

4.2.2 Modification of Histones

Histone modifications contributing to epigenetic regulation of gene transcription consist of covalent posttranslational modifications (PTMs), such as acetylation, methylation, phosphorylation, ribosylation, ubiquitination, sumoylation, or glycosylation. Histone acetylation and methylation are REDOX-sensitive and are inherited by daughter cells. Histone acetylation is regulated by ~30 histone acetyltransferases (HATs) and histone deacetylases (HDACs); epigenetic regulation of gene transcription depends on the balance between the epigenetic marks applied by HATs and HDACs. Histone methylation can either upregulate or downregulate gene expression depending on the amino acid methylated (lysine or arginine), position on the histone tail, and the number of methyl groups added. This dynamic process is regulated by more than 40 histone methyltransferases (HMTs) and demethylases. It has been shown earlier that exposure of pregnant mice to 0.5 mg/kg/day MeHg in drinking water from GD7 until PND7 results in depression-like behavior (Onishchenko et al., 2008). In addition, hippocampal neurogenesis is impaired, and the total number of granule cells in the hippocampal dentate gyrus is lower in adult mice exposed to MeHg during development (Bose et al., 2012). This model gives a concentration of Hg in the brain of about 0.9 ppm (Onishchenko et al., 2007), which is similar to the concentration found in NSCs exposed to 2.5 and 5 nM (0.4 and 0.7 ppm, respectively) (Bose et al., 2012) and with the concentration

reported in autopsy material from infants exposed to MeHg from maternal diet (up to 0.3 ppm (Lapham et al., 1995)). The decrease in BDNF expression is associated with repressive epigenetic marks, including DNA hypermethylation, increased histone H3-K27 tri-methylation, and decreased H3 acetylation at the BDNF promoter IV region. Antidepressant treatment with fluoxetine restored BDNF expression by reversing H3 acetylation at the BDNF promoter IV region (Onishchenko et al., 2008), pointing to epigenetic modification playing a role in the depression-like behavior induced by MeHg. The mechanisms behind the phenotype induced by developmental exposure to MeHg are presumably more complex than the epigenetic regulation of BDNF expression. Nevertheless, the changes in epigenetic marks in the promoter region of BDNF can account to a significant extent for the behavioral alterations, as well as for the positive effects of antidepressant treatment.

4.2.3 Noncoding RNAs

Noncoding RNAs that regulate epigenetic processes include micro-RNAs (miRNAs), long noncoding RNA (lncRNA), and circular RNA (circRNA). miRNAs are considered to play an essential role in regulating the epigenome and are affected by oxidative stress. Recent evidence demonstrated that nanomolar concentration of MeHg not only induced ROS but also altered cell viability and decreased cell proliferation that was associated with upregulation of p53R2 expression (Wang et al., 2016b). miRNAs are one of the major epigenetic alterations that have been shown to regulate specific gene expression. Wang et al. showed that MeHg-induced downregulation of miR-1285, miR-30d, and miR-25 is associated with upregulation of p53R2 expression. The overexpression of miR-25 significantly reduces the protein expression of p53 in MeHg-treated hNPCs (Wang et al., 2016b). This study indicates that MeHg-induced developmental neurotoxicity can activate diverse mechanisms simultaneously.

In summary, these data suggest that the effects of MeHg are mediated to a large extent by epigenetic changes. Exposure to nanomolar doses of MeHg alters neurodevelopmental processes, and the alterations persist beyond the time of initial exposure.

5 Conclusion

From the revised literature, it emerges that NSCs of either rodent or human origin are suitable models for mechanistic studies regarding the developmental neurotoxicity of MeHg. In agreement with earlier data, NSCs appear to be more susceptible to MeHg toxicity than differentiated neuronal and glial cells, and they recapitulate the sex-related differences in susceptibility to MeHg reported by epidemiological and in vivo experimental studies. While micromolar concentrations of MeHg induce apoptotic cell death, nanomolar levels, relevant to human exposure, affect the neurogenic potential resulting in altered proliferations, differentiation, migration, and neurite outgrowth. Notably, the effects on proliferation (cellular senescence) and differentiation are persistent, associated to epigenetic alterations. Indeed, epigenetic

modifications have been observed following exposure to nanomolar concentrations, and there is accumulating evidence to support a cross talk between oxidative stress and epigenetic mechanisms even at very low exposure concentrations. Alterations of neurodevelopment may therefore have long-term detrimental consequences that can lead to neurodevelopmental and neuropsychiatric disorders. The utilization of human NSCs (embryonic or iPSC-derived) in developmental neurotoxicity studies enables the identification of human-specific pathways that, by shedding light on the mechanisms behind early damages, could contribute to the identification of preventive and therapeutic strategies against environmental threats.

6 Cross-References

- ▶ Methylmercury and Cellular Signal Transduction Systems
- ▶ Neurotoxicity in Depression

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