The Effects of Low-Dose Methylmercury Exposure in Regulating Murine Embryonic Neural Precursor Development

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Abstract

Methylmercury (MeHg) is a global pollutant that affects millions of people worldwide. It is known to be neurotoxic, particularly during fetal development. Numerous cohort studies have shown correlations between impaired cognitive development and prenatal exposure to methylmercury by fish intake during pregnancy. However, the mechanisms responsible for MeHg-induced changes in adult neuronal function, when their exposure occurred primarily during fetal development, are not yet understood. We hypothesize that fetal MeHg exposure could affect neural precursor development leading to long-term neurotoxic effects. In this project, we exposed mouse primary cortical precursors *in vitro* to a range of methylmercury doses at nano- and subnano-molar level and examine the effect they have on embryonic neural precursor development. We observed that cortical precursor exposed to 0.25 nM MeHg showed increased neuronal differentiation, while its proliferation was inhibited. Reduced neuronal differentiation, however, was observed in the higher dose groups. RT-qPCR results also revealed that cell reprogramming was taking place for the cortical precursors exposed to 5nM MeHg treatment. Our results suggest that sub-nanomolar MeHg exposure may deplete the pool of neural precursors by increasing premature neuronal differentiation, which can lead to long-term neurological effects in adulthood as opposed to the higher MeHg doses that cause more immediate toxicity during infant development.

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Abbreviations

AMPK	5' AMP-activated protein kinase
BDNF	brain-derived neurotrophic factor
CiPSC	Chemically induced pluripotent stem cell
CC3	Cleaved Caspase-3
CNS	Central nervous system
CP	Cortical precursor
Div	Days *in vitro*
HBSS	Hanks' balanced salt solution
MeHg	Methylmercury
NCS	Neural stem cell
NGS	Normal goat serum
NPC	Neural progenitor cell
PBS	Phosphate-buffered saline
PLO	Poly-L-ornithine
pNSC	Primitive neural stem cell
PSC	Pluripotent stem cell

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1 INTRODUCTION

Methylmercury (MeHg) is a ubiquitous environmental toxicant. Of all forms of mercury, MeHg raises the most concern because of its toxicity and bioavailability in the environment. MeHg is known to be neurotoxic, particularly during fetal development. Dietary MeHg is efficiently absorbed and it distributes throughout the body, primarily targets the central nervous system (CNS) (NRC 2000). MeHg easily penetrates the placental barrier, reaching higher levels in fetal cord blood than in maternal blood (Vahter et al., 2000). Research have been conducted for decades studying the mechanisms of MeHg toxicity using different models, from acute high doses to chronic low doses. However, the mechanisms responsible for MeHg-induced changes in adult neuronal function, when their exposure occurred primarily during fetal development, are not yet understood. Furthermore, the latency in the appearance of signs and symptoms of MeHg poisoning has been well documented, of which reasons remain to be mysterious. In the following literature review, current knowledge of methylmercury, including environmental sources, human exposure, and known mechanisms are covered. Research done on prenatal MeHg exposure are emphasized and sufficient knowledge on the neural stem cell culture model are described. This is followed by the objectives, methodology, result and discussion for my research, examining the effects of low dose MeHg exposure on neural stem cells.

2 BACKGROUND

2.1 Methylmercury

2.1.1 Environmental sources of methylmercury

Methylmercury (MeHg) is a global pollutant affecting millions of people worldwide (Mergler et al., 2007; Driscoll et al., 2013). Despite the increasingly stringent regulations, the level of mercury emission, especially in the East and Southeast Asia, has been increasing over the years (Figure 2-1).

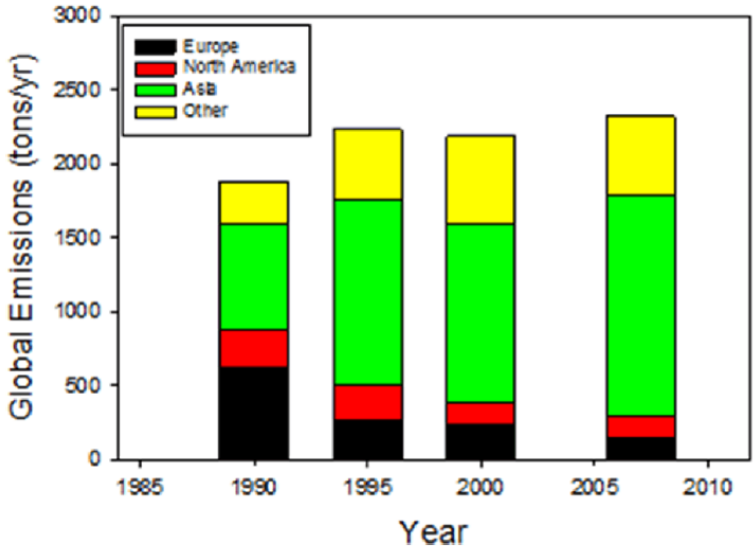


Figure 2-1. Trends in global emissions of mercury (Driscoll et al., 2013).

Environmental sources of inorganic mercury include volcanoes and forest fires, in addition to gold mining and industrial waste, as major anthropogenic sources. Once released into the atmosphere, Hg^0 can travel over large distances, which is then oxidized abiotically to Hg^{2+} when deposits onto the earth. Near-shore marine waters and inland waters are the primary sources of MeHg via methylation of Hg^{2+}. Inorganic mercury is converted to MeHg by sulfate-reducing bacteria in water sediments (Compeau and Bartha, 1985).

2.1.2 Human exposure and epidemiology

MeHg enters the aquatic food chain, which is the most common way of mercury exposure in humans. It readily enters the aquatic system and bioaccumulates in fish while it magnifies up the trophic levels (Selin, 2009). Upon consumption of contaminated fish, MeHg is almost completely absorbed in the human gastrointestinal tract, giving it a high bioavailability.

In 1998, Health Canada gave guidelines on the recommended maximum intake of methylmercury of 0.47 µg/kg bw-d for adults (~5 ppm), and 0.2 µg/kg bw-d for women of reproductive age, infants, and young children (~2 ppm). While fish consumption provides many health benefits, some species-specific fish were still found that exceeded safe exposure levels (Clarkson et al., 2003; Hachiya, 2012). Populations in certain regions that rely heavily on fish consumption are at greater risk of MeHg toxicity.

Methylmercury is toxic to many organs, but the brain is its primary target of toxicity (Clarkson et al., 2003). The neurological dysfunction caused by severe MeHg poisoning is known as Minamata disease, named after a major methylmercury outbreak in Minamata Bay, Japan. In 1956, the Chisso Inc. has been found to be releasing industrial wastewater that contain high mercury levels into the Minamata Bay for 36 years with no government regulation, causing severe environmental issues. Local fish were contaminated, and thousands of people were affected. Reports of symptoms began in the early 1950s, with only 34 adults initially diagnosed with Minamata disease (Harada, 1995). However, from the late 1950–60s, many infants were born with brain and neurological defects, while their mothers showed no to only mild symptoms of Minamata disease (Harada, 1978; Harada, 1995). Continuing research on the aging population of Minamata has found the even those who did

not exhibit symptoms during the crisis appear to be at greater risk for developing neurodegenerative diseases later in life (Harada, 1995).

Takaoka et al. (2018) conducted a survey studying the long-term effect of methylmercury exposure and found that 65% of the subjects experienced their first symptoms after 1968, when polluted waste water from the Chisso Company's factory was halted (Figure 2-2). Symptoms have even appeared in recent years. Surprisingly, they also found that the latency period of mercury poisoning became longer as the exposure became milder.

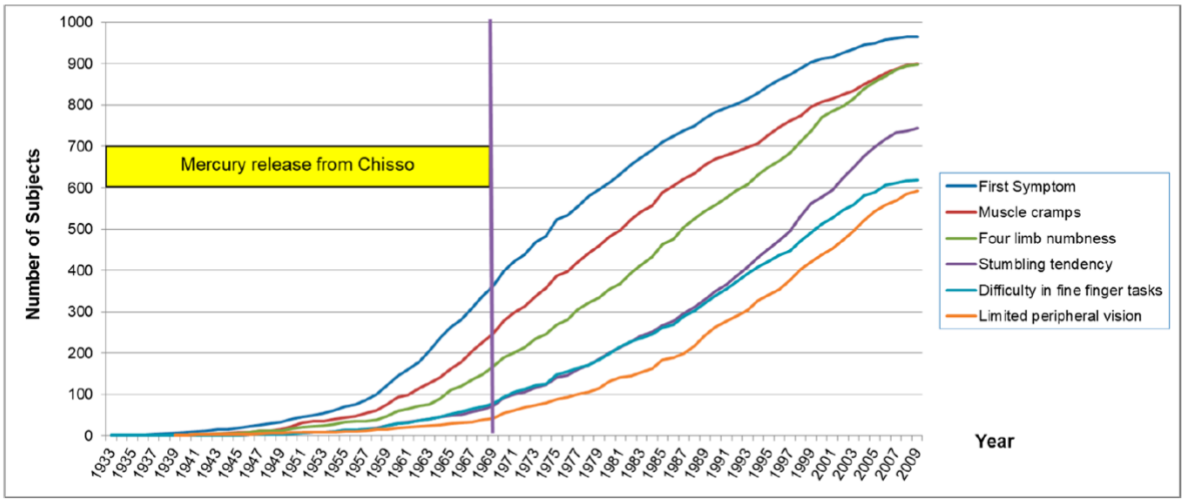


Figure 2-2. Mercury poisoning onset year of each neurological symptom (actual number). Sixty-five percent of the subjects in the exposed area experienced their first symptoms after 1968 (Takaoka et al., 2018).

The type of methylmercury exposure is mainly based on the dose and the duration of exposure. Symptoms of acute and chronic high exposure include paresthesia, dysarthria, tremor, cerebellar ataxia, gait disturbance, visual-field constriction and disturbed ocular movements, hearing loss, as well as subjective symptoms of malaise (Clarkson et al., 2003). In the worst cases, the patients went into a coma and died (IPCS-WHO 1990). The first symptoms to appear were paraesthesia and malaise. Chronic low MeHg exposure typically

shows milder symptoms or no symptoms for decades. However, it was found to be associated with increased risk of developing neurodegenerative diseases (Migliore & Coppedè, 2009). Moreover, MeHg is more toxic from prenatal exposure than in adults. The signs and symptoms due to prenatal exposure to methylmercury is referred to as congenital Minamata disease (CMD), which includes mental retardation, primitive reflexes, ataxia, disturbances in physical growth, dysarthria, and limb deformities, as well as, in some cases, hyperactivity, hypersalivation, seizures, and wandering eye (NRC 2000).

Extensive epidemiological studies have been done on low exposure MeHg toxicity in populations such as Seychelles, Faroe Islands, and New Zealand. It is found to be associated with neurological disorders and neurodegenerative diseases (Migliore & Coppedè, 2009). MeHg has been found to disrupt brain development, causing cognitive and motor disabilities. (Johansson et al., 2007). Memory loss and cognitive alterations has also been reported in adult upon MeHg exposure (Wojcik et al., 2006; Chang et al., 2008). In a recent review, the authors concluded a sure correlation between mercury exposure and the typical features of Alzheimer's disease and Parkinson's disease (Cariccio et al., 2019).

2.1.3 Mechanisms of MeHg neurotoxicity

Upon gastrointestinal absorption, MeHg rapidly binds to hemoglobin in the bloodstream. It forms complexes with the amino acid cysteine in peptides or proteins, with a half-life of about 50 days. The complexes are recognized by amino acid transporters due to molecular mimicry of L-methionine, and are readily transported across biological barriers, such as the placenta and the blood brain barrier (Aschner and Aschner, 2007).

High MeHg exposures can result in neuronal cell death, ultimately leading to brain lesions (Wakabayashi et al., 1995). The accumulation of MeHg has been shown to be region-specific, with hotspots in the cerebral cortex and cerebellum (Castoldi et al., 2000). MeHg has also been shown to have widespread sublethal effects in the brain. Basu et al. (2007) have shown that environmentally relevant concentrations of MeHg may influence the activities of multiple neurotransmitters in the cerebral cortex of river otters. In addition, Berg et al. (2010) identified 40 protein markers that are associated with molecular targets and mechanisms of MeHg-induced neurotoxicity in mammals, such as mitochondrial dysfunction, oxidative stress, altered calcium homeostasis and disruption of microtubules.

The mechanisms of methylmercury neurotoxicity have been studied from a wide range of aspects. MeHg was reported to cause oxidative stress, which affects lipid peroxidation, protein oxidation, DNA oxidation, altered calcium homeostasis, impaired mitochondria and apoptosis (Fretham et al., 2012). It also leads to altered neurotransmitters by directly inhibits proteins necessary for calcium homeostasis, glutamate transport and GABA signaling. The two modes of action can be summarized as illustrated in Figure 2-3 (Karri et al., 2016). MeHg is also known to cause dysregulated intracellular signaling, including phospholipase C (PLC), calcium signaling, and Phosphatidylinositol 3-kinases/protein kinase (PI3K/Akt) (Fretham et al., 2012). Aberrant gene expression was also observed with exposure to MeHg. MeHg activates Nrf2, increases IL-6 and activates NF-kB. It also increases late differentiation gene while decreasing early differentiation gene, leading to retarded development, with unknown mechanism.

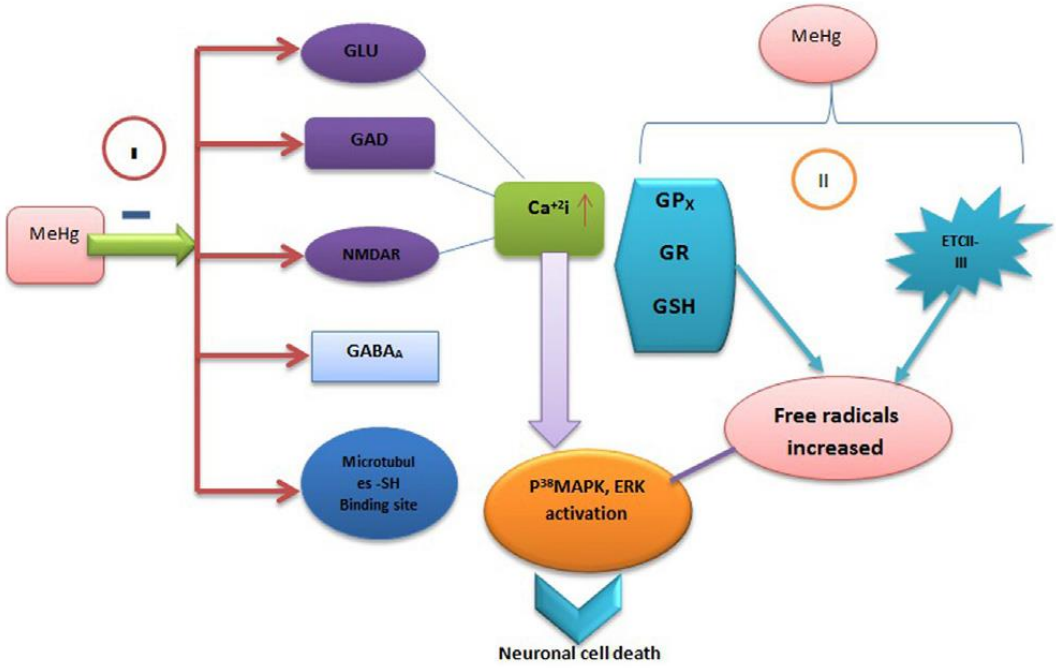


Figure 2-3. The mode of action of methyl mercury (MeHg) by two pathways: I) Neurons physiological functions and II) Reductive defensive mechanism (− sign indicates inhibition) (Karri et al., 2016).

Epigenetic regulations of methylmercury have also been studies in the recent years, mostly focused on DNA methylation. Onishchenko et al. (2008) reported that pregnant mice exposed to MeHg at a dose of 0.5 mg/kg per day from gestational day 7 through postnatal day 7 resulted in depression-like behavior in the offspring. Since reduced mRNA expression of brain-derived neurotrophic factor (BDNF) is associated with depression and anxiety, the authors examined a BDNF promoter region and identified multiple transcription-repressive epigenetic signatures, including an increase in histone H3K27me3, a decrease in histone H3 acetylation, and an increase in CpG methylation. On the other study, Desaulniers et al. (2009) treated pregnant rats with MeHg at 2 mg/kg per day from gestation day 1 until postnatal day 21, and they observed a significant decrease in the expression of DNA methyltransferases in the offspring, as well as decreased DNA methylation at brain-derived neurotrophic factor (BDNF) promoter (Desaulniers et al. 2009). Evidence for an association between mercury

and DNA methylation at the TCEANC2 region has been found in a genome-wide DNA methylation analysis (Bakulski et al., 2015). Further epigenome-wide methylation study showed that moderate prenatal mercury exposure can lead to sex-specific alterations of DNA methylation that persist throughout childhood and are associated with cognitive performance (Cardenas et al., 2017).

Recent studies showed that microRNA expression may also play a role. Shao et al. (2015) identified a key target of MeHg, MiR-19, which plays an important role in regulating key genes in the neurodegenerative disease. Wang et al. (2016) found that low-dose methylmercury-induced genes regulate mitochondrial biogenesis via miR-25 in immortalized human embryonic neural progenitor cells.

2.1.4 Prenatal exposure to methylmercury

MeHg is known to be neurotoxic and can cause permanent neurological damage in adults. The developing nervous system appears to be particularly vulnerable to MeHg (Ha et al., 2017), and the resulting behavioral deficits are persistent (Matsumoto et al., 1965). Amin-Zaki et al. (1976) reported that the levels of MeHg in fetal blood are about 25% higher than those of the mother. It has also been shown that fetuses can be affected in the absence of maternal toxicity (Matsumoto et al., 1965).

Experimental studies have shown that prenatal mercury exposure to mice results in memory disturbances and induces depression-like behavior in adult animals (Onishchenko et al., 2007). Moreover, the behavioral alterations persist into old age (Gilbert et al., 1996; Newland et al., 2004; Onishchenko et al., 2008). In humans, a cohort study on the population of the

Faroe Islands showed that prenatal exposure to MeHg was significantly associated with deficits in fine motor control, language, and learning abilities in children and adolescents (Debes et al., 2006). An epidemiological study on the Minamata population revealed an increased prevalence of psychiatric symptoms in adults who did not display overt signs of toxicity at birth (Yorifuji et al., 2011). During a recent north Quebec Inuit cohort study, Boucher et al. (2012) showed that prenatal MeHg exposure was associated with increased attention problems and ADHD behavior. Lower IQ and increased risk of intellectual disability was also observed (Jacobson et al., 2015). The association between prenatal MeHg exposure and ADHD was also reported in numerous New Bedford and Faroe Isles cohort studies. Prenatal MeHg was found to contribute most to neurodevelopmental deficits, using structural equation modelling to determine effects of MeHg exposure in the Faroese birth cohort (Grandjean et al., 2014). To understand how low-dose MeHg affects embryonic brain development, neural stem cell, or cortical precursor, is a great tool to observe any early changes at cellular level.

2.2 Neural Stem Cells and Cortical Precursors

NSCs are multipotent stem cells located in the central nervous system (CNS). They are generated from pluripotent stem cells (PSCs)(Clarke et al., 2000). They can self-renew and differentiate to produce neural progenitor cells (NPCs). The NPCs have limited capacity to proliferate, and will eventually differentiate into neurons, astrocytes and oligodendrocytes (Figure 2-4). NSCs originate from pluripotent stem cells (PSCs) *in vivo*. PSCs can self-renew and develop into all three germ layers, in other words, into all cells of the adult body (Binder et al., 2009). Some common pluripotency markers include the Oct4, Sox2, Nanog, KLF4,

and SSEA4. In some cases, PSCs can be incompletely or partially pluripotent, forming cells of all three germ layers but do not exhibit all the characteristics of completely pluripotent cells.

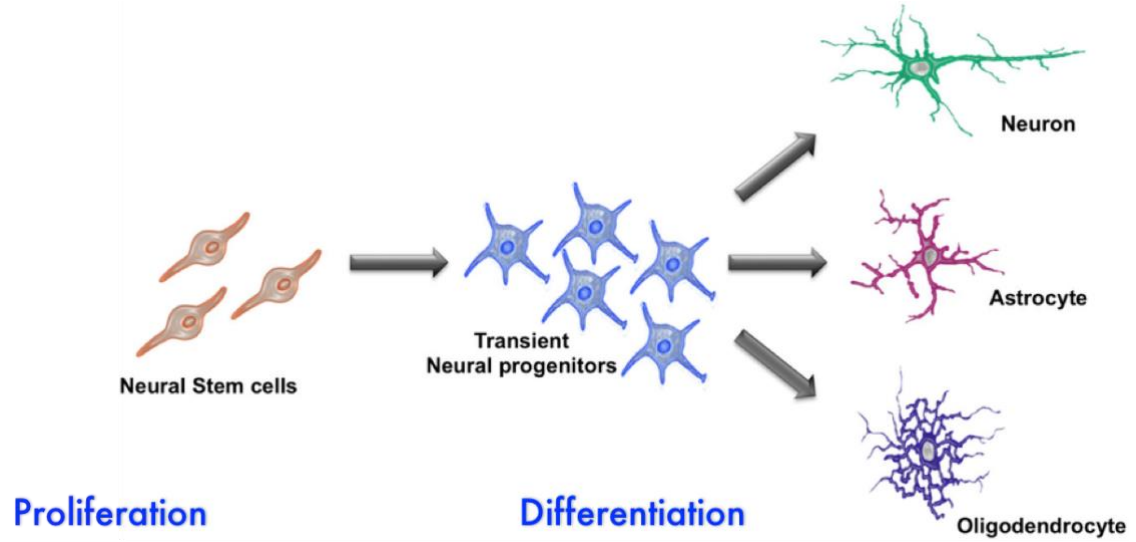


Figure 2-4. Neural stem cell properties (modified from Casarosa et al., 2014)

Cells derived from NSCs are spatially organized during well-defined period of development to ensure the correct structure of neuronal networks (Temple, 2001). Tightly regulated NSC proliferation and differentiation processes are fundamental to the establishment of brain architecture. NSC population divides symmetrically to expand during development, which is regulated by cell cycle machinery, such as cyclin-dependent kinases (CDKs) and their inhibitors (CDIs). In addition, the proliferation of NSCs is also regulated by growth factors, depending on the stage of stem cell development (Sommer and Rao, 2002). Basic FGF (bFGF, or FGF2) is required for proliferation of NSCs at early stage, while either bFGF or epidermal growth factor (EGF) is responsible at later stages (Tropepe et al., 1999). For neuronal progenitors, sonic hedgehog (Shh), FGF, and neurotrophin-3 (NT-3) are required

for mitotic division (Sommer and Rao, 2002). The brain-derived neurotrophic factor (BDNF) was also found to play an important role in NSC proliferation and differentiation (Chen et al., 2013). Finally, epigenetic regulation of gene expression has gained much attention in the recent years, and their roles in regulation of NSC proliferation and differentiation are gradually being discovered.

The model we used for our study is based on the mouse cerebral cortex development (Figure 2-5). Neuroepithelial cells are one kind of NSC residing in the embryonic cortex. They are derived from pluripotent stem cells and divide symmetrically to generate more neuroepithelial cells in early development. As the embryonic cerebral cortex develops, neuroepithelial stem cells elongate and convert into radial glial cortical precursors, which undergo three waves of neural lineage development. Radial glial cortical precursors produce neurons first through asymmetric division during mid-gestational stage, followed by the astrogenesis wave occurring at the end of gestational stage and oligodendrogenesis wave surging postnatally (Kriegstein and Alvarez-Buylla, 2009). Pax6 is specifically expressed by radial glial cortical precursors in the developing cortex, and has been shown to control radial glia differentiation in the cerebral cortex (Götz et al., 1998). Beta III tubulin is thought to be involved specifically during differentiation of neuronal cell types, which is found in the cell bodies, dendrites, axons and axonal terminations of immature neurons. Therefore, Beta III tubulin is commonly used as a marker for early-born neurons produced from cortical precursors. Astrogenesis can be identified by GFAP (glial fibrillary acidic protein), the main constituent of intermediate filaments in astrocytes (Jacque et al., 1978).GFAP is thought to help maintain astrocyte mechanical strength as well as the shape of cells (Cullen et al., 2007). Olig2 (oligodendrocyte lineage transcription factor 2) marks early oligodendrogenesis. Olig2 is a transcriptional factor that is essential for the maturation of oligodendrocyte progenitor

cells (Hwang et al., 2009). It is observed in both nucleus and the cytoplasm. Late oligodendrocyte progenitors can be identified by the cell surface antigen O4 (Schachner et al., 1981; Bansal et al., 1989). It has been commonly used as a marker specific for the oligodendroglial lineage. A subpopulation of radial glial cortical precursors retains apical contact postnatally, some of which convert into ependymal cells lining around lateral ventricle, whereas others convert into adult subventricular zone (SVZ) neural stem cells (type B cells). The type B cells maintain an epithelial organization with apical contact at the ventricle and basal endings in blood vessels.

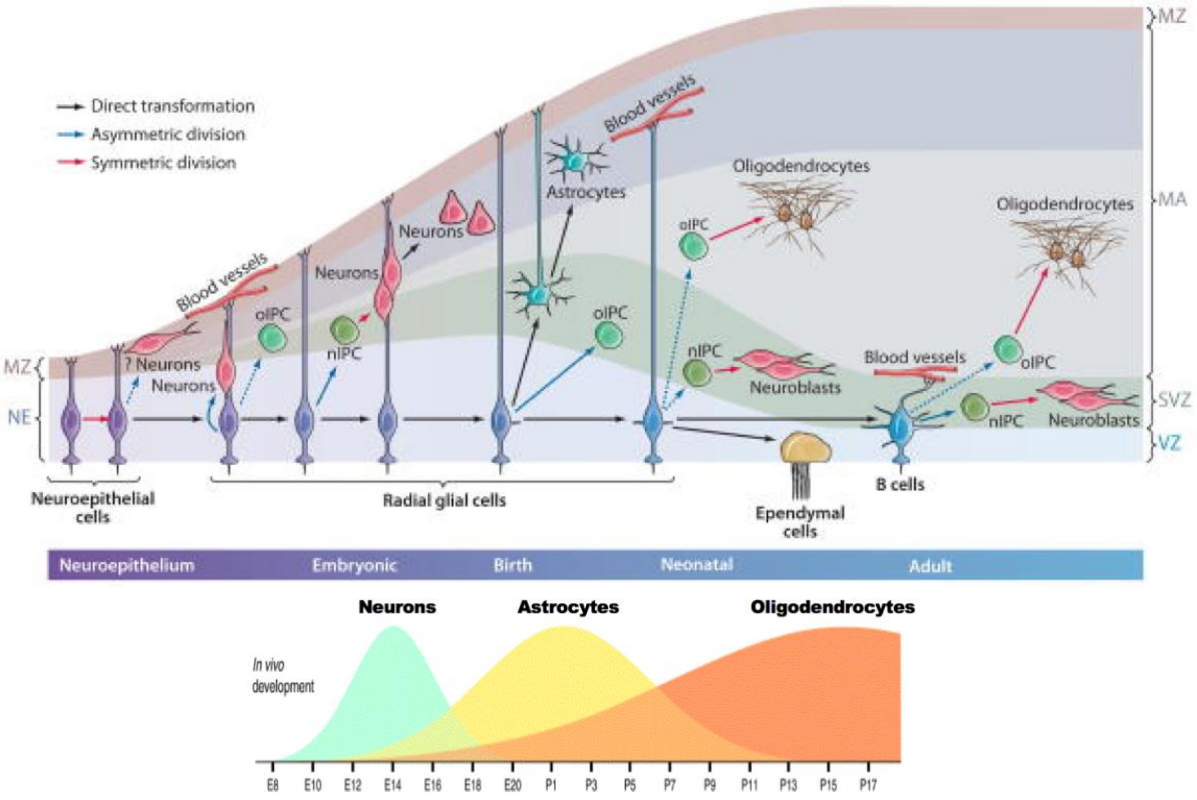


Figure 2-5. Neural stem cells (NSCs) in development and in the adult rodent brain. (Modified from Kriegstein and Alvarez-Buylla, 2009)

Generally, NSCs can be expanded *in vitro* using two different culture systems: as neurosphere (spherical clusters of cells), or as adherent monolayer cultures. In the adherent monolayer culture system, the cortical precursors (CPs) can be cultured in defined, serum-free medium supplemented with bFGF, in the presence of substrates, such as poly-L-ornithine and laminin. When plated under these conditions, the cortical precursors will attach to the substrate-coated plates, forming an adherent monolayer of cells. This adherent monolayer approach has several advantages over the neurosphere culture approach. First, the cortical precursor culture *in vitro* exactly mimics *in vivo* cortical development. Using E12 cortical precursor culture as shown in Figure 2-6, we observe only neurons being generated at 3 days in vitro (3 DIV), astrocytes occurring at 6 DIV, and oligodendrocytes showing at 9 DIV, which exactly mimic the three waves of neural lineage development (Figure 2-6). Second, the 2D monolayer allows for uniform exposure to culture media and nutrients better than the 3D neurosphere culture. Thirdly, the monolayer cortical precursor culture will allow timely and simultaneously assessing cortical precursor cell fate decision: proliferation vs differentiation at the same time. For these reasons, the adherent monolayer system is more applicable to our research purposes.

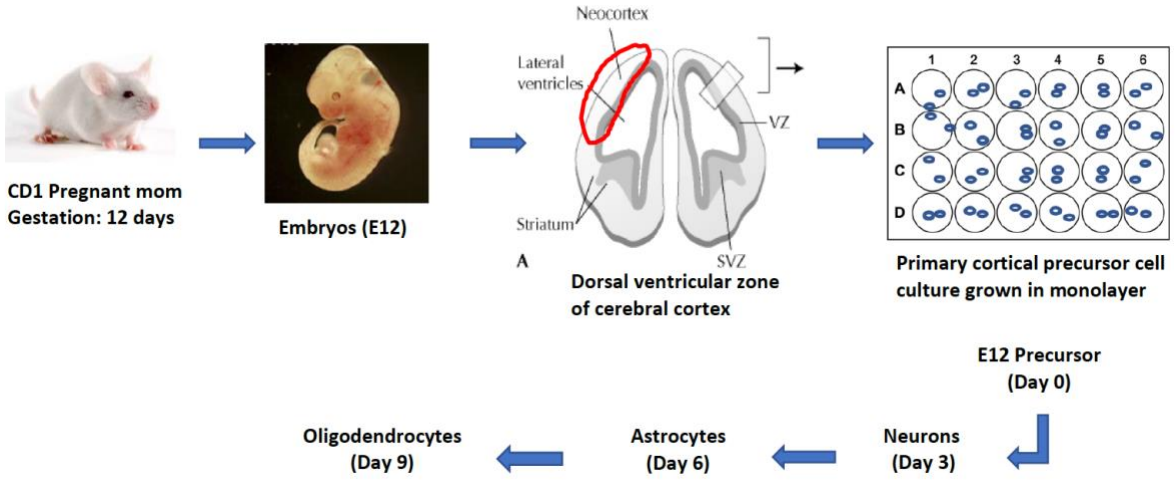


Figure 2-6. The *in vitro* approach for our mouse cerebral cortex development model (Personal communications).

2.3 Developmental Origin of Health and Diseases (DOHaD)

The idea of the “Developmental Origin of Health and Diseases (DOHaD)", previously termed the "fetal origins of adult disease" in the 1990s, postulates that exposure to environmental influences during the embryonic period is related to the risk of developing diseases in adulthood (Silveira et al., 2007). Therefore, it is important to understand not only the immediate effect of MeHg exposure on the embryos themselves, but also its potential influences that progress into adulthood. The DOHaD theory suggests that epigenetic alterations could be induced by environmental conditions during development, which are maintained in adulthood. These subtle epigenetic changes, showing no effect in early ages, can increase the risk of developing diseases later in life (Gillman et al., 2007). Therefore, there is the necessity of understanding the effect of dietary MeHg exposure on very low levels of embryonic neural development and its underlying mechanisms.

Neural stem cells have been reported to be induced to undergo apoptotic cell death by MeHg exposure. Burke et al. (2006) collected cortical precursor cells from E14.5 rat pups and treated the cells with 0.1 μM to 10 μM MeHg in adherent monolayer cell culture for 6 hr and 24 hr. The cortical precursors exhibited decreased DNA synthesis in response to MeHg exposure. They investigated the mechanisms and found that the rapid inhibition of cell proliferation in the developing brain was associated with disturbed cell cycle progression upon MeHg exposure. The G1/S transition was identified as an early target of MeHg toxicity, and cyclin E degradation was responsible for the deceased proliferation and cell death. On the other study, Tamm et al. (2006) treated the NSC line C17.2 (an multipotent NSC line originally derived from the neonatal mouse cerebellum) and primary embryonic cortical precursors from E15 rat embryos, with MeHg doses ranging from 25 nM to 2 μM. They

showed that NSCs, especially cortical precursors, are highly sensitive to MeHg. To be more specific, 250 nM and 25 nM MeHg induced similar percentages of apoptotic cells in cortical precursors. In both models, MeHg induced apoptosis via Bax activation, cytochrome c translocation, and caspase and calpain activation. Buzanska et al. (2009) used a human NSC line derived from umbilical cord blood (HUCB-NSCs) for developmental neurotoxicity testing and found that 50 nM MeHg inhibited proliferation and induced apoptosis in early-state cells. Surprisingly, 1 μM MeHg actually induced neural differentiation of HUCB-NSCs. They stated that the discrepancy of results comparing to primary embryonic cortical NSCs was due to susceptibility and cell type-specific effects between species. Watanabe et al. (2009) exposed neural progenitor cells derived from the embryonic brain to 0.1 μM MeHg and observed delayed apoptotic cell death. They also found that the cytoprotective action of antioxidants, such as N-acetyl cysteine and α-tocopherol, dramatically rescued the NPC from MeHg-induced toxicity. Ferraro et al. (2009) assessed the neurotoxic effects induced by prenatal acute treatment with MeHg on primary neuronal cultures from cerebral cortex of neonatal rats. The rats were treated with 4 and 8 mg/kg MeHg on gestational day 15, the developmental stage critical for cortical neuron proliferation. Results showed reduced cell viability and abnormal neurite outgrowth and retraction or collapse of some neurites, caused by a dissolution of microtubules.

However, in these studies, NSCs were acutely exposed to MeHg, while in reality, fetuses in the uterus are usually exposed to MeHg chronically or continuously at a much lower dose. Therefore, it is interesting to know whether very low doses of methylmercury that are non-lethal would have impact on the proliferation or differentiation of neural stem cells, and how that would influence the development of central nervous system in the long run.

3 OBJECTIVES

In this study, we investigated the effects of low-dose (nM) MeHg exposure on cortical precursor development using a mouse cerebral cortex development model. I hypothesize that a very low and non-toxic dose of MeHg perturbs the proliferation and differentiation of the embryonic cortical precursors without adverse effects on their cell viability. To test the hypothesis, I proposed to perform the following two objectives:

Objective 1: To assess cortical precursor cell fate changes at protein level upon various low doses of MeHg treatment at nano- and subnano-molar ranges;

Objective 2: To identify and quantify changes at gene expression level upon low-dose MeHg exposure.

4 RESEARCH METHODOLOGY

4.1 Animal Ethics

All animal use was approved by the Animal Care Committee of the University of Ottawa in accordance with the Canadian Council of Animal Care policies. CD1 pregnant mice were purchased from Charles River Laboratory maintained on a 12 h light/12 h dark cycle with ad libitum access to food and water until sacrificing day.

4.2 Cortical precursor culture

Primary cultures of cortical precursors were obtained from embryonic day 12 cortices dissected in ice-cold Hanks' balanced salt solution (HBSS) (Life Technologies, Carlsbad, CA, USA) from CD1 mouse (Charles River Laboratories, Wilmington, MA, USA). Embryos were transferred to ice-cold HBSS and the cerebral cortices were carefully isolated from the brain after removing the meninges. The tissue was mechanically triturated with a plastic pipette and plated at a density of 10_5 cells on coverslips pre-coated with 15% poly-L-ornithine (PLO) (Sigma, St. Louis, MO, USA) and 5% laminin (BD Biosciences, Franklin Lakes, NJ, USA) in a 24-well plate (Thermo Scientific BioLite, Waltham, MA, USA). The cortical precursors were cultured in a neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing 500 µM L-glutamine (Cambrex Biosciences, East Rutherford, NJ, USA), 2% B27 supplement (Invitrogen, Carlsbad, CA, USA), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), and 40 ng/ml FGF2 (BD Biosciences, Franklin Lakes, NJ, USA).
To investigate the effects of MeHg in cortical precursors (CPs), we exposed the primary CPs to 0.1 nM, 0.25 nM, 0.5 nM, 2.5 nM, and 5 nM MeHg for 48 or 72 h. In the control cell cultures, no MeHg was added to the culture medium. The dosing solution was freshly prepared daily from a stock solution of 4 mM using MeHgCl from Alfa Aesar (Ward Hill, MA, USA).

4.3 Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde for 10 min, then blocked with 10% normal goat serum (NGS) diluted in PBS with 0.3% Triton X-100. Primary antibodies (see Table 4-1) were diluted in 10% NGS in PBS with 0.3% Triton X-100 and incubated in a humid chamber at 4 °C overnight. Cleaved caspase-3 (CC3) was used to assess apoptotic cell death. Ki67 is a cell cycling marker used to assess cell proliferation. βIII-tubulin is expressed almost exclusively in neurons, thus is used a marker for differentiation into neurons. Pax6 is a radial glial precursor marker, used to identify cortical precursor cell population. Sox2 is expressed is both NSCs and PSCs, representing the stem cell pool. Olig2 promotes oligodendrocyte differentiation, which serves as a oligodendroglial lineage marker. GFAP is an intermediate filament expressed in astrocytes, which can be used to distinguish astrocytes from other glial cells. Nanog is a key factor that helps ESCs maintain pluripotency. Detailed information on the biological functions of each protein marker can be found in the literature review. Secondary antibodies (Alexa Fluor 555- and Alexa Fluor 488-conjugated goat antibodies) were diluted in PBST (1:500; Life Technologies) and incubated for 1 h at room temperature. Hoechst 33342 was diluted in PBS. The culture was washed three times for 5 min with PBS between each step. Nuclear staining was performed with Hoechst 33342 (1:1000; Sigma, St. Louis, MO, USA). After rinsing with PBS, the coverslips were mounted in a Lab Vision PermaFluor Aqueous Mounting Medium (Thermo Fisher, Waltham, MA, USA). All experiments were repeated at least three times.

Table 4-1. Primary antibodies used for immunocytochemistry

Antibody	Raised in	Dilution	Source	Marker for
Cleaved Caspase-3	Rabbit	1:400	Cell Signalling Tech.	Apoptosis
Ki67	Mouse	1:400	Abcam	Proliferation
βIII-tubulin	Mouse	1:1000	Covance	Differentiation
Pax-6	Rabbit	1:2000	BioLegend	Radial glial precursor
Sox2	Rabbit	1:100	Millipore	Stem cell pool
Olig-2	Rabbit	1:2000	EMD Millipore	Differentiation
GFAP	Rabbit	1:5000	Santa Cruz Biotech.	Differentiation
Nanog	Mouse	1:100	NEB	Reprogramming

4.4 Microscopy and Quantification

Digital image acquisition was performed using a Zeiss Axioplan 2 fluorescent microscope with Zeiss Axiovision software (Carl Zeiss Microscopy, Thornwood, NY, USA). Six random images over 300 cells per condition per experiment were taken for quantification.

4.5 Mercury measurements

The concentration of total mercury in the NSCs was quantified three days after MeHg exposure. The cortical precursors were plated at a density of 10_5 per well and were checked for quality under bright-field microscope before the cell pellets were harvested. Cells were harvested and centrifuged at 5 000 rpm for 10 minutes. The cell pellets were then washed with HBSS for three times. Both cell pellets and supernatants were collected. Next, the pellets and supernatants (cell culture medium and three washes) were used for total mercury

measurement using a Mercury Analyzer (MA-3000 Nippon Instruments, Bryan, TX) with a detection of 1 pg. All measurements were done in technical triplicate and biological triplicate.

4.6 Quantitative real-time PCR

Mouse cortical precursors were cultured as previously described. Two doses of MeHg, 0.25 nM and 5 nM, were selected based on previous immunocytochemistry results to show distinct difference in terms of gene expression alterations. RNA was extracted from the cortical precursor cells using the PureLink RNA Mini Kit (Thermo Fisher, Waltham, MA, USA). cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The RT-qPCR was performed with a SensiFAST SYBR Lo-ROX Kit (Bioline, London, UK) on an Mx3000P qPCR System (Agilent, Santa Clara, CA, USA). All qPCR reactions were performed using the same protocol (95°C, 30 s; 95°C, 5 s for 1 cycle; 58°C, 15 s; 72°C, 10 s for 40 cycles). All qPCRs were done in biological quadruplicate and technical duplicate. Data were analyzed using the Microsoft Excel and Prism (version 8, GraphPad Software, La Jolla, CA, USA). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an endogenous normalization control and the fold expression relative to GAPDH was determined by the delta-delta Ct method. Paired t-test between control and treatment groups was used to test for significance. PCR primer sequences were selected from published sequence data in high impact journal articles (Table 4-2). Primers were validated by running gel electrophoresis and experimental conditions were optimized (See Appendix).

Table 4-2. Primers sequences for RT qPCR. All primers were bought from Thermo Fisher.

Gene	Sequence (F)	Sequence (R)
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
βIII Tubulin	TATTCAGGCCCGACAACTTT	GTCACAATTCTCACACTCTTTCC
PAX6	TTTAACCAAGGGCGGTGAGCAG	TCTCGGATTTCCCAAGCAAAGATG
Nanog	TCCAGAAGAGGGCGTCAGAT	CAAATCCCAGCAACCACATG
Sox2	TTTGTCCGAGACCGAGAAGC	CGGGAAGCGTGTACTTATCCTT
Oct4	CCCCAATGCCGTGAAGTTG	TCAGCAGCTTGGCAAACTGTT
Klf4	ACCCACACTTGTGATTACGC	CCGTGTGTTTACGGTAGTGC
GFAP	GTACCAGGACCTGCTCAAT	CAACTATCCTGCTTCTGCTC
Olig2	CACAGGAGGGACTGTGTCCT	GGTGCTGGAGGAAGATGACT

4.7 Statistics

All immunocytochemistry data were expressed as the mean plus or minus the standard error of the mean (SEM) and were tested for statistical significance with one-way ANOVA, followed by Bonferroni's post hoc test. A two-tailed Student's t-test was used for between-group comparisons. The differences were considered significant if $p < 0.05$. All statistical analyses were performed using Prism (version 7, GraphPad Software, La Jolla, CA, USA, 2018).

RT qPCR data were expressed as the mean plus or minus the standard deviation (SD) and were tested for statistical significance with pair t-test. The differences were considered significant if $p < 0.05$. Statistical analyses were performed using Prism (version 8, GraphPad Software, La Jolla, CA, USA, 2019).

5 RESULTS

5.1 Total mercury measurement

To assess the mercury uptake in cultured cortical precursors (CPs), we collected 3 million cultured CPs at 3 days in vitro. Theorical mercury dosage and empirical mercury measurements are summarized in Table 5-1. The *in vitro* dosage was the theorical dosage that we intent to put in. The dosage can be converted to a mass unit by multiple the molar concentration by the molar mass of mercury: 200.59 g/mol. The total intracellular and extracellular Hg were the empirical measurements. Extracellular Hg dosage included mercury in the cell culture medium and wash buffers. The empirical and theorical values mostly matched, suggesting the mercury measurement were done accurately with minimal error.

Table 5-1. Theorical mercury dosage and empirical mercury measurements. Total mercury inside the cells and the total mercury in media and wash buffer was measured and estimated. All measurements were done in three technical and biological replicates. Values are mean ± SD (n=3).

Original dosage (nM)	Mass of Hg (ng)	Total Intracellular Hg (ng)	Total Extracellular Hg (pg)
0	0.00	0	0
0.1	0.04	0.02 ± 0.01	23.4 ± 17.7
0.25	0.10	0.07 ± 0.00	49.0 ± 14.9
0.5	0.20	0.13 ± 0.02	171.1 ± 15.7
2.5	1.00	0.52 ± 0.01	507.1 ± 83.3
5	2.01	0.86 ± 0.02	1007.1 ± 22.3

As illustrated in Figure 5-1 for extracellular mercury, Wash 1 appeared to contain the highest concentration of mercury, as it washes off the mercury that attached to the cell surface. Cell

culture medium came second, then the second and third wash. All mercury measurement data were very consistent over the three replicates, with only one outlier that was omitted.

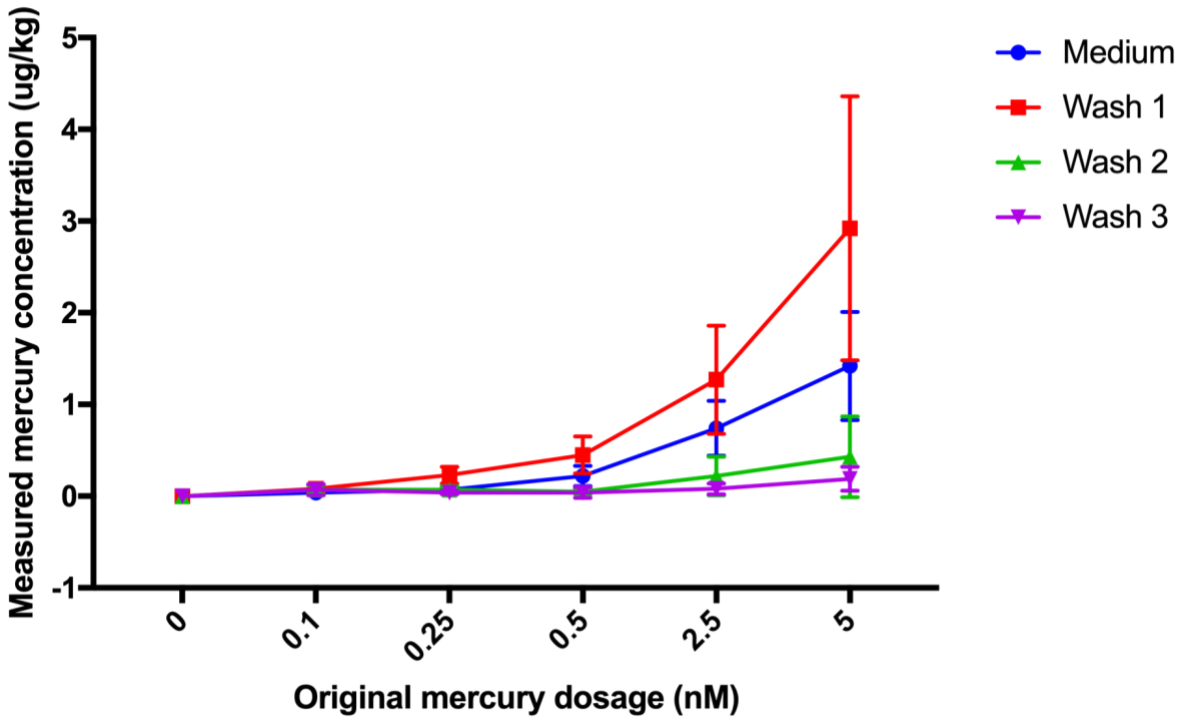


Figure 5-1. Concentration of extracellular mercury, separated by medium, first wash, second wash, and third wash. All measurements were done in three technical and biological replicates. Values are mean ± SD (n=3).

5.2. Cell Viability

To assess the effect of mercury on cortical precursor survival, we performed immunocytochemistry for cleaved caspase 3 (CC3). The results showed that the percentage of CC3+ condensed nuclei was not changed between the methylmercury treated groups and the control group (Figure 5-2 A). One-way ANOVA results showed that MeHg treatments

did not have any effect on CC3+ (Figure 5-2 B). Thus, the dosages of methylmercury used in our experiments did not the affect cell viability of the cortical precursors.

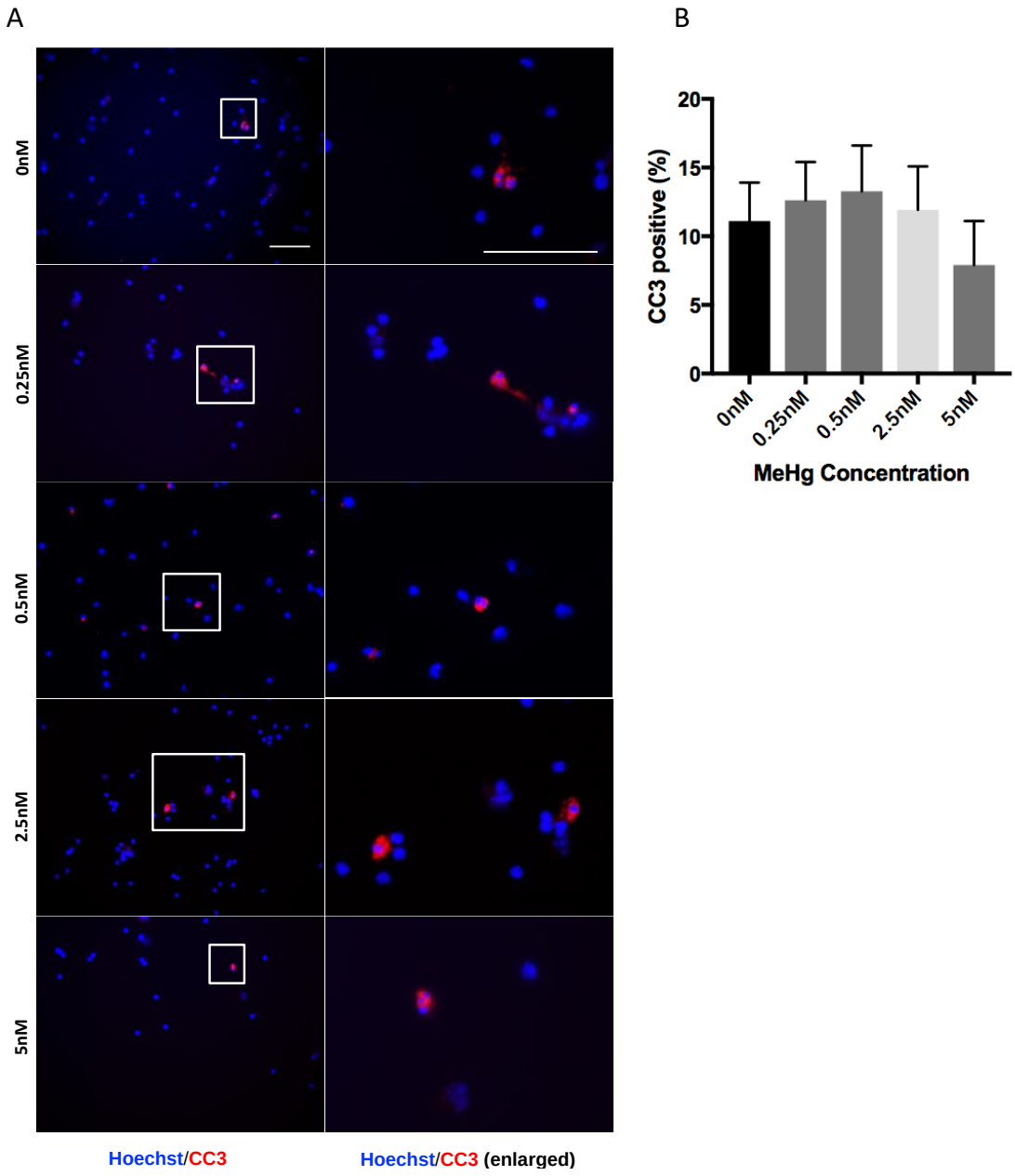


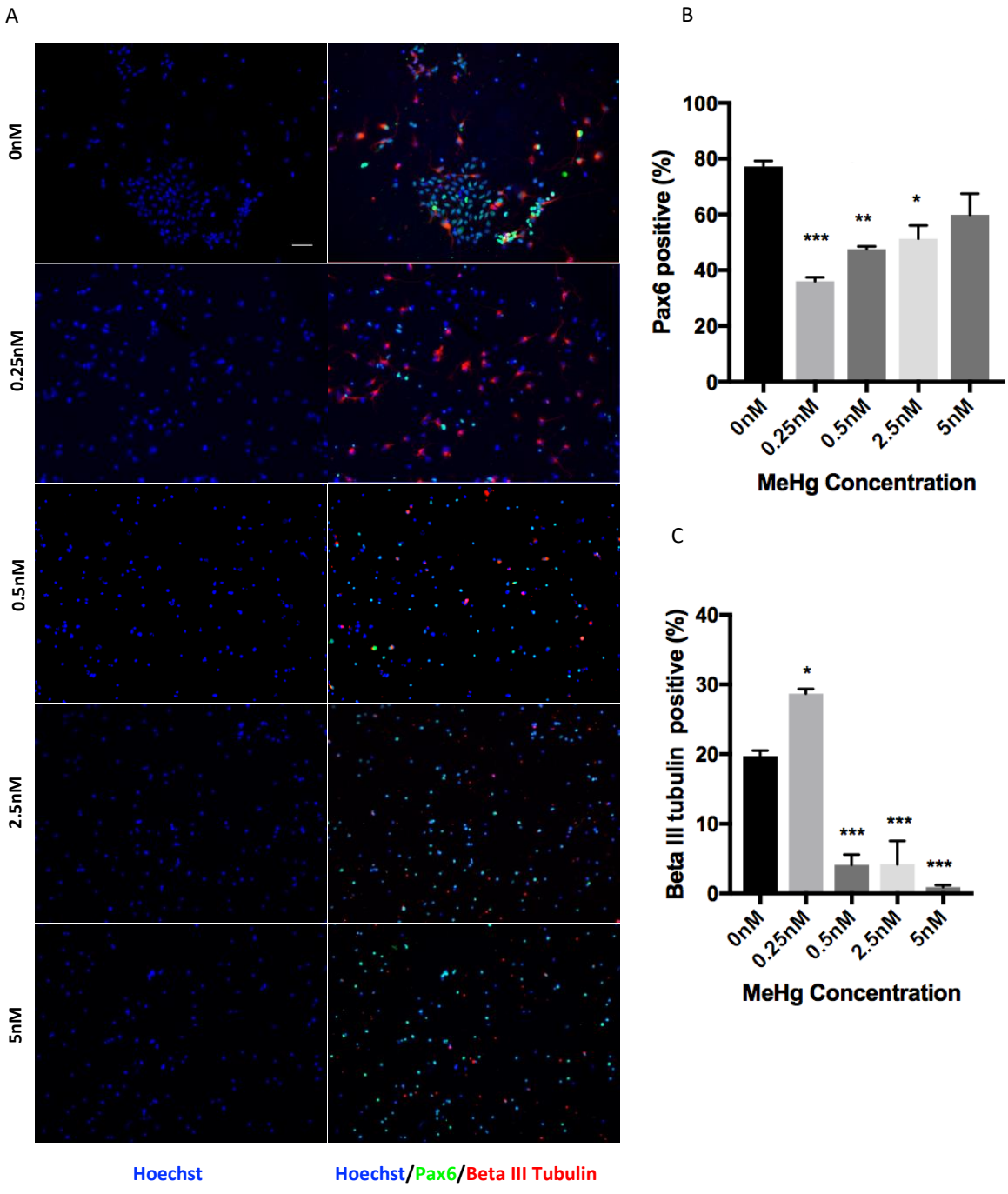
Figure 5-2. Methylmercury treatments at doses of 0.25 to 5 nM do not alter cell survival. Immunocytochemistry was performed in a 2-day cortical precursors culture. (A) Images of Cleaved Caspase-3 (CC3) (Red) and Hoechst (Blue) staining are shown. Scale bar = 50 μm. (B) The graph shows the percentage of CC3 positive cells over total live cultured cells. Values are mean ± SEM (n = 3). Statistical significance was determined by a one-way ANOVA followed by a Bonferroni's post hoc test. No significant p value was obtained for ANOVA ($F = 0.4666$, d.f. = (4,10), $P = 0.7593$).

5.3. Effects of Low-Dose MeHg Exposure to Cortical Precursors on Proliferation and Differentiation

5.3.1 Immunocytochemistry results on proliferation and neuronal differentiation

The MeHg treatments had a significant effect on the proliferation and differentiation of cortical precursors. One-way ANOVA results showed that there was a significant MeHg treatment effect for Pax 6 and Beta III tubulin staining. Immunofluorescence results showed that exposure to 0.25 nM MeHg significantly increased the percentage of new born neurons produced from E12 cortical precursors, labeled with βIII tubulin, compared to the control (Figure 5-3 A). Coincidentally, the population of Pax6 + cortical precursors was significantly decreased at 0.25 nM MeHg (Figure 5-3 B). To validate the reduced pool of cortical precursors in the culture, we performed immunocytochemistry analysis with a pan-neural stem cell marker, Sox2, and a cell cycling marker, Ki67. One-way ANOVA results showed that there was a significant MeHg treatment effect for Sox2 and Ki67 staining. The results showed that both the percentage of Sox2+ cortical precursors and Ki67+ cycling cells were dramatically decreased upon exposure to 0.25 nM MeHg (Figure 5-3 D–F). These results suggest that exposure of cortical precursors to an extremely low dose (0.25 nM) of MeHg enhances premature neuronal differentiation while reducing their proliferation. In comparison, exposure of cortical precursors to MeHg from 0.5 nM to 5 nM reduced the percentage of βIII tubulin+ new born neurons in culture (Figure 5-3 A, C). However, while the 0.5 nM and 2.5 nM treatment groups showed a significant decrease in the population of Pax6+ cortical precursors, the 5 nM treatment group did not (Figure 5-3 B). In addition, the percentage of Sox2+ NSCs and Ki67+ cycling cells were not changed in the 0.5 nM and 5 nM MeHg treatment groups (Figure 5-3 D–F). These results show that exposure to 0.5 nM

did not show the effects that we observed at the lower dose of 0.25 nM. Its effect was more similar to the effects observed at 2.5 and 5 nM MeHg, that showed significantly lower neuronal differentiation, while its proliferation recovered gradually to a level comparable with the control.



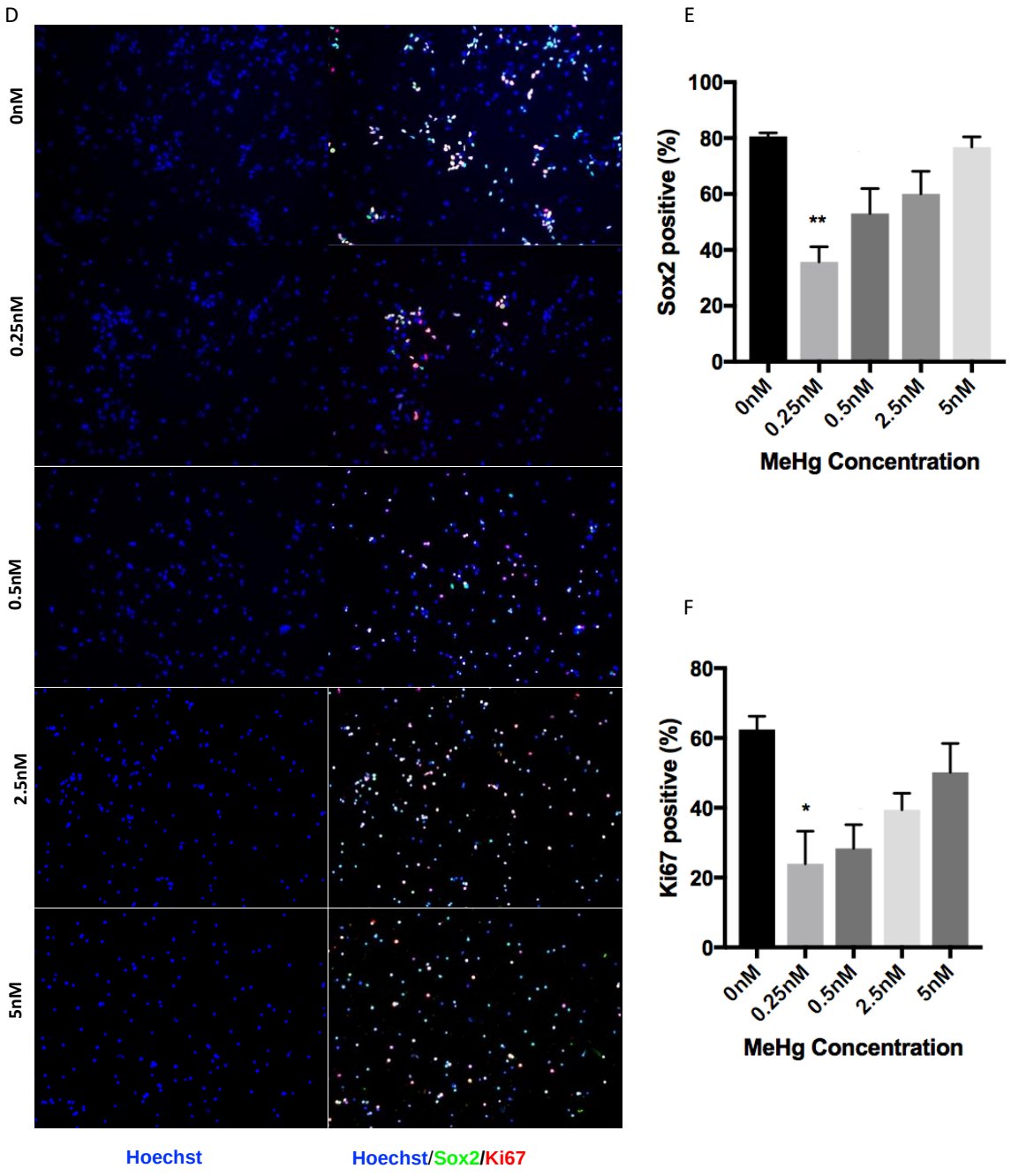


Figure 5-3. Immunocytochemistry was performed in a 3-day NSC culture. Images of (A) Pax6 (Green), Beta III Tubulin (Red), (D) Sox2 (Green), Ki67 (Red), and Hoechst (Blue) staining are shown. Scale bar = 50 μm. The bar graphs (B-C; E-F) show the percentage of immunocytochemistry-positive cells. Values are mean ± SEM (n = 3). Statistical significance was determined by a one-way ANOVA followed by a Bonferroni's post hoc test (* $p < 0.05$: vs. control). ANOVA summary: Pax6: $F = 13.56$, d.f. = (4,10), $P = 0.0005$; Beta III tubulin: $F = 50.12$, d.f. = (4,10), $P < 0.0001$; Sox2: $F = 8.849$, d.f. = (4,10), $P = 0.0025$; Ki67: $F = 5.182$, d.f. = (4,10), $P = 0.0160$.

5.3.2 Identifying the Unknown Cell Population

When co-labelling cultured CPs at 3 DIV with anti-Pax6 and anti-βIII tubulin, I observed minimal number of live cells that were negative for both Pax6 and βIII tubulin, which was consistent with previous studies (Gotz et al., 1998). Interestingly, this proportion of Pax6 and βIII-tubulin double negative cells was consistently increased to 30% upon various concentrations of MeHg treatments (Figure 5-4). To explain this phenomenon, we are examining the identity of the cells in two directions: one is differentiation into different lineages; the other direction is to check whether the precursor have been reprogrammed back to a more primitive stage, in other words, into pluripotent stem cells (Figure 5-5).

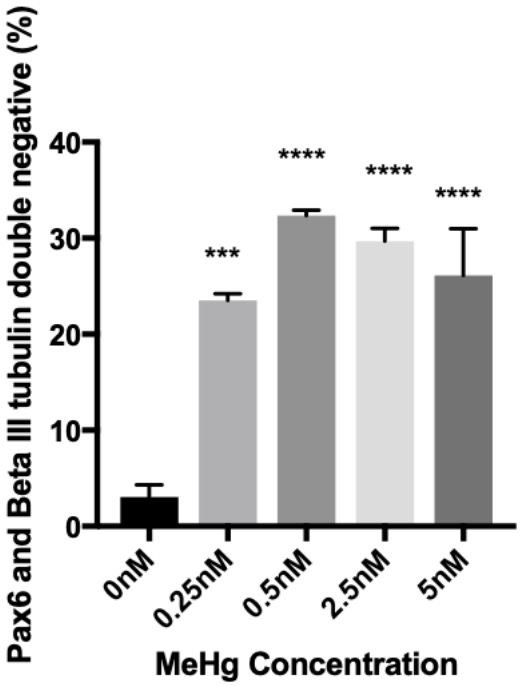


Figure 5-4. Percentage of Pax6 and βIII-tubulin double negative cell population based on immunocytochemistry result. Values are mean ± SEM (n = 3). Statistical significance was determined by a one-way ANOVA followed by a Bonferroni's post hoc test (* $p < 0.05$: vs. control).($F = 24.34$, d.f. = (4,10), $P < 0.0001$)

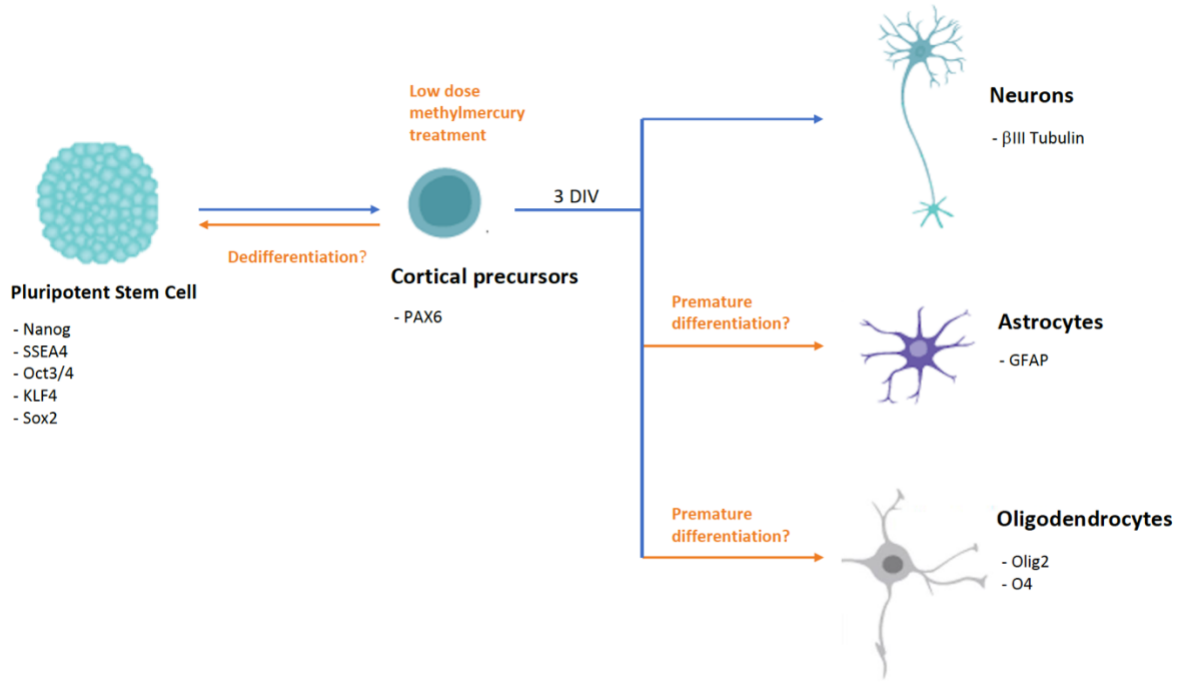


Figure 5-5. Possible paths of cortical precursor development. Cell markers unique to specific cell types are denoted.

To identify this unknown cell population, I have performed immunocytochemistry for glial markers, O4 (Figure 5-6) and GFAP (Figure 5-7). Representative pictures were selected to show the overall staining pattern. O4 and GFAP did not show any positive staining.

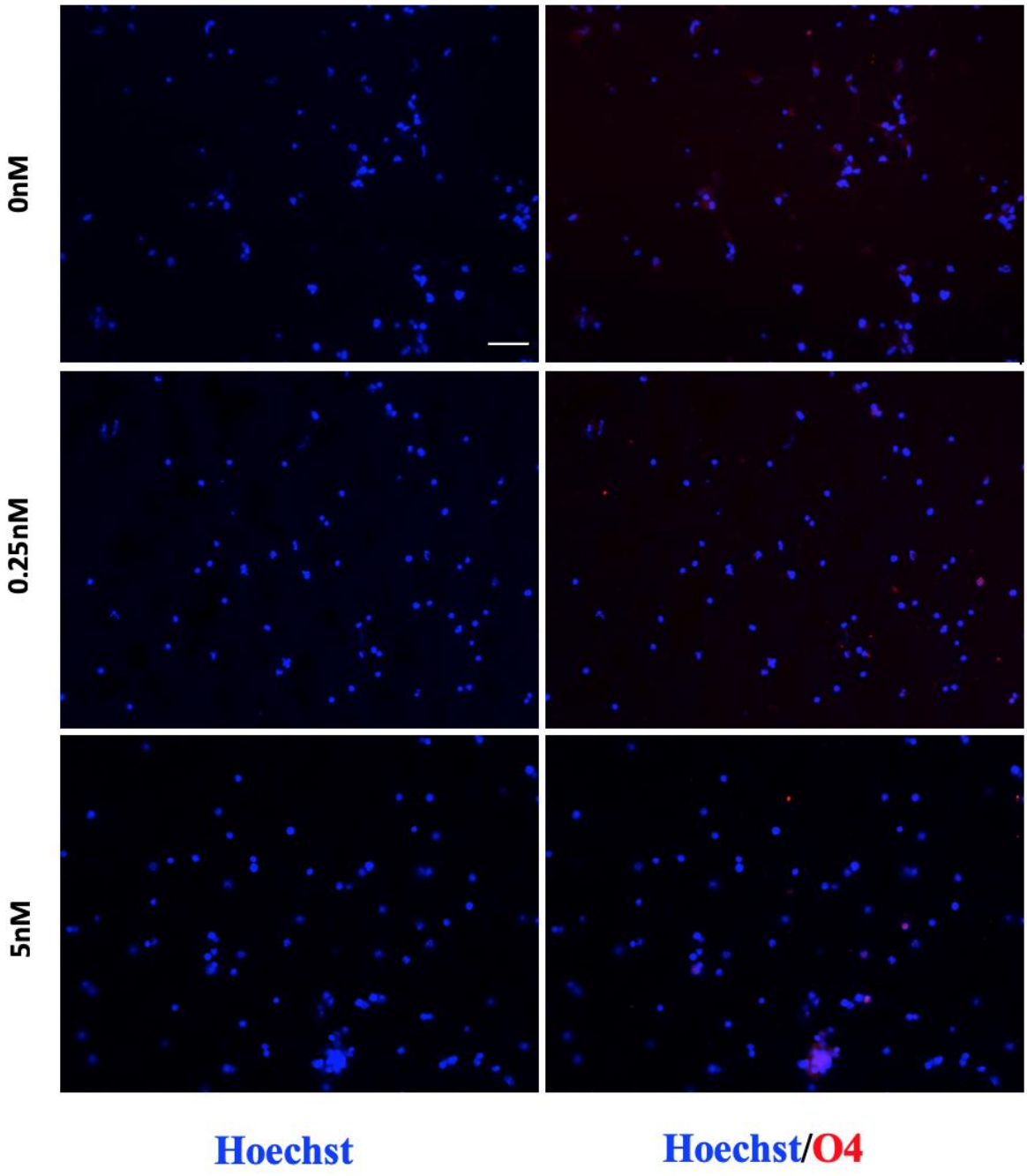


Figure 5-6. Immunocytochemistry was performed in a 3-day NSC culture. Images of O4 staining are shown for different concentrations. Scale bar = 50 μm.

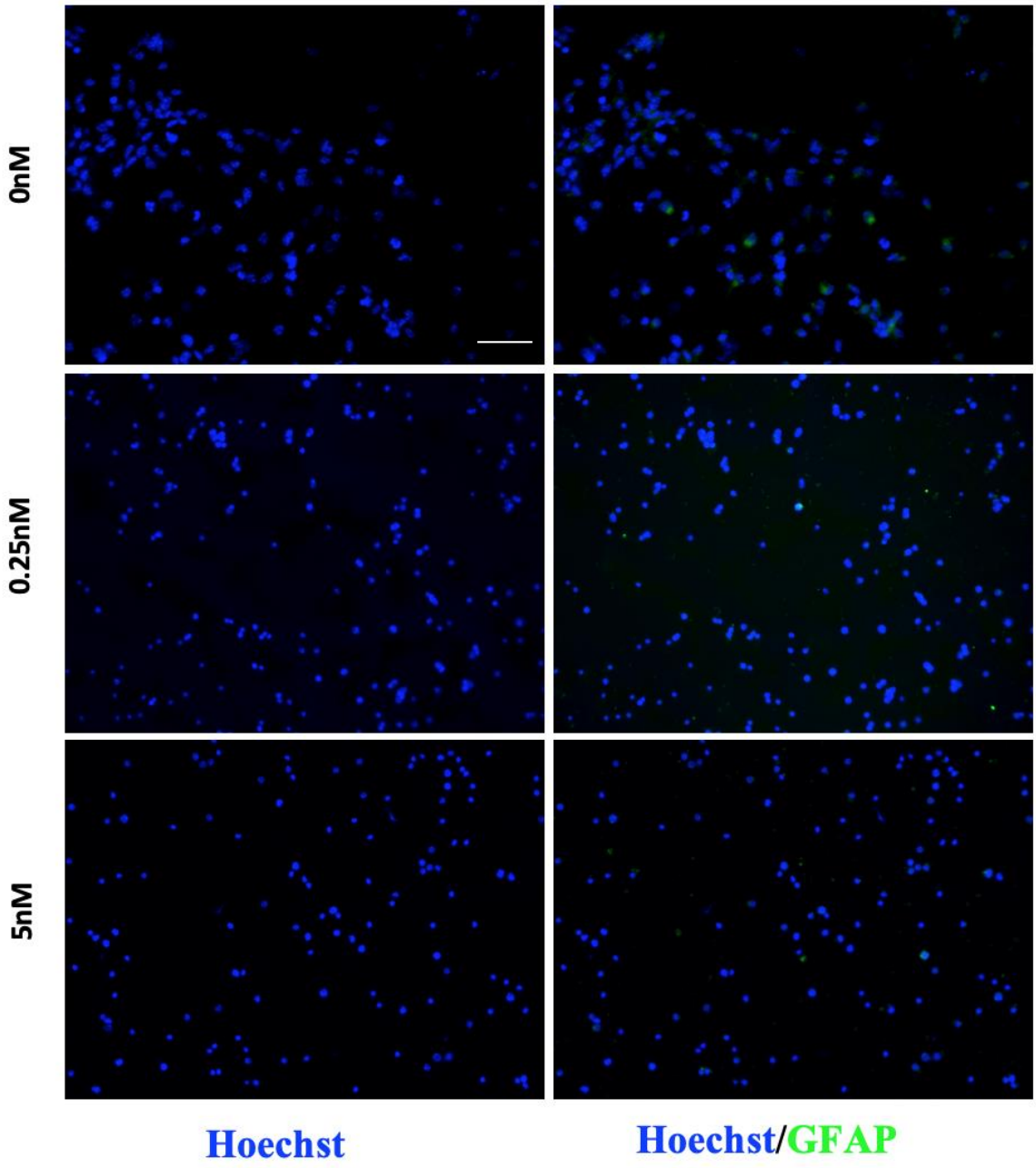


Figure 5-7. Immunocytochemistry was performed in a 3-day NSC culture. Images of GFAP staining are shown for different concentrations. Scale bar = 50 μm.

5.4 Quantitative reverse transcription PCR (RT qPCR) results

To assess the role of MeHg in regulating expression of various cell identity genes, we performed RT-qPCR analysis from 1 million cultured CPs at 3 DIV treated with either 0.25 or 5 nM MeHg. Housekeeping gene GAPDH was used as an internal loading control. Y axis represents fold changes normalized to 0nM control. 0.25nM MeHg treatment greatly suppressed Pax6, a marker for cortical precursor, as compared to control CPs ($p = 0.05$); while it significantly increased the gene expression of βIII tubulin, the marker for new born neurons ($p=0.03$) (Figure 5-8 A). This finding is in accordance with our previous immunocytochemistry results, which showed a significant decrease of proliferation and increase of neuronal differentiation. On the other hand, 5nM MeHg treatment significantly downregulate βIII tubulin gene expression ($p=0.006$), but did not alter Pax6 gene expression (Figure 5-8 B).

In addition, we performed RT qPCR to examine the alteration of gene expression on the following pluripotent stem cell markers: Nanog, Oct4, Klf4, and Sox2. Nanog, Oct4, and Sox2 cooperatively control gene expression in pluripotent stem cells and maintain their pluripotency (Johansson and Simonsson, 2010). Overexpression of KLF4 prevents neuronal differentiation and turn the neural progenitors into gliogenesis fate (Qin and Zhang, 2012). In the 0.25nM MeHg treatment group, no significant change was observed (Figure 5-8 A). Nanog, Oct4, and Klf4 expression increased in at least three biological replicates (out of four), but no significance was obtained from statistical analysis. Sox2 data contain greater variations and the trend to increase or decrease was not obvious. However, for the 5nM MeHg treated cortical precursors, Klf4 showed significant increase ($p = 0.05$) (Figure 5-8 B). Nanog expression showed a trend to increase ($p=0.09$), and the increase was seen in all four biological replicates. Oct4 mRNA level increased in three biological replicates. Based on our

results, it seems that some degree of cell reprogramming is taking place; this population of cortical precursors have obtained some pluripotent properties.

To examine whether the cortical precursors have prematurely differentiated into oligodendrocytes or astrocytes, the lineage specific makers Olig2 and GFAP were used to test for changes in gene expression. Our results showed that neither Olig2 nor GFAP had significantly changed in gene expression, which was not to our surprise. GFAP expression seemed to increase but the original Ct values were too high to be considered meaningful.

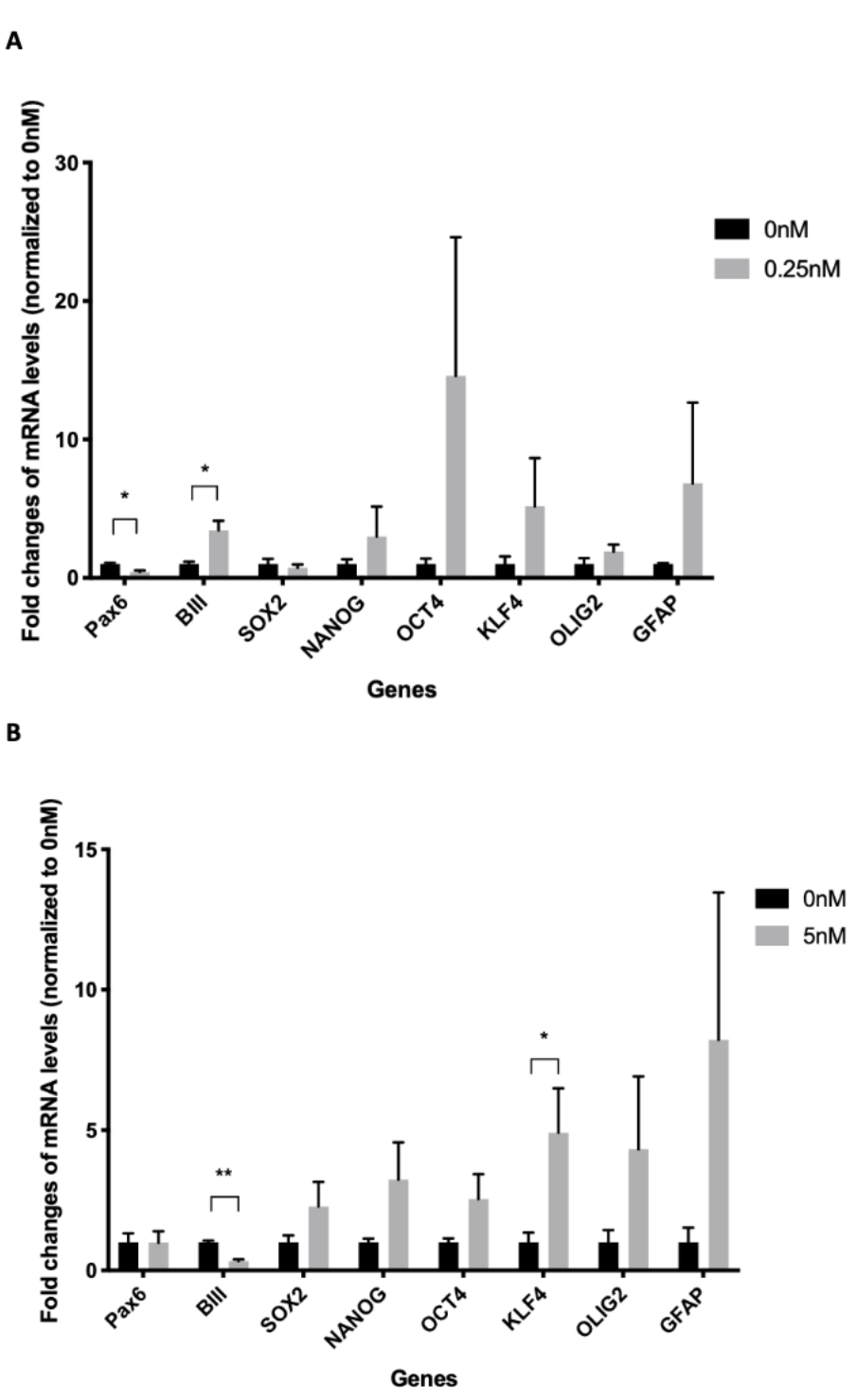


Figure 5-8. Change in gene expressions of MeHg-treated cortical precursors, expressed as fold change of mRNA levels normalized to 0nM. Cells were collected at E12 and treated with 0.25nM (A) and 5nM (B) of MeHg. The vertical axis indicates fold change for each of the genes on the horizontal axis. Statistical significance was determined by paired t-test (*:$p < 0.05$; **: $p<0.01$); Error bars: Standard error of the mean (SEM); n = 4 per condition in a single experiment.

6 DISCUSSION

6.1 Sub-nanomolar methylmercury exposure promotes premature differentiation of murine embryonic neural precursor at the expense of their proliferation

Our immunocytochemistry and RT qPCR results were consistent on the effects of MeHg on cortical precursor development. The sub-nanomolar dose of 0.25 nM MeHg increased the neuronal differentiation of cortical precursors, shown by significant increase of βIII tubulin gene expression and protein levels. On the other hand, the decrease in βIII tubulin gene expression and protein levels were observed at higher doses (5 nM) of MeHg.

This novel finding suggests that the effects of MeHg on cortical precursor development are dose-dependent. The extremely low sub-nanomolar dose of 0.25 nM MeHg increases the neuronal differentiation of cortical precursors while reducing their proliferation. On the other hand, there was a decrease in differentiation at higher doses (>0.5 nM), which is similar to the results reported in the existing literature. Similar doses of MeHg (2.5–5 nM MeHg for 48 h) were shown to inhibit the spontaneous neuronal differentiation of murine embryonic neural stem cells (Tamm et al., 2006). Fujimura & Usuki (2015) also showed that neural progenitor cell proliferation was suppressed 48 h after exposure to 10 nM MeHg, but cell death was not observed. Tamm et al. (2008) identified Notch signaling as a target for methylmercury's inhibition of neuronal differentiation at exposure levels between 2.5 and 10 nM. Bose et al. (2012) exposed E15 primary cultures of rat embryonic cortical neural stem cells to 2.5nM and 5 nM MeHg for 48 h and reported reduced cell proliferation with no effect on the cell death rate. Our results did not show a significant reduction in proliferation, but the trend was a decrease (with large error bars) (Figure 5-4 F). The stats were based on three

biological replicates, so perhaps a larger sample size could reduce the uncertainty and give a more defined outcome.

Our new findings on the increase in premature differentiation during embryonic development at a sub-nanomolar MeHg dose might be an outcome of epigenetic changes triggered by stress sensors, such as AMP dependent kinase (AMPK). Even though it is not known whether MeHg can increase AMPK activity, HgCl2 has been shown to enhance AMPK activation in the liver of mice (Kawakami et al., 2012). Hwang et al. (2010) have shown that inhibition of AMPK subunit increases methylmercury toxicity, suggesting that AMPK dephosphorylation could be a biological response induced by the extremely low dose of MeHg. Our previous work has shown that AMPK activation can stimulate a signaling-directed epigenetic pathway, atypical protein kinase C (aPKC)-mediated S436 phosphorylation of CREB-binding protein and histone acetyltransferase, to promote the neuronal differentiation of embryonic and adult neural precursor cells (Wang et al., 2012; Fatt et al., 2015). Moreover, it has been reported that the levels of cysteine and glutathione (GSH) as well as the GSH/GSSG ratio in neural stem cells progressively decreased in association with neuronal differentiation (Trivedi et al., 2017). Since it is well known that MeHg decreases GSH, this may also be a potential mechanism for the observed effects.

This enhanced neuronal differentiation by sub-nanomolar MeHg could have significant biological consequences. The premature embryonic neurogenesis can lead to depletion of the neural precursor cell pool at the adulthood, which ultimately decreases adult neurogenesis resulting in neurological functional impairment (Figure 6-1). Juliandi et al. (2015) have shown that prenatal treatment of valproic acid in mice can enhance neurogenesis and reduce the proliferation of neural precursor cells (NPCs), leading to the depletion of the NPC pool.

This depletion may cause a slower differentiation of the residual NPCs throughout lifetime. In contrast, Gallaher et al. (2013) showed that a maternal IL-6 surge aberrantly affected embryonic precursors, ultimately causing an expanded adult forebrain NPC pool and enhanced olfactory neurogenesis in offspring, months after fetal exposure. The possibility that different doses of prenatal exposure to MeHg can have different effects on the adult NPC pool and its associated neurological effects is an interesting avenue to be explored in the future.

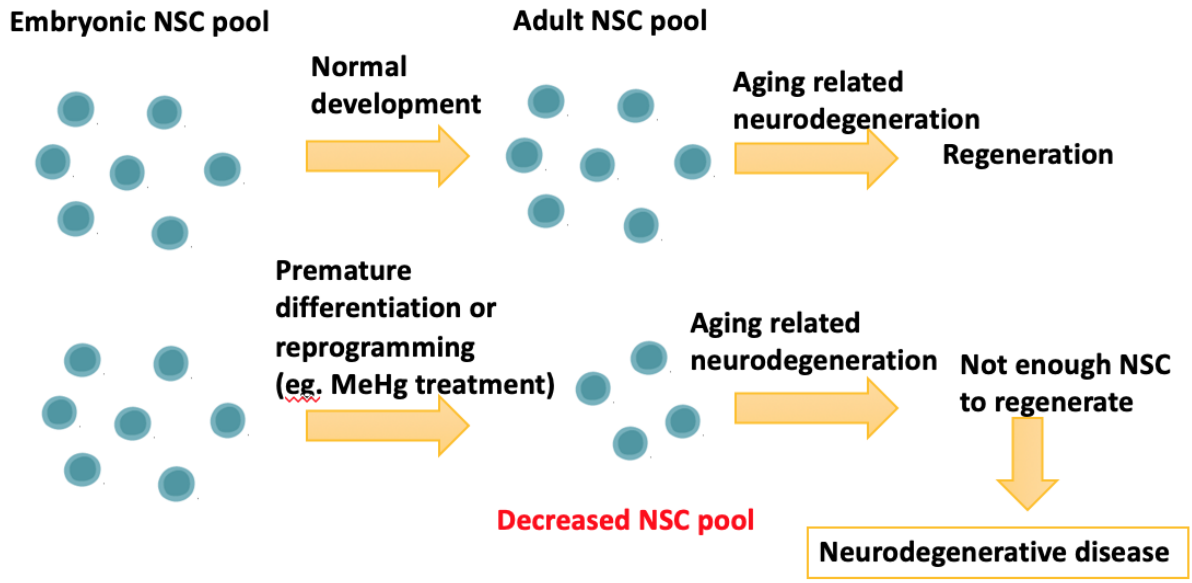


Figure 6-1. Adult neural stem cell pool model. The embryonic NSC pool that persists into adulthood is responsible for the regeneration of aging related neurodegeneration. Premature differentiation or reprogramming reduces adult NSC pool, leaving insufficient amount of NSC for the regeneration that is crucial for aging related neurodegeneration, ultimately contributing to neurodegenerative disease and neurological disorders.

While the doses used in this in-vitro experiment cannot be extrapolated to MeHg doses during human fetal development, the different effects observed between the sub-nanomolar

range and the nanomolar range has biological significance. An analysis of autopsied brain tissue from infants prenatally exposed to methylmercury showed that the mercury levels detected were approximately 129.6nM to 1.5 μM (Lapham et al., 1995). Sakamoto et al. (2018) measured total Hg concentrations in the cord blood of 54 healthy Japanese pregnant women, with no particular exposure to any Hg compounds at Fukuda Hospital (Kumamoto City, Kumamoto, Japan) from 2006 to 2007, and reported a mean Hg concentration of approximately 36.2 nM . Proteomic study conducted by Vendrell et al. (2010) showed a significant increase of non-phosphorylated cofilin, an important protein that regulates actin dynamics, with 60 nM MeHg exposure for 10 days in primary culture of mice cerebellar granule neurons. Neurobehavioral effects of *in utero* exposure to MeHg in monkeys show impaired temporal discrimination in offspring with as low as 997 nM of MeHg (Rice, 1992). This means that the nanomolar range of exposure is environmentally relevant.

Based on our results, we propose a novel model for low-dose MeHg exposure to neural precursor cells, as shown in Figure 6-2. Extremely low-dose MeHg exposure may also have a detrimental effect on embryonic neural development, which may lead to neurodevelopmental disorders or neurodegeneration later in life.

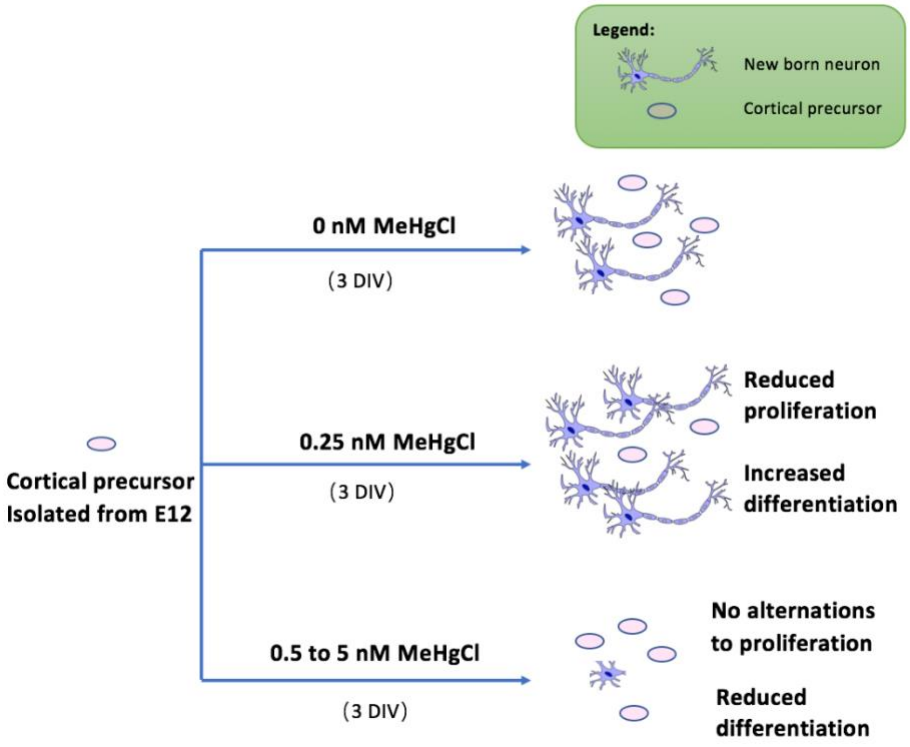


Figure 6-2. Proposed model for low-dose MeHg exposure to NSCs. Under normal circumstances, cortical precursor cells undergo both differentiation and proliferation. Upon administration of 0.25 nM MeHgCl, the cell population show reduced proliferation and increased differentiation. Cell population dosed with 0.5 nM to 5 nM MeHgCl show inhibited differentiation and gradual recovery of proliferation.

6.2 Nanomolar methylmercury exposure reprograms neural precursors, changing their cellular identity.

Our study also explored the possibility of MeHg toxicity altering neural stem cell fate, as we tried to identify a subpopulation of cells that were neither neural precursors nor neurons upon mercury treatment. One of the pluripotent stem cell genes, Klf4, was significant upregulated upon 5nM mercury exposure, suggesting that some degree of cellular reprogramming may likely take place and the cortical precursors dedifferentiated into a more primitive stage. Corresponding to the altered genes expression, I also found that the morphology of the cultured CPs were changed to tiny, round dotted cells that were alive. Nanog, Sox2 and Oct4 expression showed a trend to increase, but had not yet reach a significance.

Huangfu et al. (2008) reported that valproic acid, a histone deacetylase inhibitor, enables reprogramming of primary human fibroblasts with only two factors, Oct4 and Sox2. The two factor-induced human induced embryonic stem (iPS) cells resemble human ES cells in pluripotency, global gene expression profiles and epigenetic states. Their results support the possibility of reprogramming through purely chemical means. Hou et al. (2013) generated all chemical generation of mouse iPSCs from mouse embryonic fibroblasts (MEFs) using a combination of seven small-molecule compounds VC6TFZ: VPA, CHIR99021 (CHIR), 616452, Tranylcypromine, Forskolin (FSK), 2-methyl-5-hydroxytryptamine (2-Me-5HT), and D4476. The chemically induced pluripotent stem cells (CiPSCs) exhibited similar global gene expression profiles as mouse ESCs. This study provided the proof of principle that by using small molecules, whereas ectopic expression of pluripotent genes is not necessary for cell fate reprogramming. Three years later, Ye et al. (2016) reported the use of chemical cocktail “VC6TFE5”, a modified version of VC6TFZ, to generate CiPSCs from mouse neural stem cells. This suggests that a similar molecular mechanism underlying the chemical

reprogramming might be conserved amongst diverse cell types. Long et al. (2015) also discovered that a commonly used biological reagent, bromodeoxyuridine (BrdU), was able to generate CiPSC from mouse fibroblasts with several chemical cocktails, and the minimal combination was BrdU, CHIR99021, Repsox, and Forskolin. The CiPSCs generated resemble ESCs in terms of their gene expression, epigenetic status, in vivo definition and chimera generation. The mechanisms underlying chemical reprogramming are largely elusive. Whether low level of MeHg is able to trigger similar pathways with the chemical cocktails used for generating CiPSCs would be an interesting direction for future research.

What we did not observe was the change in Sox2 expression, which was characterized by a dramatic decrease at protein level during our previous study. This may involve a mechanism by which the regulation is at protein level, instead of mRNA level. Further experiment investigating mechanisms such as protein trafficking could be done to elucidate the phenomenon. Not all pluripotent stem cell genes were upregulated, which means the cells were most likely not yet been reprogrammed back to pluripotent stem cell; there is also a stage in-between pluripotent stem cell and definitive neural stem cell, which is the primitive neural stem cell. Hitoshi et al. (2004) investigated the characteristics of the primitive neural stem cell and found that these cells possess self-renewal capacity and neural multipotentiality, which are cardinal features of the neural stem cell. However, it also retains some nonneural properties, suggesting that they are likely in a transient phase. The research done by Reeve et al. (2016) studied targeted activation of primitive neural stem cells (pNSCS) in the mouse brain. pNSCs are the earliest NSCs to appear in the developing forebrain. They persist into the adult forebrain where they can generate all cells in the neural lineage and therefore hold great potential for brain regeneration. The researchers discovered that the pNSCs can be activated by targeting the cell surface proteins C-Kit and ErbB2.

Methylmercury may also activate similar pathways that push the cortical precursors back to a more primitive stage, of which the exact mechanism require further investigation.

7 CONCLUSION

Our study investigated the effects of nanomolar and sub-nanomolar levels of methylmercury exposure on mouse cortical precursor development. We found that 1) sub-nanomolar methylmercury exposure promotes premature differentiation of murine embryonic neural precursor at the expense of their proliferation; 2) nanomolar methylmercury exposure reprograms neural precursor cell and changes their cellular identity. Since normal development of the central nervous system requires cell proliferation and differentiation to occur in a well-coordinated manner, any subtle changes during developmental stage could have huge impact later on in life. Our research significantly contributes to the understanding of methylmercury neurotoxicity at the neural stem cell level, and gives implications of how the influence during developmental stage could progress into adulthood and affect our health. The strength of our research is that we used the ultra-low and environmentally relevant dose to explore MeHg toxicity in relation to neural stem cell fate, instead of examining the conventional cytotoxicity. The study contains both qualitative and quantitative approaches that were consistent. Limitations include small sample size (n=3 or 4) for statistical measurement, lack of true mechanistic study and time constraints. Improvements could be done for study design and time management. Future direction would be focused on identifying the exact molecular pathways that are responsible for the changes observed, and that how these changes to neural stem cells actually affects brain development later in the life using *in vivo* models.

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Appendix

Appendix A

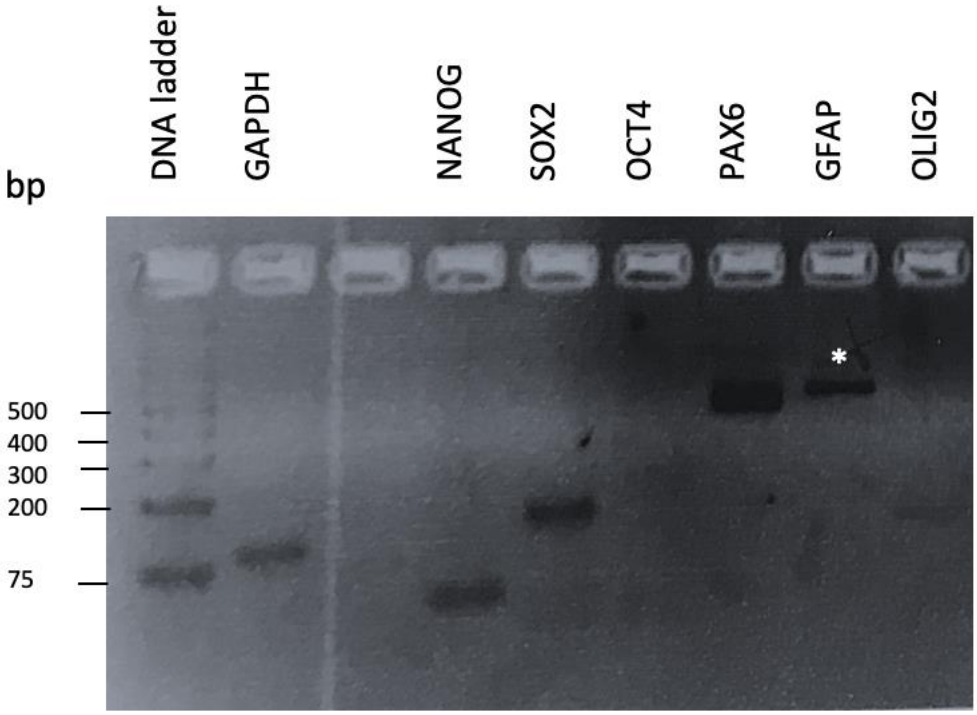

Figure A1. Agarose gel electrophoresis (3% agarose) of PCR amplified products using untreated cortical precursor cDNA. GeneRuler 1kb plus DNA Ladder was used.
*: Non-specific band.

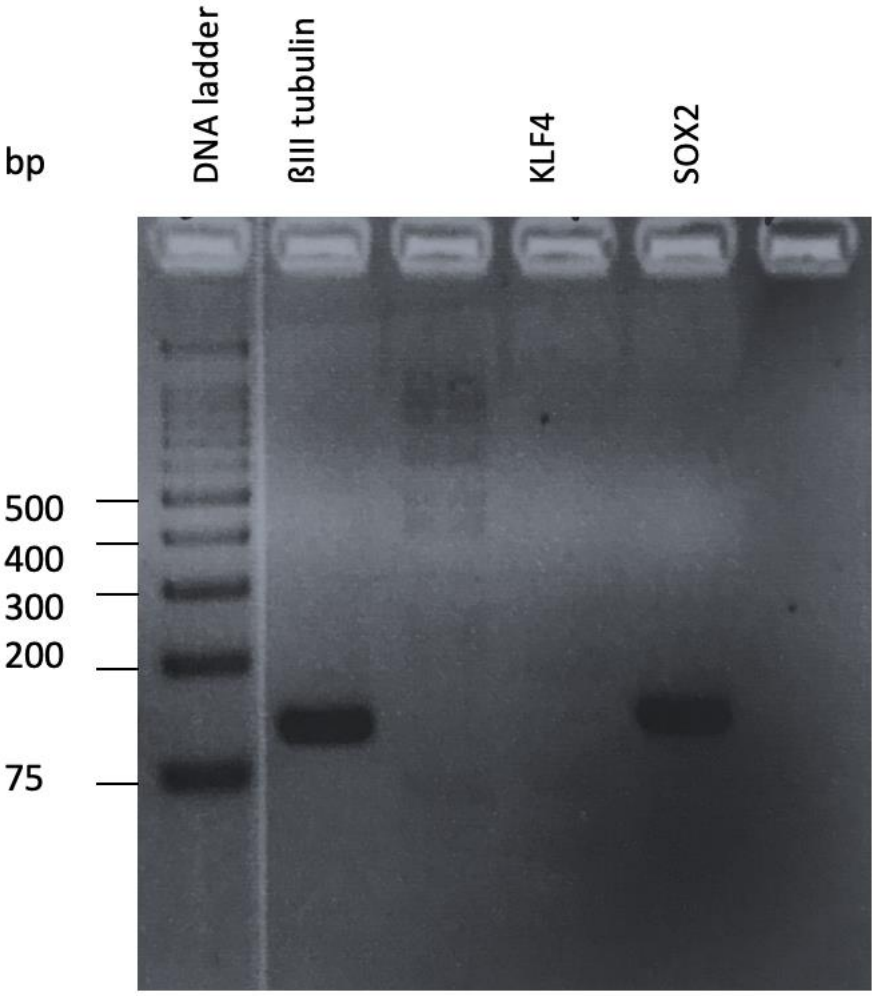

Figure A2. Agarose gel electrophoresis (3% agarose) of PCR amplified products using untreated cortical precursor cDNA. GeneRuler 1kb plus DNA Ladder was used.

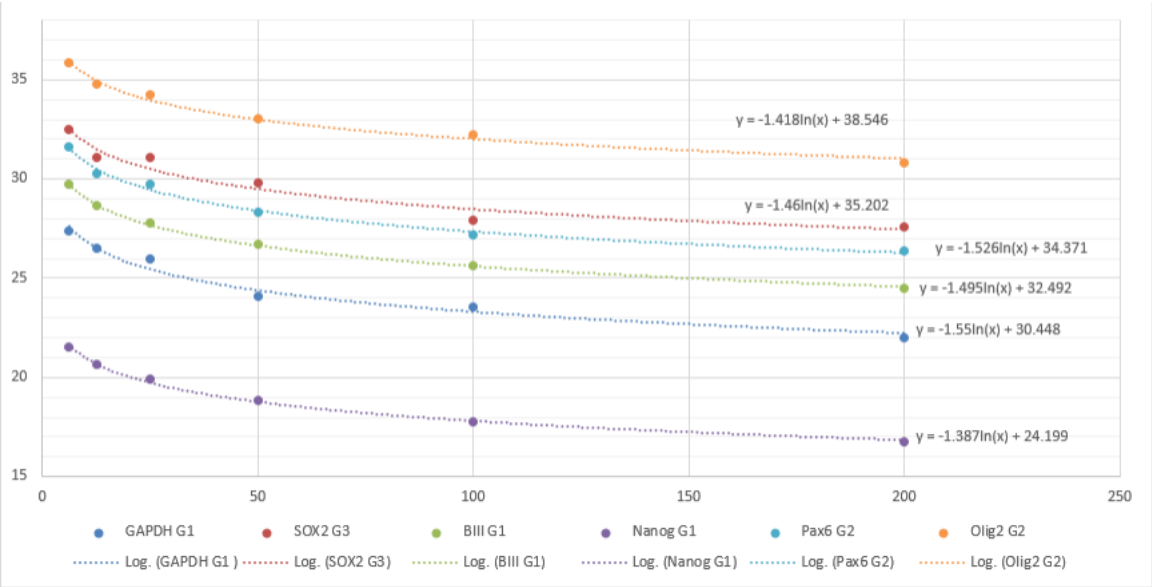

Figure A3. Standard curve of SYBR green-based RT qPCR amplification for six selected genes. The two-fold serial dilutions are close to linear over six logs. Efficiency (E) = $10^{-1/\text{slope}}$.