Inherited Effects of Low-Dose Exposure to Methylmercury in Neural Stem Cells

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Methylmercury (MeHg) is an environmental contaminant with recognized neurotoxic effects, particularly to the developing nervous system. In the present study, we show that nanomolar concentrations of MeHg can induce long-lasting effects in neural stem cells (NSCs). We investigated short-term direct and long-term inherited effects of exposure to MeHg (2.5 or 5.0nM) using primary cultures of rat embryonic cortical NSCs. We found that MeHg had no adverse effect on cell viability but reduced NSC proliferation and altered the expression of cell cycle regulators (p16 and p21) and senescence-associated markers. In addition, we demonstrated a decrease in global DNA methylation in the exposed cells, indicating that epigenetic changes may be involved in the mechanisms underlying the MeHg-induced effects. These changes were observed in cells directly exposed to MeHg (parent cells) and in their daughter cells cultured under MeHg-free conditions. In agreement with our in vitro data, a trend was found for decreased cell proliferation in the subgranular zone in the hippocampi of adult mice exposed to low doses of MeHg during the perinatal period. Interestingly, this impaired proliferation had a measurable impact on the total number of neurons in the hippocampal dentate gyrus. Importantly, this effect could be reversed by chronic antidepressant treatment. Our study provides novel evidence for programming effects induced by MeHg in NSCs and supports the idea that developmental exposure to low levels of MeHg may result in long-term consequences predisposing to neurodevelopmental disorders and/or neurodegeneration.

Key Words: neural stem cells; developmental neurotoxicity; epigenetics; stereology; proliferation; senescence.

Methylmercury (MeHg) is an environmental contaminant with recognized neurotoxicity, particularly to the developing nervous system (Grandjean, 2007). Human exposure to MeHg occurs mainly via consumption of fish and sea food in adults and via maternal blood and milk early in life. Chronic prenatal exposure to low levels of MeHg can cause alterations in motor function, language development, attention, and memory capabilities in children and adolescents (Debes et al., 2006). Moreover, a recent epidemiological study on a population in Minamata revealed an increased prevalence of psychiatric symptoms, including impaired intelligence and mood and behavioral dysfunctions, in adults of different ages who did not present overt toxicity signs at birth after intrauterine exposure (Yorifuji et al., 2011). These observations call for studies shedding light on the plausible mechanisms underlying the long-lasting consequences of the damage induced by MeHg exposure early in life.

Neural stem cells (NSCs) play an essential role in the developing nervous system, and their significance in the mature brain has also been widely discussed (Sahay and Hen, 2007, Zhao et al., 2008). In vitro culture of NSCs provides an excellent possibility for studying adverse effects on critical neurodevelopmental processes, such as proliferation and differentiation. Moreover, the proliferative ability of NSCs provides a tool for studying mitotically inherited effects in vitro. Importantly, NSCs show higher sensitivity to toxicants compared with differentiated neural or glial cells (Ceccatelli et al., 2010). Therefore, NSCs represent a relevant in vitro model for mechanistic studies and toxicity assessment in the field of developmental neurotoxicology.

Cytotoxicity has been observed upon MeHg exposures to concentrations varying from 25nM to 100pM depending on the NSC type used, and these experiments have contributed significantly to the knowledge on mechanisms leading to cell death and overt neurotoxicity (Johansson et al., 2007). However, studies on the effects of lower doses seem to be more relevant in relation to environmental exposures occurring in the human population. In our previous studies, we showed that as low as nanomolar (2.5–10nM) concentrations of MeHg can affect proliferation and differentiation of rodent embryonic NSCs and that activation of Notch signaling is involved in the inhibition of neuronal differentiation in the exposed cells (Tamm et al., 2006, Tamm et al., 2008a). Similar effects of 25nM MeHg exposure on neural differentiation were observed in mouse embryonic NSCs (Theunissen et al., 2011).

In the present work, we investigated long-lasting effects of low-dose MeHg exposure using a model that we have developed to study heritable effects in vitro (Bose et al., 2010). This

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model permits detection of the programming effects of the subtoxic exposure that can be inherited from one cell generation to another. In addition, we used an in vivo perinatal MeHg exposure model, earlier shown to produce long-lasting behavioral deficits (Onishchenko et al., 2008), to analyze possible detrimental effects on proliferating and mature populations in the adult hippocampus, one of the few neurogenic areas in the adult brain.

MATERIALS AND METHODS

Embryonic cortical NSC culture and exposure procedures. All experiments involving work with laboratory animals were approved by the local Animal Ethics Committee (Stockholm Northern Ethics Board of Animal Experimentation). Primary cultures of NSCs were prepared as previously described (Bose et al., 2010). Cells were obtained from embryonic cortices (n = 6–8/cell preparation) dissected in Hank’s Balanced Salt Solution (HBSS) (Life Technologies) from timed-pregnant Sprague Dawley rats (n = 20) (Harlan Laboratories, The Netherlands) at E15 (the day of copulatory plug defined as E0). The tissue was mechanically dispersed, and meninges and larger cell clumps were allowed to sediment for 10 min. The cells were plated at a density of 40,000/cm² on a dish precoated with poly-L-ornithine and fibronectin (both from Sigma). Cells were maintained in enriched N2 medium with 10ng/ml basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN) added every 24 h, and the medium was changed every other day to keep cells in an undifferentiated and proliferative state. The cells were mechanically passaged via scraping in HBSS. Afterwards, the cells were gently mixed in N2 medium, counted, and plated at a desired density. Under these culture conditions, NSC doubling time was ~20 h. To investigate the long-lasting effects of MeHg, we exposed P1 NSCs to 2.5 or 5.0nM MeHg (CH3HgOH, ALFA, Johnson Matthey, Karlsruhe, Germany) for 48 h (Fig. 1A). The exposure was performed by replacing the culture medium with medium containing MeHg. In the control cell cultures, no MeHg was added to the replacement medium. The parent (P1) cells were harvested at the end of the exposure to MeHg. Mitotically heritable effects were investigated in daughter cells (D2 and D3) (see Figure 1A), also cultured in the presence of bFGF, at different time points, depending on the type of analysis. For counting and evaluation of cell viability, cells were harvested in HBSS, stained with 0.4% trypan blue solution (Sigma Aldrich), and analyzed under a phase-contrast microscope using a Neubauer improved counting chamber. Cells with a damaged cell membrane (necrotic cells) stained blue, whereas cells with intact plasma membrane (healthy or apoptotic cells) remained unstained. Apoptotic nuclei were detected by using an appropriate secondary Texas Red-conjugated antibody for 1 h at RT (1:20; Alexa, Invitrogen). Cell nuclei were counterstained with Hoechst 33342 (1 mg/ml) for 5 min. After rinsing with PBS, coverslips were mounted onto slides with Vectashield (Vector Laboratories, Inc., Burlingame, CA). Images were captured using a Nikon microscope system. All experiments were performed in triplicate and repeated at least three times.

Mercury measurements. The concentration of total mercury was quantified in the MeHg-exposed P1 (day 5 after 48-h exposure), D2 (day 2 in the passage 2), and D3 (day 2 in the passage 3) NSCs. Cells were harvested in HBSS and centrifuged at 5000 rpm for 3 min, and cell pellets were washed with PBS. Next, the pellets were used for total mercury measurement using the cold vapor atomic-absorption technique following alkaline digestion as previously described (Magos, 1971).

5-ethyl-2′-deoxyuridine incorporation and staining. NSCs were grown on coverslips in a 12-well plate for 48 h in presence of bFGF. Then cells were incubated with 10µM of 5-ethyl-2′-deoxyuridine (EdU) for 90 min and fixed in 4% paraformaldehyde (PFA) (Sigma Aldrich) for 15 min at room temperature (RT), followed by washing with PBS. EdU visualization was performed using Click-IT EdU imaging kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Fixed cells were incubated for 30 min with azide-conjugated Alexa Fluor 488 dye in Tris buffer supplemented with 4µM CuSO4. Cells were then washed three times with PBS. For subsequent DNA staining, cell nuclei were counterstained with Hoechst 33342 for 5 min. After rinsing with PBS, coverslips were mounted onto slides with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Images were performed by counting at least 100 cells/coverslip in triplicates.

Extraction of total RNA, complementary DNA synthesis, and qPCR. Total RNA was isolated from NSCs using the RNeasy Mini Kit (Quagen, WQ, Stockholm, Sweden). Integrity and concentration of extracted RNA were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA (cDNA) was prepared using 1 µg total RNA and 0.5 µg of oligo dT primer following the instructions of the Superscript II first strand cDNA synthesis kit (Invitrogen). Amplification reactions were performed with 1 µl cDNA, SYBR Green Master Mix (Applied Biosystems, Stockholm, Sweden), and 0.2µM of forward and reverse primers. The reaction volume was adjusted to 25 µl with DEPC water. Negative control samples contained water instead of cDNA. qPCR was performed using an ABI Prism 7000 Sequence Detection System with SDS version 2.1 software (Applied Biosystems). The PCR cycle conditions were 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min (40 cycles). To evaluate the amplification of a specific sample, final melting curve (from 60°C to 95°C) was added under continuous fluorescence measurements. All expression values were normalized against the housekeeping gene hypoxanthine phosphoribosyltransferase.
(Hprt) (ΔΔCT = CT_{target gene}–CT_{Hprt}). Relative expression levels were calculated as ΔΔCT = ΔCT_{target}–ΔCT_{Hprt} and relative expression changes were calculated as 2^{−ΔΔCT}. All experiments were conducted in triplicates and repeated at least three times. PCR primer sequences are available in the Supplementary table 1.

Global DNA methylation assay. DNA was prepared using the GeneElute Mammalian Genomic DNA Mini prep Kit (Sigma Aldrich) according to the manufacturer's instructions. RNA quality and concentration were measured by NanoDrop 1000 spectrophotometer (Thermo Scientific). Global DNA methylation was determined with a MethylFlash Methylated DNA Quantification Kit (Epigenetek, New York, NY) as instructed by the manufacturer, including methylated DNA standard as positive control. Global cytosine methylation levels were determined by measuring optical density in a microplate reader at 450 nm. The percentage of cytosine methylation was calculated in MeHg-treated cells relative to untreated control cells, according to the manufacturer’s instructions.

Animals and treatments. Pregnant C57BL/6/Bkl mice (Scanbur BK, Sollentuna, Sweden) were exposed to MeHg at the dose of 0.5 mg/kg/day via the drinking water from E7 till day 7 after delivery as described elsewhere (Onishchenko et al., 2007). Control females received tap water. Only male offspring were included in the following studies. We used the same antidepressant treatment protocol as in our previous study (Onishchenko et al., 2008). At the age of 10 weeks, control and MeHg-exposed males were divided in groups receiving either fluoxetine treatment or vehicle for 3 weeks. Fluoxetine (SalutasPharma GmbH, Gerlingen, Germany) was dissolved in the drinking water from E7 till day 7 after delivery as described elsewhere (Onishchenko et al., 2007). Control females received tap water. Only male offspring were included in the following studies. We used the same antidepressant treatment protocol as in our previous study (Onishchenko et al., 2008).

After cryoprotection in buffered sucrose solutions (10% for 24 h and 30% for 3–2 days), the brains were frozen and cut on a cryostat in 20-μm-thick coronal sections. We used a systematic uniform random sampling, collecting every second section in rostro-caudal direction throughout the entire hippocampal formation. Sections used for counting neurons were dehydrated in ethanol solutions of increasing concentrations, stained with cresyl violet (Sigma), cleared in ethanol and xylene, and finally mounted with Entellan (Merck) and coverslipped. For immunohistochemical stainings, tissue sections were incubated with anti-Ki-67 antibody (1:700, Novocastra Laboratories Ltd, UK) overnight, rinsed with PBS, incubated by 4% PFA with picric acid. Postfixation was done in the same solution for 2 h. After cryoprotection in buffered sucrose solutions (10% for 24 h and 30% for 3–2 days), the brains were frozen and cut on a cryostat in 20-μm-thick coronal sections. We used a systematic uniform random sampling, collecting every second section in rostro-caudal direction throughout the entire hippocampal formation. Sections used for counting neurons were dehydrated in ethanol solutions of increasing concentrations, stained with cresyl violet (Sigma), cleared in ethanol and xylene, and finally mounted with Entellan (Merck) and coverslipped. For immunohistochemical stainings, tissue sections were incubated with anti-Ki-67 antibody (1:700, Novocastra Laboratories Ltd, UK) overnight, rinsed with PBS, incubated with a secondary anti-rabbit alkaline phosphatase-conjugated antibody for 1 h at RT, developed using an alkaline phosphatase substrate kit (Vector Laboratories, Inc.), and coverslipped with mounting medium.

Stereological analysis. Cresyl violet–stained neuronal cells in the entire dentate gyrus (DG) were counted on six hippocampal sections (every 12th section, 240-μm interval) at ×100 magnification using the neuronal nucleolus as sampling unit in a Olympus BH2 microscope linked with a CAST-system (Computer Assisted Stereological Toolbox; Olympus, Albertshund, Denmark). Sections were analyzed using the optical fractionator method as described elsewhere (Janson and Møller, 1993). The height of the dissector was determined after excluding “lost caps.” The subgranular zone was defined as the layer adjacent to the granule cell layer, extending approximately three cell diameters. Penetration of the Ki-67 antibody throughout the section thickness was evaluated. To obtain enough Ki-67–positive cells, the sampling fraction was increased.

Statistical analysis. One-way ANOVA followed by Bonferroni’s post hoc test was performed to detect statistical significance (p < 0.05), unless other is specified.

RESULTS

In Vitro Experiments

The MeHg-induced effects were investigated in parent NSCs (P1) exposed to 2.5 or 5.0 nM MeHg for 48 h and in daughter cells (D) from passage 2 (D2) and 3 (D3), which had never been directly exposed to MeHg (Fig. 1A).

Determination of Mercury Concentration in NSCs

It is known that MeHg binds to cellular sulfhydryl groups covalently and can persist in the cells even after replacement of the medium (Gruenwedel et al., 1981). Therefore, we measured the total amount of mercury in the exposed P1 cells and in D2 and D3 cells. Mercury concentrations in P1 NSCs after exposure to 2.5 or 5.0 nM MeHg were approximately 9- or 15-fold higher than in control, respectively (Fig. 1B). There was a drastic decline in mercury content in D2 cells compared with P1, and there was no difference in the mercury level between D3 and control cells (Fig. 1B).

The Effect of Low-Dose MeHg Exposure on NSCs Proliferation

MeHg exposure significantly reduced the total number of cells (Fig. 2A), which maintained normal morphology (Supplementary figs. 1A–C). EdU was used as a cell proliferation marker incorporated in cells during the S-phase of the cell cycle. We observed a decrease in the number of EdU-positive cells in the MeHg-exposed P1 cells, and this effect persisted in following passages (D2-D3) (Fig. 2B; Supplementary figs. 1D–F). To ensure that the MeHg concentrations used would not
To induce cell death, we evaluated cell morphology of the exposed cells. Neither necrosis, as shown by the trypan blue exclusion test, nor apoptosis, evaluated by Hoechst 33342 staining, was induced by the used MeHg concentrations (Figs. 2C and D).

**Long-Lasting Upregulation of p16 and p21 Induced by MeHg Exposure**

Cyclin-dependent kinase inhibitors p16 and p21 restrict the G1/S-phase transition of the cell cycle. qPCR analysis revealed a significant MeHg-induced upregulation of p16 and p21 messenger RNA (mRNA) expression in P1 NSCs (p16 fold change: 2.5nM, 2.12±0.17; 5.0nM, 1.89±0.20) (Fig. 3A) (p21 fold change: 2.5nM, 1.63±0.05; 5.0nM, 2.03±0.21) (Fig. 3B) that persisted in D2 cells (p16 fold change: 2.5nM, 1.6±0.20; 5.0nM, 1.53±0.10; p21 fold change: 2.5nM, 1.59±0.14; 5.0nM 1.7±0.06) and D3 cells (p16 fold change: 2.5nM, 1.77±0.15; 5.0nM, 1.87±0.21; p21 fold change: 2.5nM, 1.52±0.12; 5.0nM, 1.5±0.05).

**MeHg Induces Expression of Senescence Markers**

The expression of senescence markers after exposure to MeHg was evaluated by qPCR and immunocytochemistry. qPCR analyses revealed that MeHg repressed Bmi1 (polycomb ring finger oncogene) mRNA expression in P1 (fold change 2.5nM, 0.35±0.08; 5.0nM, 0.22±0.05), D2 (fold change 2.5nM, 0.30±0.05; 5.0nM, 0.167±0.017), and D3 cells (fold change 2.5nM, 0.50±0.04; 5.0nM, 0.48±0.03) (Fig. 4A) and upregulated high mobility group A1 (Hmga1) in D2 cells (fold change 2.5nM, 1.31±0.12; 5.0nM, 2.54±0.47) and D3 cells (fold change 2.5nM, 2.08±0.12; 5.0nM, 2.80±0.72) (Fig. 4B). MeHg-induced changes in global heterochromatin levels were determined by immunocytochemical stainings against heterochromatin protein 1 gamma (HP1γ). We found that MeHg exposure led to a concentration-dependent enrichment of HP1γ in so-called senescence-associated heterochromatin foci (SAHFs) in D3 cells (Fig. 4C).

**MeHg Alters Mitochondrial Genes Expression**

High concentrations of MeHg have been shown to impair mitochondrial function (Mori et al., 2007, 2011). We evaluated...
the effects of low-dose exposure to MeHg on mitochondrial gene expression using qPCR analysis. We found a downregulation of NADH dehydrogenase 3 (Nd3) and cytochrome b (Cytb) genes encoding mitochondrial respiratory chain proteins: Nd3 (P1 fold change: 2.5nM, 0.34 ± 0.06; 5.0nM, 0.35 ± 0.70; D2 fold change: 2.5nM, 0.26 ± 0.04; 5.0nM, 0.22 ± 0.03; D3 fold change: 2.5nM, 0.24 ± 0.06; 5.0nM, 0.09 ± 0.02) (Fig. 5A) and Cytb (P1 fold change: 2.5nM, 0.25 ± 0.04; 5.0nM, 0.16 ± 0.02; D2 fold change: 2.5nM, 0.33 ± 0.07; 5.0nM, 0.19 ± 0.03; D3 fold change: 2.5nM, 0.27 ± 0.05; 5.0nM, 0.11 ± 0.02) (Fig. 5B).

**FIG. 5.** MeHg exposure alters the expression of mitochondrial genes. A long-lasting significant decrease in Nd3 (A) and Cytb (B) mRNA expression was induced by MeHg in all passages (P1, D2, D3) of the NSCs. Values are shown as mean ± SEM. *, statistical significance (p < 0.05, ANOVA followed by Bonferroni’s post hoc test) versus control.

**FIG. 6.** Global DNA methylation and methyltransferase Dnmt3b expression in MeHg-exposed P1 cells and D2 cells. (A) The percentage of 5-methylcytosine (5mC) of total DNA was decreased in MeHg exposed P1 cells and D2 cells. (B) Dnmt3b mRNA expression was decreased by MeHg-exposure at either concentration in the P1 NSCs but only the highest concentration of MeHg downregulated Dnmt3b expression in D2 cells. Values are shown as mean ± SEM. *, statistical significance (p < 0.05, ANOVA followed by Bonferroni’s post hoc test) versus control.

**FIG. 7.** Perinatal exposure to MeHg leads to a decrease in proliferation of subgranular cells and a reduction of total numbers of DG neurons in adult mice. These effects were attenuated by fluoxetine (FLX) known to increase neurogenesis. (A) The number of Ki-67-positive cells tended to be lower in the subgranular zone (SGZ) of the hippocampi of MeHg-exposed 13-week-old mice compared with control. A 3-week fluoxetine treatment increased the number of Ki-67 cells in the MeHg exposed mice. Statistical analyses were performed with ANOVA employing Fisher’s post hoc test, #p = 0.066 versus control, n = 3–4. (B) The number of cresyl violet-stained neurons was decreased in the DG of MeHg-exposed 13-week-old mice but reversed after a 3-week fluoxetine treatment. Statistical analyses were performed with ANOVA employing Fisher’s post hoc test, *p < 0.05 versus control, n = 4. Values are shown as mean ± SEM.

**MeHg Modifies Global DNA Methylation**

To evaluate modifications of global DNA methylation, genome wide 5-methylcytosine was quantified in control and MeHg-exposed P1 NSCs, as well as in daughter cells. There was a significant decrease in global DNA methylation in P1 NSCs and D2 cells exposed to MeHg (2.5–5.0nM) (Fig. 6A). The reduction in DNA methylation in the NSCs exposed to 5.0nM MeHg was associated with decreased Dnmt3b mRNA expression in P1 NSCs but only the highest concentration of MeHg downregulated Dnmt3b expression in D2 cells (fold change: 5.0nM, 0.730 ± 0.077) and D2 cells (fold change: 5.0nM, 0.783 ± 0.058) (Fig. 6B), whereas 2.5nM MeHg exposure decreased mRNA expression of Dnmt3b only in P1 NSCs (fold change: 0.776 ± 0.022). However, exposure to MeHg did not induce changes in mRNA expression of Dnmt1 and Dnmt3a in P1 or D2 cells (Supplementary figs. 2A and B).

**In Vivo Experiments**

**Effects of MeHg exposure on cell division and neuronal number** in vivo. In our previous work, we observed that perinatal exposure to MeHg induced long-lasting behavioral deficits, including depression-like behavior, which could be reversed by chronic treatment with the antidepressant fluoxetine. Taking into account literature data on the association between depressive disorder and hippocampal neurogenesis, as well as our in vitro results showing reduced cell division rate after MeHg exposure,
we evaluated the proliferation rate of the neural progenitor cells in the hippocampi of MeHg-exposed mice. We found a clear trend showing a reduction in the number of Ki-67–positive cells in the subgranule zone of the hippocampal DG in the MeHg-exposed animals (Fig. 7A). Due to a high variation in the number of proliferating cells between animals, this difference did not reach significance ($p = 0.066$, ANOVA followed by Fischer’s post hoc test). Interestingly, the total number of neurons in the DG of MeHg-exposed mice was significantly lower compared with unexposed controls ($0.82 \pm 0.14 \cdot 10^6$ vs. $1.19 \pm 0.03 \cdot 10^6$ per DG, respectively, $p < 0.05$) (Fig. 7B), but the neuronal loss was restored after fluoxetine treatment. Antidepressant administration to unexposed controls did not produce significant changes in the number of neurons in the DG.

**DISCUSSION**

The present study demonstrates that exposure to nanomolar concentrations of MeHg induces heritable effects including reduced NSC proliferation, alterations in the expression of genes related to cell cycle regulation, cellular senescence, and mitochondrial function. Additionally, a decrease in global DNA methylation indicates that epigenetic mechanisms are involved in the heritable effects of MeHg.

We have studied the effects of low-dose MeHg exposure using an in vitro NSC model, which permits the evaluation of both direct and long-lasting inherited alterations. Exposure of NSCs to 2.5 or 5nM of MeHg for 48 h led to total mercury accumulation at levels of 0.43 or 0.69 µg/g wet weight, respectively, in the directly exposed cells. These concentrations are close to mercury levels observed after perinatal exposure in vivo (0.9 µg/g in mouse brains), leading to learning impairments and depression-like behavior later in life (Onishchenko et al., 2007). These levels are also in the same order of magnitude as those detected in autopsy brain tissues from infants born in fish-consuming populations (0.026–0.295 µg/g) (Lapham et al., 1995). Intracellular mercury content was rapidly declining after exposure termination, and total mercury amount in the D3 cells was equal to unexposed control.

We found that exposure of NSCs to MeHg at environmentally relevant doses reduced total cell number in several cell generations. Notably, the exposure did not cause cell death of NSCs either by apoptosis or necrosis. Thus, the reduction in cell number was due to a decrease in NSCs proliferation, which was confirmed by a lower amount of EdU-positive cells. This is in agreement with previous findings by Xu et al. (2010) that showed induction of cell cycle arrest in rat embryonic cortical NSCs after 48 h exposure to 2.5–5nM MeHg. Our study provides evidence that compromised proliferative status can be observed not only as a direct but also as an inherited effect of low-dose MeHg exposure.

Gene expression analyses revealed a persistent increased expression of the cell cycle regulating genes p16 and p21 in the in vitro model. These genes encode proteins that represent potent inhibitors of cyclin-dependent kinases and cyclins in various cell types, and their overexpression is known to cause cell cycle arrest (Stein et al., 1999). Involvement of p21 in retarding cell cycle progression following MeHg exposure in the micromolar dose range has been shown earlier in primary embryonic neuroepithelial cells (Faustman et al., 2002). Increased expression of p21 at mRNA and protein level was found in mouse brains after chronic exposure to MeHg (Ou et al., 1999). Interestingly, we also observed that MeHg decreased extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) phosphorylation, which is required for G1-/S-phase transition, whereas total amount of the protein was not altered (data not shown). These findings clearly point to altered regulation of proliferation in the exposed NSCs. The upregulation of p16 and p21 genes and the inhibition of ERK phosphorylation have been linked to senescence (Huang et al., 2008). The reduced proliferation and the altered expression of cell cycle regulating genes that we have observed in our model point to premature senescence being a consequence of MeHg exposure. Other hallmarks of cellular senescence are represented by SAHFs that contain a variety of chromatin proteins, such as K9-methylated histone H3, macro H2A, HP1, and HMGA proteins (Narita, 2007). We have detected an increased expression of the Hmga1 gene and an elevated expression of HP1γ protein in MeHg-exposed daughter NSCs. Furthermore, we have also detected a heritable downregulation of Bmi1 in all passages of MeHg-exposed NSCs. There is growing evidence pointing to an important role of Bmi1, the main component of the Polycomb repressive complex 1, in proliferation and senescence of NSCs (Subkhankulova et al., 2010, Wang et al., 2010). Recent work by Yadiri et al. (2011) shows that conditional expression of Bmi1 constitutes a sensitive tool for regulation of the self-renewal potential of NSCs both in vitro and in vivo. All these mechanisms may be involved in the detrimental effects of MeHg on the developing nervous system.

Mitochondrial dysfunction resulting in overproduction of reactive oxygen species has been shown to be one of the mechanisms underlying MeHg-induced cytotoxicity (Mori et al., 2007). Disruption of mitochondrial function following MeHg exposure has been observed in brain mitochondrial preparations (Franco et al., 2010) and in cultures of neural cells and in in vitro models where it was associated with induction of apoptosis (Mori et al., 2011, Tamm et al., 2008b). Our data demonstrate that transcriptional repression of the mitochondrial respiratory chain enzymes of complexes I and III occurs upon exposure to subtoxic concentrations, which do not affect cell survival, and these alterations persist in the following cell generations. Presumably, these alterations may also contribute to the development of cellular senescence in accordance with the mitochondrial hypothesis of ageing (Navarro and Boveris, 2007).

In recent years, epigenetic alterations have emerged as a plausible mechanism underlying toxic effects. Global DNA hypomethylation has been observed in different tissues after
exposure to various environmental toxicants, and it has been proposed as a biomarker of the toxic exposure (Baccarelli and Bollati, 2009, Skinner et al., 2011). In particular, the level of global DNA methylation in the brain tissue of wildlife predators exposed to MeHg via fish has been found to be inversely correlated with Hg concentration (Plisner et al., 2010). We have detected a decrease in global DNA methylation in the NSCs following exposure to nanomolar concentrations of MeHg. Similarly to the other induced effects, alterations in the DNA methylation status were retained by the daughter cells. It is known that DNA methylation can be affected via altered activity of DNA methylating enzymes and/or the ratio between methyl donor S-adenosylmethionine to its demethylated product S-adenosylhomocysteine regulated by methionine synthase (Ulrey et al., 2005). Waly et al. (2004) showed that inorganic and ethylated mercury inhibited the responsiveness of the methionine synthase to neurochemical stimulation in human neuroblastoma cells, thus potentially interfering with methylation reactions, including DNA methylation. Whether similar alterations can also be induced by MeHg is not yet known but theoretically plausible. A recent study demonstrated that inhibition of DNMTs led to an increased expression of p16 and p21, followed by G1-phase cell cycle arrest, a decreased cell proliferation rate, and an induction of cellular senescence, as well as reduction of neural differentiation of human umbilical cord blood–derived multipotent stem cells (So et al., 2011). We have found that mRNA expression of DNMT3b, an enzyme responsible for de novo methylation, was decreased in the cells directly exposed to MeHg, and this alteration persisted in the next cell generation. Interestingly, mRNA expression of DNMT1, a maintenance methylation enzyme, was not affected by the exposure. There is growing evidence showing an association between early events altering the epigenetic status of neural cells and long-term behavioral deficits (Bagot and Meaney, 2010). In particular, in our previous work, we showed an association between depression-like behavior and epigenetic changes at the promoter of the BDNF gene after perinatal MeHg exposure (Onishchenko et al., 2008). Altogether, present and previous data point to a critical role of epigenetic changes in neurotoxicity.

The biological relevance of our in vitro findings was confirmed by in vivo data demonstrating a more than 30% decrease in the total number of neurons in the hippocampal DG of adult mice exposed to MeHg during the perinatal period. Because neuronal numbers in fluoxetine-treated MeHg-exposed mice did not differ from controls, the reduced number of mature neurons after perinatal MeHg exposure is likely to reflect complex issues linked with proliferation, survival, and differentiation of neuronal precursors (David et al., 2010). In analogy with this interpretation, we found a trend to a reduction in the number of proliferating cells in the subgranule zone of the hippocampus, a neurogenic niche in the adult brain, in the MeHg-exposed animals. Decreased neurogenesis has been linked to pathogenesis of depression in experimental and clinical studies (Ming and Song, 2011; Sahay and Hen, 2007). Notably, we observed approximately a fivefold increase in the number of Ki-67–labeled cells in the MeHg-exposed mice after the antidepressant treatment that also ameliorated depression-like behavior in the exposed animals as shown in our previous work (Onishchenko et al., 2008).

In conclusion, our study provides novel evidence for heritable programming effects induced by MeHg in NSCs and supports the idea that developmental low-dose exposure results in persistent changes leading to long-lasting alterations at morphological and functional levels, which potentially predispose to neurodevelopmental disorders and/or neurodegeneration.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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