ALTERATIONS IN PROLIFERATION AND DIFFERENTIATION OF NEURAL STEM CELLS INDUCED BY ADVERSE NEURODEVELOPMENTAL FACTORS

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Stockholm 2014
To my family
Predisposition to diseases can be acquired during early stages of development and resolve into an actual disorder later in life. Early life programming defines the association between challenges during pregnancy that result in altered fetal growth, and developmental and adult disorders. This thesis aimed at studying the effects on neurogenesis triggered by a deranged milieu induced by neurodevelopmental insults, namely excess glucocorticoids (GC) or methylmercury (MeHg). We investigated the effects of exposure to the synthetic GC analog dexamethasone (Dex) on proliferation and differentiation of human progenitor cells (hNPC) grown as neurospheres. We found that Dex decreases hNPC proliferation and differentiation by up-regulating DKK1, a known inhibitor of the canonical Wnt signaling, via a glucocorticoid receptor (GR)-mediated mechanism. We then focused on the effects of Dex in rat cortical neural stem cells (NSCs) and found that exposed cells exhibited a decreased proliferation, increased expression of senescence markers, a down-regulation of mitochondrial genes and global DNA hypomethylation associated with a down-regulation of DNA methyltransferases (Dnmt) 1 and 3a. These effects were heritable, being present also in “daughter” NSCs never directly exposed to Dex. Global DNA hypomethylation was also found in the cortex of 3 day-old mouse pups that were exposed to Dex in utero.

We used the same experimental design to investigate the effects of the environmental contaminant MeHg (at nanomolar concentrations) on NSCs. MeHg had no effect on cell viability, but reduced the proliferation rate and, similarly to Dex, induced a senescence phenotype associated with down-regulation of mitochondrial genes and global DNA hypomethylation. These changes were also detected in “daughter” NSCs that were never directly exposed to MeHg. Long-lasting effects on NSCs proliferation were also observed in the hippocampal subgranular zone of adult mice exposed to low doses of MeHg during development. The reduced proliferation had a measurable impact on the total number of neurons in the hippocampal dentate gyrus and it could be reversed by treatment with the antidepressant fluoxetine. We further studied the programming effects of GC in Dex-exposed NSCs by genome-wide analysis of differentially methylated DNA regions (DMRs). DMRs occurred in the promoter regions of 575 genes as compared to 1479 in control cells. We selected genes identified as DMR-enriched and found that Dkk1, Dkk3, Txnip and Cyba were up-regulated in Dex-exposed proliferating NSCs, and that the changes persisted in daughter cells. We found that the Dex-induced DNA hypomethylation was associated with an up-regulation of Tet1-3 factors and a down-regulation of Dnmt3a in both NSCs and postnatal mouse cortex. In Dex-exposed NSCs the expression of Dkk1 was up-regulated by promoter demethylation in a Tet3-dependent fashion. These effects were also heritable. The Dex-mediated down-regulation of Dnmt3a was also dependent on Tet3 expression. In conclusion, our studies show that epigenetic modifications play a critical role in the reprogramming effects exerted by neurodevelopmental insults, such as exposure to excess GC or MeHg.
LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their Roman numbers:


* Equal contribution
ADDITIONAL RELEVANT PUBLICATIONS


- Spulber S, Onishchenko N, Bose R, Ceccatelli S. Behavioural and molecular changes induced by prenatal exposure to dexamethasone in mice. Manuscript
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<tr>
<td>11β-HSD2</td>
<td>11 beta hydroxysteroid dehydrogenase type 2</td>
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<tr>
<td>BMI1</td>
<td>Polycomb ring finger oncogene</td>
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<tr>
<td>Carboxy-H2DCFDA</td>
<td>5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate</td>
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<td>Cyt b</td>
<td>Cytochrome b</td>
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<td>D</td>
<td>Daughter NSCs</td>
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<td>Dex</td>
<td>Dexamethasone</td>
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<td>Dkk</td>
<td>Dickkopf</td>
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<td>DMNQ</td>
<td>2,3-dimethoxy-1,4-naphthoquinone</td>
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<td>DMR</td>
<td>Differentially methylated DNA regions</td>
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<td>DNMTs</td>
<td>DNA methyltransferases</td>
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<td>EdU</td>
<td>5-ethynyl-2´-deoxyuridine</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>GC</td>
<td>Glucocorticoids</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<td>GC responsive elements</td>
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<td>HMGA1</td>
<td>High Mobility Group A1</td>
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<td>hNPC</td>
<td>human neural stem/progenitor cells</td>
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<td>HP1γ</td>
<td>Heterochromatin protein 1 gamma</td>
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<td>MeHg</td>
<td>Methylmercury</td>
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<td>MR</td>
<td>Mineralocorticoid receptor</td>
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<td>ND3</td>
<td>NADH dehydrogenase 3</td>
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<td>NSCs</td>
<td>Neural stem cells</td>
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<td>OD</td>
<td>Optical density</td>
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<td>P1</td>
<td>Parent NSCs</td>
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<td>PF</td>
<td>Paraformaldehyde</td>
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<td>q-PCR</td>
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<td>Reactive oxygen species</td>
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<td>Senescence-associated heterochromatin foci</td>
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<td>siRNA</td>
<td>Short interfering RNA</td>
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<td>SGL</td>
<td>Subgranular layer</td>
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<td>SVZ</td>
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1 INTRODUCTION

The development of the brain is a strictly regulated sequence of events, and any interference with the regulatory pathways may result in long term alterations. Predisposition to diseases can be acquired during early stages of development and can result in an actual disorder later in life (Barker, 1995), as more recently formulated in the FeBAD theory (fetal basis of adult disease) (Barlow et al., 2007). The concept of early life programming associates challenges during pregnancy and altered fetal growth with developmental and adult disorders (Seckl, 1998). Low birth weight increases the risk of cardiovascular, metabolic, neuroendocrine and cognitive disorders in adulthood (Dobbing, 1993; Meaney et al., 2007; Räikkönen et al., 2008; Seckl and Meaney, 2006; Thompson, 2001; Wiles et al., 2005). Adverse developmental conditions may result in structural and functional changes in the brain that will translate into behavioral alterations. Sensory or social stimuli, maternal care, stress, but also exposure to drug treatments or toxic compounds, may change the expression of key genes involved in neural development and plasticity (Ceccatelli et al., 2013; Lemaire et al., 2000; Weinstock, 2008). This thesis is focused on the mechanisms behind the adverse neurodevelopmental effects exerted by exposure to excess glucocorticoids (GC) or to methylmercury (MeHg).

1.1.1 Glucocorticoids

GC are lipophilic steroid hormones secreted by the adrenal gland that are critical for development and adaptation to stress. In humans, the endogenous production starts around mid-gestation (week 18), and peaks about 6 weeks before birth to induce the terminal maturation of the lungs and prepare the fetus for the extrauterine life (Mastorakos and Ilias, 2003). In the nervous system, GC are essential for initiating terminal maturation, remodeling of axons and dendrites, and for cell survival (reviewed in (Owen et al., 2005)). However, they can have adverse effects on the nervous system if secreted in excess (Fukumoto et al., 2009; Gould et al., 1998; Matthews et al., 2004; Sapolsky, 1999; Weinstock, 2008; Wong and Herbert, 2005).

GC bind to intracellular receptors, namely glucocorticoid receptors (GR), and mineralocorticoid receptors (MR). GR are expressed from early embryonic stages in most fetal tissues, including the placenta. In contrast, MR are detected only in the later stages of development (Brown et al., 1996; Diaz et al., 1998). Both the GR and the MR are transcription factors that regulate the expression of many other genes and signaling pathways including Wnt signaling (Matrisciano et al., 2011). Edwards et al. (1993) demonstrated that fetal GC levels are much lower (~10-fold) than maternal levels due to the placental 11βHSD2 that converts the active GC (cortisol and corticosterone) into their inactive metabolites. However, a small proportion (10-20%) of maternal glucocorticoids can cross the placenta (Benediktsson et al., 1997). A high amount of GC can reach the developing fetus in cases of maternal infection and inflammation, hypoxia, severe maternal stress, genetic deficiency of 11βHSD2 (Cottrell and Seckl, 2009; Seckl, 2004), or because of exogenous administration of GC analogs (Seckl and Holmes, 2007). Recent evidence shows that high level of maternal GC (measured in blood, saliva or urine) can slow fetal growth (Reynolds, 2013). In addition, human and
animal studies reported that low birth weight is associated with alterations in the HPA axis activity after birth (Davis et al., 2012; Reynolds, 2013; Smith et al., 2011), which might be explained by a persistently impaired central negative feedback sensitivity (Davis et al., 2012). Importantly, it appears that alterations in GC signaling and its regulation are responsible for the reprogramming effects of \textit{in utero} growth retardation (Wyrwoll and Holmes, 2012).

\subsection{1.1.2 Methylmercury}

Methylmercury (MeHg) is one of the most dangerous environmental pollutants (Gilbert and Grant-Webster, 1995), as shown by the devastating consequences of the industrial pollution that occurred in the late 1950’s in Minamata and Niigata regions in Japan (Harada, 1995). 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, mercury can travel over large distances. Inorganic mercury is converted to MeHg by sulphate-reducing bacteria in water sediments. MeHg enters into the aquatic food chain, which is the most common way of mercury exposure in humans, due to the accumulation of MeHg in fish. Upon consumption of contaminated food, MeHg is almost completely absorbed in the human gastrointestinal tract. It rapidly binds to hemoglobin in the bloodstream, where it forms complexes with free cysteine or with cysteine residues in peptides and proteins, and has a half-life of about 50 days. The complexes are recognized by amino acid transporters because of molecular mimicry of methionine, and are readily transported across biological barriers, such as the placenta and the blood-brain barrier (Aschner and Aschner, 2007).

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 (Harada, 1995), and the resulting behavioral deficits are persistent (Grandjean, 2007). Experimental studies have shown that prenatal exposure in 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, the exposure to MeHg during development impairs the acquisition of fine motor control, language, and learning abilities in children and adolescents (Debes et al., 2006). A recent 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). The raise in public awareness will hopefully prevent the occurrence of environmental disasters involving massive and prolonged exposure to MeHg. However, it became apparent that dietary exposure to doses previously considered “safe” can induce subtle behavioral and cognitive deficits (Grandjean et al., 1998).
1.1.3 Neural stem cells

Over the past ten years, our group has introduced the use of neural stem cells (NSCs) as an in vitro model for the investigation of the mechanisms of neurodevelopmental toxicity (Ceccatelli et al., 2013; Tamm et al., 2006).

NSCs are multipotent cells located in the central nervous system (CNS). They are able to self-renew and proliferate to produce more restricted undifferentiating cells - neural progenitor cells. The latter have limited capacity to proliferate and eventually undergo terminal differentiation into neurons, astrocytes and oligodendrocytes (Figure 1-1). NSCs are identified in all embryonic brain regions. NSC derived cells have a specific spatial organization during well-defined periods of development to enable the establishment of neuronal networks (Temple, 2001). Therefore, tightly regulated NSC proliferation and differentiation processes are fundamental for the establishment of brain architecture.

During development, NSC population expands by symmetric division. The mitotic cell division is regulated by cell cycle machinery which employ different cyclin-dependent kinases (CDKs) and their inhibitors, including Cdkn1a (p21) and Cdkn2a (p16). These intrinsic mechanisms maintain proper timing of the basic cycle at each check point by the activation or inactivation of the CDK proteins. Interestingly, it has been demonstrated recently that Cdkn1a regulates NSC proliferation also in the adult brain (Marqués-Torrejón et al., 2013). In addition to the cell cycle machinery, the proliferation of NSCs is also regulated by growth factors depending on the stage of stem cell development (Sommer and Rao, 2002). At early stages of development, basic FGF (bFGF) is essential for proliferation. At later stages, either bFGF or epidermal growth factor (EGF) can maintain the proliferation of NSCs (Tropepe et al., 1999). More restricted progenitor cells, such as glial progenitors, require platelet-derived growth factor (PDGF) and FGF for mitotic division, while neuronal progenitors require sonic hedgehog (Shh), FGF, and neurotrophin-3 (NT-3) (reviewed in (Sommer and Rao, 2002)).

The more restricted neural progenitor cells are generated by asymmetric division of NSCs. They proliferate, migrate, and further differentiate into neurons and glial cells within distinct regions of the developing and adult brain (Kwan et al., 2012; Steiner et al., 2004; Violeta et al., 2013). Part of the NSC population in the telencephalon also migrates to contact with the ventricular surface and change shape to become radial glia. The radial glia differentiate and give rise to neurons and astrocytes (Götz and Barde, 2005; Noctor et al., 2001). The mechanism of asymmetric division and subsequent differentiation of NSCs is closely related to cell cycle regulating systems (Ohnuma and Harris, 2003). Bone morphogenetic protein (BMP) and retinoic acid can act as specific inhibitors of proliferation by regulating the levels of CDK inhibitors, such as p16 and p21. The neurotrophic factors, in particular brain-derived neurotrophic factor (BDNF), expressed during early development may also play an important role in NSC proliferation and differentiation (Chen et al., 2013). Last but not least, the epigenetic control of gene expression plays a crucial role in the regulation of NSC proliferation and differentiation.
In the adult brain, only two regions preserve limited pools of NSCs throughout life: the subventricular zone (SVZ) of the lateral ventricle, and the subgranular layer (SGL) of the dentate gyrus. Specifically, NSC proliferation in the SVZ generates neuroblasts which migrate through the rostral migratory stream and further differentiate into olfactory bulb neurons. NSCs in the SGL continue to generate granule cells that migrate and integrate functionally into the granular layer of the dentate gyrus, thereby supporting the plasticity of the hippocampal formation, a feature that is crucial for learning and memory. While NSCs can be derived from either developing, or adult mammalian brains, the dynamic properties of NSCs are somewhat different. For example, adult NSCs divide slowly and generate a more restricted cell population than embryonic NSCs. While the function of neurogenesis in the olfactory bulb is apparently different than that of hippocampal neurogenesis, the net effect of neurogenesis is the maintenance of plasticity in brain regions that are essential for survival and continuous adaptation to the environment (Imayoshi et al., 2008).

Figure 1-1. The differentiation of neural stem cells isolated from the developing forebrain depends on growth factors. FGF is essential for maintaining the stem cell population.

In this thesis, we used primary culture of human neural progenitor cells (hNPC) from gestational week 16 to 19, and rat embryo telencephalic NSCs isolated at embryonal day (E) 15. In rodents, the telencephalon starts to form around E10.5. Neurogenesis starts on E12, and is followed by gliogenesis starting from E16 (astrocytes), and continues postnatally (oligodendrocytes). In our model systems, hNPCs can proliferate in a defined media with EGF and bFGF, while rat NSC are grown in presence of bFGF alone. Both hNPCs and rat NSCs can differentiate into neurons, astrocytes and oligodendrocytes following either spontaneous or forced differentiation. The
spontaneous differentiation is induced by simply omitting the addition of growth factors (bFGF and EGF) (Moors et al., 2012; Tamm et al., 2008). The forced differentiation is initiated by cell type specific growth factors, such as BDNF, CNTF and T3 for neurons, astrocytes and oligodendrocytes respectively (Gross et al., 1996; Johe et al., 1996).

1.1.3.1 Wnt signaling

A range of signaling pathways are involved in the patterning and the development of different brain regions. The Wnt signaling pathway plays an important role in the development of the CNS, from neural tube formation, to segmentation and development of the forebrain, midbrain, and cerebellum (Hari et al., 2002; McMahon and Bradley, 1990; Mukhopadhyay et al., 2001; Zechner et al., 2003). Defects in Wnt signaling are associated with neurodevelopmental disorders including schizophrenia, mood disorders, and autism (De Ferrari and Moon, 2006; Proitsi et al., 2008). At the cellular level, Wnt signaling is involved in NSC maintenance, proliferation, and fate determination, as well as in axon guidance, dendrite development, and synapse formation (reviewed in (Michaelidis and Lie, 2008)).

Nineteen members of the Wnt family have been identified to date, and are classified based on their action in the signaling pathways as Wnt1 class (Wnt1, 2, 3, 3a, 8, 8a, etc.) and Wnt5a class (Wnt4, 5a, 5b, 6, 7a, 11, etc.). The Wnt1 class activates the canonical signaling pathway (i.e. β-catenin dependent) (Clevers, 2006) and the Wnt5a class activates two non-canonical signaling pathways (i.e. β-catenin independent) (Klein and Mlodzik, 2005; Kohn and Moon, 2005). It has been reported that only the canonical signaling pathway is involved in the regulation of neural development (Daneman et al., 2009; Michaelidis and Lie, 2008). Therefore, we will further discuss only the canonical pathway (Figure 1-2).

Wnt signaling is initiated by Wnt binding to frizzled receptors and recruiting the LRP5/6 co-receptor to phosphorylate and activate three domains of Dishevelled (Dvl) and Axin, respectively. The activated Dvl and Axin inactivate GSK-3β and remove it from the protein complex that continuously binds, and targets β-catenin for degradation. Thus, β-catenin is released and translocates to the nucleus, where it binds to the lymphoid enhancer factor (Lef)/T Cell Factor (Tcf) to form an active transcription complex which leads to target gene transcription. In the absence of Wnt, the scaffolding protein complex of adenomatous polyposis coli (APC), axin (Ikeda et al., 1998; Yamamoto, 1999) and the serine/threonine kinase GSK (glycogen synthase kinase)-3β binds free cytoplasmic β-catenin for phosphorylation. The phosphorylated β-catenin then binds to β-TrCP (β-transducin repeat-containing protein) for ubiquitination by E3 ubiquitin ligase complex, then undergoes proteasome-mediated degradation (Liebner and Plate, 2010). The canonical Wnt signaling is regulated by secreted antagonists, such as Dickkopf 1 (Dkk1), frizzled-related protein families (sFRPs) and WIF (Wnt inhibitory factor)-1 (Kawano and Kypta, 2003; MacDonald et al., 2009; Moors et al., 2012). The inactivation of Wnt signaling blocks β-catenin mediated gene transcription by targeting it for proteosomal degradation.
Figure 1-2. The canonical Wnt signaling switch. OFF mode is the default, and is also induced by Dkk1 binding to the LRP5/6 receptor to prevent the activation of the signaling cascade. ON mode is triggered by Wnt binding to frizzled receptor, followed by the recruitment of LRP5/6.

1.2 ALTERATIONS INDUCED BY TOXIC INSULTS

Unfavorable changes in the micro environment lead to intracellular modifications, including impairment of mitochondrial function, occurrence of oxidative stress and premature senescence, which can persist long after the insult (Ahlbom et al., 2000). It is conceivable that epigenetic mechanisms may have a major contribution to the whole process.

1.2.1 Mitochondrial alterations; oxidative stress; senescence

1.2.1.1 Mitochondrial dysfunction and oxidative stress

Mitochondria are double membrane-enclosed organelles that produce ATP in the electron transport chain (ETC) complexes (I-IV). When electrons leak during ATP production, they react with O$_2$ to produce the superoxide anion (O$_2^-$), which can further produce other ROS (H$_2$O$_2$, and OH) via enzymatic or non-enzymatic reactions (Zhang and Gutterman, 2007). The majority of superoxide anions are reduced to water.
by different antioxidant defense mechanisms. Antioxidants, such as glutathione, glutathione peroxidase, superoxide dismutases, NADP dehydrogenase, and Vitamins E and C, are mostly present in the mitochondria (McGowan et al., 1995). However, 1-4% of oxygen is converted to ROS and low level of ROS are required for physiological signaling during development (Sena and Chandel, 2012). The small G protein Ras is activated by ROS (Pennisi, 1997), and various transcription factors, such as NFkB, p53 and AP-1, have been shown to be modulated by ROS (Morel and Barouki, 1999).

While complex I and III of the mitochondrial respiratory chain are the major source of superoxide, the deficiency of antioxidant capacity and mitochondrial respiratory chain defects result in high levels of ROS which can damage mitochondrial DNA (Chen et al., 2003; Poyton et al., 2009). Thereby dysfunctional mitochondria may further stimulate the abnormal production of ROS (Sastre et al., 2003)

**Oxidative stress** is defined as an imbalance between the generation of ROS and the antioxidant defense mechanisms. Oxidative stress causes oxidative damage to proteins, lipids and DNA in cells, and induces apoptosis, necrosis or cellular senescence, depending on the levels of ROS production. ROS can break single-strand DNA and activate poly(ADP-ribose)-polymerase (PARP) (Schraufstatter et al., 1986). Mitochondrial dysfunction and oxidative stress-induced by radiations and toxic chemicals may play an important role in the pathogenesis of numerous diseases including neurodegenerative disorders. ROS formation is increased in Parkinson’s disease and Alzheimer’s disease due to impaired activity of complex I and complex IV, respectively (Chagnon et al., 1995; Schapira, 1998). The accumulation of oxidative damage and the decrease in mitochondrial ATP production are two underlying concepts of the mitochondrial hypothesis of aging (Navarro and Boveris, 2007).

1.2.1.2 Senescence

Leonard Hayflick and Paul Moorhead (1961) were the first to describe that normal (diploid) human fibroblast cultures stop proliferating after a certain number of passages (50-55). This phenomenon was called cellular senescence, more specifically replicative senescence; it is due to growth arrest at G1/S-phase of the cell cycle and is dependent on telomerase activity and telomere length: when the telomere length drops below a minimum length, they lose the function to protect the DNA from damage (d’Adda di Fagagna et al., 2003; Harley et al., 1990). Senescence can also be induced by a variety of environmental extrinsic factors (premature senescence) including ultraviolet light (Scharffetter-Kochanek et al., 1997), oxidative stress, chemotherapy and radiotherapy (Lee et al., 1999; Di Leonardo et al., 1994; Robles and Adami, 1998) in different types of stem cells (Burova et al., 2013; Kim et al., 2011). Upon undergoing senescence, the cells become large, flat and multinucleated: a cellular morphology that differs from proliferating, quiescent, or terminally differentiated cells (Figure 1-3). Both replicative and premature senescence are reversible and are linked to age-associated tissue dysfunction, reduced regenerative capacity, and neurodegenerative diseases (Beauséjour et al., 2003; Naylor et al., 2013). They also share a number of common features including morphological changes, DNA damage, oxidative stress, and activation of specific signaling pathways (p53/p21 and pRb/p16). The mechanism of DNA damage differs between replicative and premature senescence: while replicative
senescence is induced by telomere attrition and subsequent DNA damage (d’Adda di Fagagna et al., 2003), the DNA damage in premature senescence is independent from telomeres and telomerase activity (Di Leonardo et al., 1994; Robles and Adami, 1998).

**Figure 1-3.** Premature senescence is induced by various insults that can cause DNA damage, oxidative stress, and up-regulation of p16 and p21. Stem cells receiving senescence signals stop proliferating and start displaying the senescence-associated secretory phenotype (SASP).

DNA damage- or oxidative stress-induced premature senescence often results in a flat cell morphology (Chen et al., 2001; Parrinello et al., 2003). A recent report demonstrated that ROS-mediated senescence is linked with DNA damage and induction of p21 expression (Passos et al., 2010). In addition, oxidative stress activates either directly or indirectly p53/p21 and/or pRb/p16 pathway (Itahana et al., 2003; Iwasa et al., 2003). The cell cycle arrest due to activation of p53/p21 and/or pRb/p16 pathways is commonly found in all types of senescent cells (Brandl et al., 2011; Chen and Ames, 1994; Dumout et al., 2000; Pascal et al., 2005). Another widely used marker is SA-β-GAL, derived from lysosomal β-D-galactosidase, which is typically
increased in senescent cells (Dimri et al., 1995). Campisi (2005) reported that secreted factors like cytokines and chemokines could also induce cellular senescence, and increased secretion of a range of chemokines, cytokines and proteases form the senescence-associated secretory phenotype (SASP). Finally, epigenetic markers can also be used as senescence markers. Recent reports demonstrate that global DNA hypomethylation is associated with senescence in primary culture of human embryonic lung fibroblasts (Zhang et al., 2008a), and inhibition of DNMTs induces senescence of human umbilical cord blood-derived stem cells (hUCB-SCs) (Kang, 2011).

Experimental evidence indicates that premature senescence is reversible. Primary cells are sensitive to oxygen levels in the culture medium. Therefore, premature senescence can be prevented by lowering the oxygen levels in the cell and tissue culture conditions (Parrinello et al., 2003). Moreover, cells treated with antioxidants or free radical scavengers may also delay the appearance of senescent phenotypes (Chen et al., 1995). The recovery from senescence has also been implemented through various signaling pathways. For example, inactivation of p53 and low expression p16 can increase proliferation of senescent cells (Beauséjour et al., 2003). A recent report demonstrated that genetic inactivation of p16<sup> Ink4a</sup> in BubR1 progeroid mice delayed aging by preventing the formation of p16<sup> Ink4a</sup>-positive senescent cells (Baker et al., 2008). However, deletion of p16<sup> Ink4a</sup> in humans can be impracticable because it may result in cancer.

### 1.3 EPIGENOME AND EPIGENETICS

The DNA code is fixed for life and instructs to build all the parts of the body. DNA is wrapped around scaffold proteins called histones to form the chromatin. Both DNA and histones bear chemical tags. DNA methylation and histone modifications (acetylation and methylation) are major epigenetic mechanisms that regulate gene expression and function. The epigenome is defined as a layer of structural modifications that do not alter the DNA sequence, but shapes the physical structure of the chromatin and thereby modulates the transcription of DNA into RNA. The epigenome differs between cell types, although the genome is the same throughout the organism. Such chromatin modifications, and the ensuing alterations in gene function that do not involve alterations in the DNA sequence, are heritable across mitosis and meiosis, and make the object of epigenetics (reviewed in (Feil and Fraga, 2011)).

#### 1.3.1 DNA methylation

DNA methylation is one of the most important epigenetic marks, and plays a critical role in development (Bestor and Coxon, 1993; Bird, 2002; Cedar and Bergman, 2009; Suzuki and Bird, 2008). It is catalysed by DNA methyltransferases (Dnmt1,-3a and -3b), which transfer a methyl group from S-adenyl-methionine (SAM) to the fifth carbon of a cytosine residue to form 5-methyl cytosine (5mC). The catalytic activity of Dnmts can be categorized as maintenance, or de novo methylation. The Dnmts have a
similar structure, with an N-terminal regulatory domain and a C-terminal catalytic
domain, but they have different expression patterns and functions (Xie et al., 1999; Yen
et al., 1992). Dnmt1 is expressed in mammalian tissues including the brain (Goto et al.,
1994), and its activity has been described as the major maintenance methyltransferase
(i.e. it rather methylates hemimethylated CpG sites than non-methylated CpG)
(Pradhan, 1999; Ramsahoye et al., 2000). It copies methylation patterns from parental
DNA to the next generation DNA strand and repairs DNA methylation (Mortusewicz et
al., 2005). Knocking out Dnmt1 in mice is lethal at embryonic stages (Li et al., 1992)
due to massive loss of DNA methylation. Interestingly, Dnmt1 deficient mouse
embryonic stem cells are viable (Chen et al., 1998) in vitro, but the differentiation
results in massive cell death. These findings clearly indicate that Dnmt1 plays a critical
role in cellular differentiation as well as in dividing cells. Dnmt3a and Dnmt3b are
known as de novo methylation enzymes, i.e. they methylate unmodified CpG
dinucleotides and establish a new methylation pattern in the mammalian genome
(Figure 1-4) in addition to methylating hemimethylated DNA as Dnmt1 does (Jones
and Liang, 2010; Okano et al., 1999). Dnmt3a is mostly expressed in differentiated
tissues, and knockout mice are viable up to 4 weeks after birth (Okano et al., 1999).
Dnmt3b is poorly expressed by most differentiated tissues, and knockout causes
embryonic lethality in mice (Okano et al., 1999; Xie et al., 1999).

Figure 1-4. Biochemical mechanism of DNA methylation. SAM (S-adenosyl-L-
methionine) is the main donor of methyl groups for the methylation of cytosine in the
DNA strands. The transfer of a methyl group from SAM to cytosine is catalyzed by
Dnmts (DNA-methyl transferases).

Dnmt3a and 3b bind DNA via a conserved PWWP domain (Ge et al., 2004). The
question on how they target specific regions for de novo methylation remains open. So
far two hypotheses have been proposed: one hypothesis is based on the silencing of
DNA region specific Dnmts by RNA interference (RNAi) (Morris et al., 2004), but
there is no evidence supporting it in mammalian cells; the second hypothesis is that
transcription factors regulate de novo DNA methylation. Transcription factors can bind
DNA sequence either to methylate DNA or to protect the DNA sequence from
methylation. In case of methylation, specific transcription factors bind to DNA and
recruit Dnmts, which can bind to either those transcription factors, or repressor complexes to target DNA methylation (Brenner et al., 2005). Transcription factors can also protect DNA sequence from de novo methylation (Gebhard et al., 2010; Lienert et al., 2011; Straussman et al., 2009). Evidence shows that CpG islands are protected from methylation by transcription factor binding (Brandis et al., 1994; Lienert et al., 2011; Macleod et al., 1994; Straussman et al., 2009). Taken together, these results may suggest that transcription factors are probably key regulators of de novo DNA methylation via Dnmts.

### 1.3.2 DNA demethylation

Methylated DNA can be demethylated by removing the methyl group from 5mC. Active and passive pathways (Figure 1-5) are mainly involved in the process of demethylation (Williams et al., 2012). In the passive pathway, the methyl group of 5mC is simply removed in the daughter strand after successive rounds of DNA replication without any enzymatic modification of the DNA base, due to the functional failure of Dnmt1 (Fan et al., 2001). In the active pathway, 5mC is oxidized by ten-eleven translocation (Tet) oxygenases and the first product is 5-hydroxymethylcytosine (5hmC), which is a key intermediate in active demethylation pathways (Ito et al., 2010; Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). 5hmC can be converted to unmodified cytosine following either the active or passive pathway. First, the hydroxyl group can simply be removed from 5hmC by the passive way of DNA demethylation. Second, 5hmC can undergo further enzymatic modifications to 5faC and 5caC catalyzed by Tet enzymes, and can then be converted back to cytosine by thymine DNA glycosylase (TDG), followed by base excision repair (BER) (Kohli and Zhang, 2013; Shen et al., 2013). An additional mechanism of demethylation is by deamination of either 5mC or 5hmC, followed by BER (Branco et al., 2012; Wu and Zhang, 2011).

The Tet protein family consists of three members (Tet1, Tet2 and Tet3) that have similar structure and functions. All Tet proteins share domains for the binding of iron and oxoglutarate, and contain unique DNA binding domains (catalytic domain; cysteine-rich) and DSBH regions, which exhibits 2-oxoglutarate (2-OG)- and iron (II)-dependent dioxygenase activity. Tet1 and Tet3 contain a CXXC zinc finger in the regulatory domain (N-terminal), which is known as the CpG binding domain. Tet2 does not contain a CXXC domain, but the adjacent IDAX (inhibition of the Dvl and axin complex protein) (CXXC4) protein contains a CXXC domain which is very similar to those in Tet1 and Tet3 (Long et al., 2013). Despite structural similarity of Tet CXXC domains with several other proteins, such as Dnmt1, the function of Tet proteins is different (Frauer et al., 2011); while Dnmt1 CXXC domain binds almost exclusively to hemimethylated DNA, Tet proteins oxidise methylated and hydroxymethylated cytosine bases (Long et al., 2013; Risner et al., 2013). In addition, the distinct expression patterns of Tet enzymes indicate that they may have unique functions during development and in specific cell types (Liu et al., 2013; Long et al., 2013).
Figure 1-5. The mechanisms of DNA demethylation. 5mC is oxidized by Tet enzymes and gives rise to 5hmC which is further oxidized to 5fC and 5caC. 5mC and all its derivatives (5hmC, 5fC, and 5caC) are converted to unmodified cytosine by base excision repair (BER). 5mC can also be modified to cytosine by deaminase followed by BER.

The expression and activity of Tet proteins, and consequently the levels of 5hmC, vary between cell types and tissues. The expression of Tet1 and levels of 5hmC are high in embryonic stem cells, and gradually decrease during differentiation into embryoid bodies (Ito et al., 2011; Nestor et al., 2012; Tahiliani et al., 2009; Tollervey and Lunyak, 2012). Tet3 knockout mice are embryonic lethal (Gu et al., 2011), but knockdown of either Tet1 or Tet2 produces viable adult mice with various developmental defects (Dawlaty et al., 2013). The expression patterns of Tet2 and Tet3 in adult rodent tissues are rather similar (Ito et al., 2010), but the global 5hmC content does not correlate with the expression of Tet proteins (Nestor et al., 2012). The highest levels of 5hmC are found in the brain (around 0.80%), particularly in cortex,
hippocampus and hypothalamus (Globisch et al., 2010; Jin et al., 2011; Kriaucionis and Heintz, 2009; Münzel et al., 2010).

1.3.3 DNA methylation and demethylation in disease

Epigenetic mechanisms are involved in neural development, particularly for the cell type specification. Neuronal activity, cognition and memory formation are also regulated via epigenetic mechanisms. Therefore, a finely tuned balance between DNA methylation and demethylation is required for the appropriate development and function of the nervous system.

Dnmts have different patterns of expression in relation to the developmental stage. Dnmt1 is expressed throughout development as well as in the adult brain, while Dnmt3b is detected for a short period during neuronogenesis (from E11.5 to E14.5). Genetic deletion of Dnmt1 decreases DNA methylation in mitotic NSCs but not in postmitotic neurons (Fan et al., 2001). In addition, Dnmt1 deletion in neuronal progenitor cells decreases DNA methylation and upregulates the gliogenic JAK-STAT pathway that leads to premature astrocyte differentiation (Fan et al., 2005). Dnmt1 conditional knock-out results in DNA hypomethylation in the embryonic mouse cortex and hippocampal cells. In addition, it increases neuronal cell death between E14.5 and PND 21 and causes multiple defects in postnatal maturation of neurons (Hutnick et al., 2009).

Dnmt3a is strongly expressed in NSCs, postmitotic neurons in the CNS, and in oligodendrocytes. Astrocyte differentiation exhibits relatively weak or no Dnmt3a expression either in vitro or in vivo (Feng et al., 2005). In the postnatal brain, Dnmt3a is expressed particularly in the regions that retain the neurogenic potential, namely the SVZ and the hippocampal SGL. Loss of Dnmt3a decreased neuronal differentiation and induced premature astrocyte and oligodendrocyte differentiation in postnatal mouse NSCs and embryonic stem cells via down-regulation of neurogenic genes and up-regulation of gliogenic genes (Wu et al., 2010, 2012). Moreover, Dnmt1 or Dnmt3a conditional knockout mice exhibit deficits in learning and memory in adulthood (Hutnick et al., 2009; LaPlant et al., 2010).

The functions of demethylation during neural development are not fully elucidated. While 5hmC is a key intermediate for demethylation, it can also be a stable epigenetic mark (Hahn et al., 2013; Xu et al., 2012). Recent reports demonstrate that 5hmC-mediated epigenetic regulation is important in the developing human brain and its dysregulation may lead to neurodevelopmental disorders (Wang et al., 2012). For example, loss of 5hmC may impair neurogenesis, neuronal function and survival in Huntington’s disease (Wang et al., 2013). A recent study has demonstrated that Tet3 is most highly expressed in the developing mouse brain compared to Tet1 and Tet2. Neuronal differentiation is associated with increased expression of Tet2 and Tet3, and higher 5hmC levels, while Tet2 and Tet3 deficiency results in incomplete or blocked neuronal differentiation (Pfeifer et al., 2013). Overexpression of Tet3 disrupts olfactory receptor expression and the targeting of axons to the olfactory bulb (Colquitt et al.,...
In addition, Tet1 and Apobec1 are found to be important for neuronal activity, region specific demethylation and subsequent gene expression in the dentate gyrus (Kaas et al., 2013). While genetic deletion of Tet1 impairs hippocampal neurogenesis accompanied by poor learning and memory (Zhang et al., 2013), increased expression of TET1 and decreased expression of deaminase enzymes (APOBEC3A and APOBEC3C) with concomitant increased levels of 5hmC, are found in the parietal cortex of psychotic patients (Dong et al., 2012).
2 AIMS

The major aim of this thesis was to investigate the effects on neurogenesis that can be triggered by a deranged milieu during development. We focused on two different types of neurodevelopmental insults, namely excess glucocorticoids and methylmercury.

Specific objectives were:

- To study the effects on neural stem cell proliferation and differentiation;
- To study whether the effects were heritable;
- To investigate the mechanisms and the occurrence of epigenetic changes;
- To study the programming effects of glucocorticoids on the epigenome in neural stem cells.
3 MATERIALS AND METHODS

3.1 IN VITRO MODELS AND METHODS

3.1.1 Human neural progenitor cell culture.

Human neural progenitor cells (hNPC) (Lonza Verviers SPRL) from three different preparations (gestational week 16, 16.5 and 19) were cultured as neurospheres as previously described (Moors et al., 2010). Briefly, spheres were cultured in DFB medium (Dulbecco’s modified Eagle medium and Hams F12 (2:1) supplemented with B27 (Invitrogen), 20ng/ml EGF (Invitrogen) and 20ng/ml rhFGF (R&D Systems) at 37°C with 5% CO2.

3.1.1.1 hNPC exposed to Dexamethasone (Dex)

In Paper I, hNPC grown as neurospheres with a diameter of 0.25 – 0.3 mm were plated on poly-D-lysine/laminin coated cover glasses and exposed to 1µM Dex. For co-exposure experiments with Mifepristone (1µM) or anti-human DKK1 neutralizing antibody (25µg/ml), hNPC were pre-incubated for 30 minutes before Dex was added. To exclude effects from hormones included in the B27 supplement, DFB medium was exchanged with DFN medium (Dulbecco modified Eagle medium and Hams F12 (2:1) supplemented with N2) (Invitrogen).

3.1.2 Neural stem cells culture from rat embryos

Primary cultures of neural stem cells (NSCs) were prepared as previously described (Ilkhanizadeh et al., 2007; Tamm et al., 2008). Cells were obtained from embryonic cortices (n=6-8/cell preparation) dissected in HBSS (Life technologies) from timed-pregnant Sprague Dawley rats (n=20) (Harlan Laboratories, The Netherland) 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 dish precoated with poly-L-ornithine and fibronectin (both from Sigma). Cells were maintained in enriched N2-medium with 10 ng/ml basic fibroblast growth factor (R&D systems, Minneapolis, MN, USA) added every 24 h and medium changed every other day to keep cells in an undifferentiated and proliferative state. Cells were passaged by detaching via scraping in HBSS. Afterwards, the cells were gently mixed in N2 medium, counted, and plated at a desired density. With this culture conditions, the doubling time was ~20 h.
Figure 3-1. The experimental model. After plating, the NSCs were cultured for 5 days before passaging to obtain parent cells (P1). After 3 days in culture, P1 cells were exposed to insults (Dex or MeHg) for 48 h. To investigate the long-lasting (inherited) effects, P1 NSCs were passaged to get daughter cells (D). D2=daughter NSCs from passage 2; D3=daughter NSCs from passage 3.

3.1.2.1 NSC exposed to Dex

In Paper II we investigated the long-lasting effects of Dex in NSCs. We exposed P1 NSCs to 1 µM Dex for 48 h, the harvested the cells as follows: direct effects were investigated in P1 cells that were harvested at the end of the exposure to Dex; inherited effects were investigated in daughter NSCs (D) that were never directly exposed to Dex (Figure 3-1). D2 and D3 NSCs were harvested at different time points, depending on the type of analysis.

For the investigation of GR-dependent mechanisms, P1 NSCs were incubated with 200 nM mifepristone for 30 min prior to the exposure to Dex. To induce oxidative stress we used 2, 3-Dimethoxy-1,4-naphthoquinone (DMNQ) (Calbiochem). D2 NSCs (72 h after passaging) were exposed to 3 µM DMNQ for up to 24h.

In Paper IV, we studied Dex long-lasting effects, we exposed P1 NSCs to Dex (1 µM) for 48 h. P1 cells were harvested at the end of the exposure to Dex. Inherited effects were investigated in D3 NSCs

3.1.2.2 NSC exposed to methylmercury (MeHg)

In Paper III we investigated the long-lasting effects of MeHg using a similar experimental design as in Paper II. We exposed P1 NSCs to 2.5 nM or 5.0 nM MeHg for 48 h. 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 P1 cells were harvested at the end of the exposure to MeHg. Mitotically heritable effects were investigated in D2 and D3 cells (also cultured in the presence of bFGF) at different time points, depending on the type of analysis.
3.1.3 Cell morphology and viability

3.1.3.1 Trypan blue

Cell viability (Papers II and III) were detected by staining with 0.4% Trypan blue solution (Sigma Aldrich) and analysed under a phase-contrast microscope using an improved Neubauer counting chamber. Cells with a damaged cell membrane (necrotic cells) stained blue, while cells with intact plasma membrane (healthy or apoptotic cells) remained unstained.

3.1.3.2 Tunnel assay

To evaluate the nuclear morphology (Paper II), NSCs (P1, D2 and D3) were grown on poly-L-ornithine/fibronectin coated coverslips and fixed in 4% PF for 1 h at 4°C. After washing with PBS, cells were stained with Hoechst 33342 (1 µg/ml) for 5 minutes at RT, then rinsed with PBS. Apoptotic cells were identified by the condensed chromatin. TUNEL assay was performed to detect single strand DNA breaks. NSCs were fixed as described above and then incubated with TUNEL-reaction mixture (0.07% Triton X-100, 2.5 mM CoCl2, 5 µM fluorescein-12-UTP, 5 U/µl terminal transferase, 0.2 mM potassium cacodylate, 0.25 mg/ml BSA, 25 mM Tris–HCl pH 6.6) (Roche, Bromma, Sweden) at 37°C for 1 h. After mounting, cells were analyzed with a fluorescent microscope and at least 100 nuclei were counted per coverslip. All experiments were performed in triplicates and repeated at least three times.

3.1.4 NSC proliferation and differentiation

3.1.4.1 NSC proliferation

For proliferation assays, 5-6 spheres (Paper I) per donor were exposed in DFN medium supplemented with 20ng/ml EGF (Invitrogen) and 20ng/ml rhFGF (R&D Systems) for 24 h followed by immunocytochemical analyses. NSCs (Paper II and III) prepared from rat embryo at embryonic day 15 (E15) were grown in N2 medium supplemented with 10ng/ml bFGF every day until we analyzed cells. We used both ki67 staining (see details in immunocytochemistry and Table 1) and 5-ethynyl-2’-deoxyuridine (EdU) incorporation and staining

3.1.4.2 EdU incorporation and staining

NSCs were grown on cover slips in a 12 well plate for 48 h in presence of FGF. Then cells were incubated with 10 µM of 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 TBS supplemented with 4 mM CuSO_4. 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, USA). Images were captured using a Nikon microscope system.
3.1.4.3 NSC differentiation

Differentiation assays (Paper I) were performed with 5-6 neurospheres per donor in DFN without growth factor supplementation for 4 days before immunocytochemical analyses. To differentiate NSCs (Paper IV), cells were mixed in N2 medium and plated at low density (500cells/cm²) on coverslips coated with poly-L-ornithine and fibronectin, and grown in the presence of bFGF. After 48h, the medium was changed without adding bFGF to promote spontaneous differentiation and cells were allowed to differentiate for 7 days in bFGF free medium. Next, NSCs were fixed in 4% PFA for 15 min at room temperature, followed by washing in PBS. These fixed cells were used for immunocytochemical analyses.

Immunocytochemistry

Primary antibodies were diluted in PBS containing 0.3% Triton X-100 and 0.5% bovine serum albumin (BSA; Boehringer Mannheim). NSCs were incubated with primary antibodies (see Table 1) overnight in a humid chamber at 4°C. Cells were then rinsed with PBS and incubated with appropriate secondary FITC- or Texas-red-conjugated antibodies for 1 h at room temperature (RT) (1:200; Alexa, Invitrogen). Cell nuclei were counterstained with Hoechst 33342 (1 µg/ml, Sigma Aldrich). After rinsing with PBS, coverslips were mounted onto slides with Vectashield® mounting medium (Vector Laboratories, Inc, USA). Images were captured using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Hamamatsu digital camera (C4742-95-10sc, Hamamatsu Photomics Norden AB, Solna, Sweden).

Table 3-1 Primary antibodies used for immunocytochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Raised in</th>
<th>Dilution ratio</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin</td>
<td>Mouse</td>
<td>1:200</td>
<td>Chemicon</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rabbit</td>
<td>1:800</td>
<td>Dakocytomation</td>
</tr>
<tr>
<td>β-Tubulin III</td>
<td>Mouse</td>
<td>1:400</td>
<td>Convance</td>
</tr>
<tr>
<td>Ki67</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Novoceastra Laboratories</td>
</tr>
<tr>
<td>HP1γ</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>CNPase</td>
<td>Mouse</td>
<td>1:200</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

3.1.5 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 centrifuge at 5 000 rpm for 3 minutes and cell pellets were washed with phosphate buffered saline (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).
3.1.6 Immunoblotting

Total protein content was isolated from human NPC (Paper I) and rat NSCs (Paper III). To prepare protein lysates, cells were collected and centrifuged with 2000 rpm at 4°C for 10 min. Cell pellets (Paper I) were lysed in a buffer containing 125 mM TRIS, 4 % (w/v) SDS, 20 % (v/v) glycerol, 100 mM DTT and 0.2 % (w/v) bromophenol blue. In Paper III, cells were lysed by using a lysis buffer (10mM EDTA, 2mM DTT and 1mM Pefabloc) followed by sonication.

Whole cell lysates were separated applying 6 % SDS-PAGE and transferred to a nitrocellulose membrane (Amersham, Little Chalfont, Bucks, UK). The membrane was blocked in Tris-buffered saline containing 0.01 % Triton (v/v) and 5 % (w/v) non-fat dry milk for 1 hour at 4°C, followed by incubation with primary antibodies overnight at 4°C. The membranes were then rinsed with PBS and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Equal loading was verified with anti-GAPDH (Nordic Biosite, Täby, Sweden). The membranes were rinsed again and developed with ECL reagents. Signals were visualized by exposure to high performance chemiluminescent film. The films were scanned and densitometric quantification of the bands was done in Velocity (Improvision, Perkin Elmer; Massachusetts, USA).

3.1.7 Quantitative real-time PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions including on-column DNase digestion (RNase-free DNase Set, Qiagen) for 30 minutes at 25°C. cDNA was synthesized from equal amounts of RNA by using Superscript II first strand cDNA synthesis kit (Invitrogen) according to the manufacturer’s protocol. Product accumulation was measured by real-time PCR analyses based on SYBR Green detection via ABI Prism 7000 Sequence Detection System with SDS software (version 2.1; Applied Biosystems Inc). Expression levels were normalized to the housekeeping genes β-actin and HPRT ($\Delta \Delta cT = cT_{\text{target gene}} - cT_{\text{housekeeping gene}}$, which showed no Dex-induced changes in gene expression (data not shown). Relative expression levels were calculated as $\Delta \Delta cT = \Delta cTDex - \Delta cTcontrol$ and expression changes were calculated as $2^{-\Delta \Delta cT}$. PCR reactions contained 1µl cDNA, 0.2µM Primers each and SybrGreen PCR Master Mix (Applied Biosystems Inc. Amplification protocol: 10 min 95°C (AmpliTaq Gold Activation); 15 sec 95°C, 1 min 56-60°C (40 cycles)). Product specificity was determined via melting curve analyses (temperature ramp from 60°C to 95°C) and agarose gel electrophoresis.

3.1.8 siRNA knockdown

We used two different approaches for gene knock down. In Paper II, siRNA targeting rat GR (CAU GUU AGG UGG GCG UCA A) and negative siRNA control sequence (UUG ACG CCC ACC UAA CAU G) were purchased from Qiagen. siRNA was
delivered by using a Nucleofactor device and the Neucleofactor kit according to the supplier’s protocol (Amaxa, Lonza, Switzerland). Three hours after nucleofection, NSCs were treated with 1 µM Dex for 24 h. Then cells were harvested for gene expression analysis.

In Paper IV, we used a Smartpool mix of 4 siRNA targeting rat Tet3 and negative siRNA control purchased from Dharmacon. NSCs were grown in N2 medium with 10 ng/ml bFGF for 24h. Then N2 medium were replaced with a smart pool mix and media supplement. Cells were incubated with smart pool mix for 72h in presence of bFGF and treated with 1 µM Dex for the last 24h. The cells were then harvested for gene expression analysis.

### 3.1.9 Measurement of intracellular ROS levels

D2 NSCs were seeded in 96-well plates. 72 h after seeding, cells were exposed to 3 µM DMNQ for 8 h, then washed with HBSS buffer and incubated with 10 µM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H$_2$DCFDA) (Image-iT$^\text{TM}$ Live Green Reactive Oxygen Species Detection Kit, Molecular Probes) for 30 min under 5% CO$_2$ atmosphere. The nonfluorescent carboxy-H$_2$DCFDA permeates live cells and is deacetylated by nonspecific intracellular esterases. In the presence of ROS, the reduced fluorescein compound is oxidized and emits bright green. The fluorescence intensity of the cells from each well was analyzed by Fluoroskan Ascent FL (Thermo Scientific Wilmington, DE, USA) at an excitation wavelength of 495 nm and emission wavelength of 529 nm, followed by determination of the protein content of the respective well using NanoDrop 1000 spectrophotometer (Thermo Scientific Wilmington, DE, USA). The data are expressed as fluorescence intensity related to the protein content. All experiments were performed in triplicates and repeated at least three times.

### 3.1.10 Chromatin immunoprecipitation (ChIP) assay

For ChIP analysis, dissociated hNPC were plated on poly-D-lysine/laminin coated 10 cm dishes (1x106 cells) and stimulated with 1µM Dex or solvent control for 90 minutes. Subsequently, formaldehyde was added (1%, 10 minutes). The reaction was stopped by glycine (0.125 M, 5 minutes). After washing, cells were resuspended in 400µl lysis buffer (50mM HEPES, 1mM EDTA, 0.5mM EGTA, 140mM NaCl, 10% glycerol, 0.5% NP-40, 0.25% Triton x-100, 1mM PMSF) containing Complete Protease Inhibitor Cocktail (Roche). Chromatin was sheared (fragments around 500bp – 1kb) by sonication for 15 minutes using a Diagenode Bioruptor. Similar concentrations of chromatin-containing supernatants obtained after centrifugation were incubated overnight with 2µg anti-GR antibody (sc-8992, Santa Cruz) or control antibody (anti-14 3-3ß antibody, sc-629, Santa Cruz) at 4°C. Next, samples were incubated with 50µl Protein-A/G Sepharose beads (Santa Cruz, sc-2003) for 2 hrs at 4°C under gentle agitation. Cell pellets were washed twice with ChIP wash buffer (10mM Tris-HCl, 1mM EDTA, 0.5mM EGTA, 200mM NaCl, 1mM PMSF and
Complete Protease Inhibitor Cocktail), once with ChIP wash buffer containing 100µg/ml salmon sperm DNA, thrice with ChIP wash buffer containing 500mM NaCl and 100µg/ml salmon sperm DNA followed by a final washing with ChIP wash buffer. Subsequently, cell pellets were resuspended in TE-SDS buffer (10mM Tris-HCl, 1mM EDTA, 1% SDS) and incubated overnight at 65°C. Precipitated DNA was purified using a PCR purification kit (Promega). For quantification, 2µl DNA were amplified by real-time PCR as described above with the following primer sequences: DKK1 promoter, 0.5kb: LP: GGCAACTGAAAGGACCTCAAA; RP: GGGTGATAGCCTCTGAAAAA; 1kb: LP: ATGAGGAAGTCAGGCGCTAA; RP: AGTGGTGGCTAATGTTGAG; 1.5kb: LP: TCTCCCTTTCACACATCCCACA; RP: TTTTTGGTAGATGGGTGGGTT; MT2A promoter Fwd: CAGAGTTTGCTACGACTTC; Rev: TGCTGCTTGCAATTCACCTT; A selected sequence located ~7.5 kb downstream of the last exon of the human DUSP1 gene was used as a negative control (Fwd: TTCAGAAGGTCGGTT; Rev: CATCCCTGCAAGAAC)

3.2 ANIMAL EXPOSURE AND IN VIVO METHODS

3.2.1 Animals and treatments

Pregnant C57BL/6/Bkl mice (Paper III) (Scanbur BK, Sollentuna, Sweden) were exposed to MeHg at the dose of 0.5 mg/kg/day via the drinking water from E7 until 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. 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 drinking water at the concentration of 0.08 mg/mL.

In Paper IV, Dex dissolved in sterile saline was injected subcutaneously to pregnant female C57Bl/6 mice (0.05 mg/kg/day; injection volume 10 ml/kg) from gestational day (GD) 14 until delivery (GD19-20). The control females were injected with an equivalent amount of saline.

3.2.2 Tissue preparation and analyses

In Paper III, the mice were deeply anesthetized and perfused transcardially with PBS followed 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 2-3 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 cover-slipped. For immunohistochemical staining, tissue
sections were incubated with anti-Ki-67 antibody (1:700, Novocastra Laboratories Ltd, UK) overnight, then 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, Burlingame, CA) and cover-slipped with mounting medium.

In Paper IV, the male offspring were killed by decapitation on postnatal day (PND) 3 and the brain was rapidly dissected on ice and stored at -80°C until processing. RNA and DNA were extracted from dissected cortex of male offspring. RNA was used for the analysis of gene expression by qPCR. Global DNA methylation and hydroxymethylation were performed on DNA samples.

### 3.2.2.1 Stereological analysis

Cresyl-violet stained neuronal cells in the entire dentate gyrus were counted on six hippocampal sections (every 12th section, 240 µm interval) at 100x magnification using the neuronal nucleolus as sampling unit in a Olympus BH2 microscope linked with a CAST-system (Computer Assisted Stereological Toolbox; Olympus, Albertslund, Denmark). Sections were analyzed using the optical fractionator method as described elsewhere (Janson and Møller, 1993). The height of the disector was determined after excluding the top and bottom of the section with non-uniform cell distribution to avoid bias from ‘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.

### 3.3 EPIGENETIC ANALYSES

#### 3.3.1.1 Genomic DNA extraction

DNA was prepared using the XL GenDNA Extraction Module Kit (Diagenode, Belgium) according to the manufacturer’s instructions. Quality and quantity of DNA was measured using Nano-Drop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Quant-iTPicoGreensDNA Reagent and Kits (Invitrogen, USA).

#### 3.3.1.2 Global DNA methylation and hydroxymethylation assay

DNA was prepared using the GeneElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Sweden) according to the manufacturer’s instructions. DNA quality and concentration was measured by NanoDrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Separately global DNA methylation and hydroxymethylation were measured using two different quantification Kits (Epigenetek, New York, NY) as instructed by the manufacturer including standard as positive control. Global cytosine methylation and hydroxymethylation levels were determined by measuring optical density (OD) in a microplate reader at 450 nm. The data were calculated according to the manufacturer’s instructions.
3.3.1.3 Methyl-DNA immunoprecipitation sequencing (MBD-seq)

DNA was sonicated using Bioruptor TM 200 (Diagenode) at high frequency with 30sec off/on. The average length of sonicated DNA was 200bp which was determined by the gel electrophoresis. We used 1.2 µg of sonicated DNA for subsequent MBD2 enrichment using methylMinerTM Methylated DNA Enrichment Kit (Life technologies). Briefly, first 10µl of Dynabeads M-280 streptavidin was cleaned by 1XBbind/Wash buffer and 3.5µg of BMD-biotin protein was mixed with clean Dynabeads on a rotating mixer for 1 hour. Then DNA fragments were incubated with the coupled MBD-beads for overnight at 4ºC. After removing non-captured DNA as supernatant, captured DNA was isolated by NaCl gradient elution (0.5M and 1M). The accuracy of the assay was confirmed by using kit supplied control DNA. Isolation of hypermethylated (0.5M and 1M) and non-methylated DNA (supernatant) were confirmed by quantitative real time PCR analysis using Tsh2b (methylation specific primer.) or Gapdh (non-methylation specific primer) and those primer sequences were bought from Diagenode. The recovered DNA was quantified by Qubit™(Invitrogen) and 50ng of immunoprecipitated DNA was used for library preparation using a kit from New England Biolabs (NEB# E6240S/L). Subsequently the library was analyzed by HiSeq 2000. Then the sequence tags were aligned to the rat genome (assembly rn4) with the Bowtie alignment tool. To avoid any PCR bias we allowed only one read per chromosomal position. Next, the peaks (hypermethylated regions) were identified using MACS software (Feng et al., 2011; Zhang et al., 2008b) and the rat CpG islands (CGIs) were downloaded from the UCSC database.

3.3.1.4 Methylation-specific PCR (MSP)

To validate the methylation status of the selected regions, we used MSP in control and Dex exposed NSCs. MSP was used for gene promoter methylation described elsewhere (Herman et al., 1996). Briefly, DNA samples were treated by bisulfite for conversion of unmethylated cytosine to uracil but not methylated cytosine using a MethylCodeTM Bisulfite Conversion Kit (Invitrogen). Primers were designed using MethPrimer program (Li and Dahiya, 2002), which produced two different set of primers for the methylated and unmethylated sequences. Next, bisulfite-treated DNA was amplified by MSP using EpiMark Hot Start Taq DNA Polymerase (New England Biolabs) according to manufacturer’s protocol. After amplification, MSP products were visualized on an E-gel 2 % with SyBR Safe (Invitrogen) and methylated and unmethylated bands were observed in control versus treatment.

3.3.2 Statistical analyses

One-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test was performed. Student’s t-test (Paper II and IV) was used for between-group comparisons. The significance value was set at p<0.05.
4 RESULTS

4.1 PAPER I

Dickkopf 1 mediates glucocorticoid-induced changes in human neural progenitor cell proliferation and differentiation

In this study, we investigated the effects of exposure to Dex on cell proliferation and differentiation using human neural progenitor cells (hNPCs). For proliferation assays, 5-6 spheres per donor were exposed to 1 µM Dex in presence of growth factors (20 ng/ml EGF and 20 ng/ml rhFGF) for 24 hours followed by immunocytochemical analyses. Differentiation assays were performed with 5-6 neurospheres per donor in medium without growth factors for 4 days.

4.1.1 DKK1 mediates the alterations in hNPC proliferation and differentiation

Immunocytochemical analyses of hNPCs cultured with EGF and FGF showed that Dex exposure decreased the number of Ki67-positive cells as compared with controls. Analyses with markers for NSCs (nestin), glia cells (GFAP), and neurons (Tuj1) showed that Dex interferes with hNPC spontaneous differentiation resulting in a significant decrease of cells positive for nestin or Tuj1 and a concomitant increase in the number of cells positive for GFAP. No CNPase-positive cells were detected in either control or Dex-treated cells.

Wnt signaling is essential for NSC proliferation and differentiation (Ille and Sommer, 2005; Michaelidis and Lie, 2008). We investigated whether Dex inhibits the Wnt signaling pathway and found that Dex induced an up-regulation of DKK1, a member of the dickkopf family and a potential inhibitor of the Wnt signaling. To further investigate the mechanisms of Dex-stimulated DKK1 induction, we pre-exposed neurospheres to the GR antagonist mifepristone (RU486). Mifepristone inhibited the Dex-induced increase of DKK1 expression. To determine whether the GR directly interacts with the DKK1 promoter, we performed a ChIP analysis. The data from that ChIP analysis confirmed that GR bind to the DKK1 promoter after Dex treatment. Thus, DKK1 mediates Dex-induced alterations in hNPC proliferation and differentiation.

4.1.2 Confirmation of the mechanism by functional assays

We found that recombinant DKK1 mimics Dex-induced changes in proliferation and differentiation. Additionally, blocking DKK1 abolish Dex effects in proliferation and differentiation. Thus, these data indicate that Dex-induced alterations are regulated by an increased expression of DKK1. We therefore analyzed the effects of GC or DKK1 exposure on known Wnt targets. Cyclin D1 and ID2 are direct targets of canonical Wnt
signaling, with effects on stem cell proliferation and maintenance (Panhuysen et al., 2004; Willert et al., 2002). Neurospheres exposed to 1µM Dex or 300 ng DKK1 showed reduced levels of Cyclin D1 and ID2 expression suggesting that DKK1 has a critical role in the Dex-induced inhibition of proliferation and neuronal differentiation with a concomitant increase in glial cells.

4.2 PAPER II

Glucocorticoids induce long-lasting effects in neural stem cells resulting in senescence-related alterations.

We investigated the long-term effects of GC on rat neural stem cell (NSC) proliferation and differentiation and elucidated the underlying molecular mechanisms. In the proliferating condition, primary cultures of NSCs derived from rat embryos were exposed to Dex (1 µM) for 48 h in presence of bFGF (10ng/ml) from day 3 to day 5 in passage 1. Heritable effects were investigated in daughter cells (D2 and D3, i.e. from passages 2 and 3, respectively), which were never directly exposed to Dex.

4.2.1 Long-lasting alterations in NSC proliferation and differentiation

We first observed alterations in cell morphology by phase contrast microscopy after Dex exposure (Figure 4-1). Moreover, we found that Dex exposure led to a significant reduction in total cell number, as observed in P1 cells, as well as in D2 and D3 cells, without a concomitant increase in necrotic or apoptotic cells. Next, immunocytochemical staining against Ki67 (an established proliferation marker) showed that Dex exposure significantly decreased proliferation in P1 NSCs and this effect persisted in D2 and D3.

![Figure 4-1](image.png)

Figure 4-1. Live cell imaging revealed that control cells grow in clusters with tight cell-to-cell contacts, while Dex-exposed P1 NSCs had more processes and tend to grow widely separated.
To clarify whether the reduction in cell proliferation was associated with an increase in differentiation, we performed immunocytochemical analyses in control and Dex P1, D2 and D3 cells using markers for NSCs (nestin), early neurons (TuJ1) and glia progenitor cells (GFAP,) in proliferating NSCs. Nearly 100% of the control, as well as Dex P1, D2 and D3 cells, were nestin positive, whereas no TuJ1- or GFAP-positive cells were detected at any passage.

### 4.2.2 Dex induces the expression of senescence markers in NSCs

Gene expression analyses by q-PCR in P1 cells revealed a significant Dex-induced up-regulation of the cell-cycle regulating genes p16 and p21 that persisted in D2 and D3 NSCs. Pre-exposure to the GR antagonist Mifepristone (RU486) significantly inhibited the p16 and p21 up-regulation, without altering GR expression, pointing to a GR-mediated mechanism. siRNA nucleofection to knock down GR blocked Dex-induced p16 and p21 up-regulation significantly. In light of the fact that p16 and p21 up-regulation has been linked to cellular senescence, we analysed Dex-induced effects on the expression of senescence markers by q-PCR and immunocytochemistry. Interestingly, Dex repressed Bmi1 (polycomb ring finger oncogene) expression in P1 and D3 NSCs and upregulated high mobility group A1 (Hmga1) in D2 and D3 cell. In contrast, we did not detect significant changes in Hmga2 expression. In addition, Dex led to an enrichment of heterochromatin protein 1 gamma (HP1g) in so-called senescence-associated heterochromatin foci (SAHF).

### 4.2.3 The senescent cells are sensitive to oxidative stress

Cellular senescence has been associated with impaired mitochondrial functions. Interestingly, gene expression analyses by q-PCR showed a long-lasting down-regulation of mitochondrial genes NADH dehydrogenase and we then wanted to test the hypothesis that the long lasting senescence and mitochondrial alterations would be associated with a higher vulnerability to oxidative stress. We challenged daughter NSCs with the oxidative stress inducer 2,3-dimethoxy-1,4-naphthoquinone (DMNQ, 3 mM) and observed an increased ROS production in Dex-NSCs, compared with control cells, which were associated with a higher incidence of cell death, as shown by the two fold increase in the number of apoptotic NSCs.

### 4.2.4 Dex induces global DNA hypomethylation.

Methylation measurements revealed a significant decrease in global DNA methylation in P1 and D2 NSCs associated with a decreased expression in Dnmts. In parent cells, a significant down-regulation in Dnmt1, Dnmt3a, and Dnmt3b mRNA levels was detected, whereas D2 cells showed a decrease in Dnmt1 and Dnmt3a expression.
4.3 PAPER III

Low-dose exposure to methylmercury induces premature senescence and epigenetic changes in neural stem cells

Here 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. NSCs were exposed to subtoxic doses of MeHg (2.5 nM and 5.0 nM) for 48 h in the presence of bFGF from day 3 to day 5 in passage 1. To study the heritable effects, we used passage 2 and passage 3 cells, which were never exposed to MeHg directly. In addition, we investigated the possible detrimental effects on proliferating NSCs in the adult hippocampus in a model of perinatal exposure to MeHg, which we have previously shown to induce long-lasting behavioral deficits (Onishchenko et al., 2008).

4.3.1 MeHg induces long-lasting alterations in NSC proliferation

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 5.0nM MeHg were approximately 9- or 15-fold higher than in control, respectively. 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.

To investigate the MeHg effects in NSCs, we performed trypan blue exclusion test followed by microscopic analysis for analyzing cell morphology, proliferation and differentiation. We found that MeHg exposure significantly reduced the total number of cells, which maintained normal morphology. 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). To ensure that the MeHg concentrations used would not induce cell death, we evaluated cell morphology of the exposed cells. We found that the MeHg concentrations we used induced neither necrosis, as shown by the trypan blue exclusion test, nor apoptosis, evaluated by Hoechst 33342 staining.

4.3.2 MeHg induces the expression of senescence markers in NSCs

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 that persisted in D2 cells and D3 cells. Notably, p16 and p21 are also potential senescence markers that are upregulated in various senescent cells (Chen et al., 2006; Herbig et al., 2004; Robles and Adami, 1998). The expression of other 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, D2 and D3 cells as well as upregulated high mobility group A1 (Hmga1) in D2 cells and D3 cells. MeHg-induced changes in global heterochromatin levels were determined by immunocytochemical staining 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 (SAHF) in D3 cells. Additionally, we found a down-regulation of NADH dehydrogenase 3 (Nd3) and cytochrome b (Cytb) Genes, suggesting alterations of mitochondrial function that might be associated with senescence.

4.3.3 MeHg induces global DNA hypomethylation

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). The reduction in DNA methylation in the NSCs exposed to 5.0nM MeHg was associated with decreased Dnmt3b mRNA expression in P1 NSCs and D2 cells, whereas 2.5nM MeHg exposure decreased mRNA expression of Dnmt3b only in P1 NSCs. However, exposure to MeHg did not induce changes in mRNA expression of Dnmt1 and Dnmt3a in P1 or D2 cells.

4.3.4 MeHg-induced alterations in mouse hippocampus

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 SGL of the hippocampal dentate gyrus in the MeHg-exposed animals. Interestingly, the total number of neurons in the dentate gyrus of MeHg-exposed mice was significantly lower compared with unexposed controls, 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 dentate gyrus.
4.4 PAPER IV

Glucocorticoid programming of neural development involves a Tet3-dependent regulation of Wnt signaling and DNA methylation

In this project, we used the model we established in Paper II. Here we mainly focused on epigenetic mechanisms induced by Dex in NSCs, by genome-wide investigation of differentially methylated DNA regions (DMRs), promoter specific DMR-enriched genes which we then analysed in both in vitro and in vivo models. Differentiation assays were performed with NSCs exposed to 1µM Dex for 48h in absence of FGF. Then, Dex was removed by replacing medium without adding FGF and cells were allowed to differentiate for the following 5 days in Dex- and FGF-free medium. Finally, we investigated the functional interaction between DNA methyltransferases and DNA demethylating enzymes, as well as epigenetic regulation of Wnt signaling.

4.4.1 Epigenetic alterations induced by Dex exposure in NSCs

To study the global relationships between the response of NSCs to GC and chromatin modifications, we investigated genome wide DNA methylation by high-throughput sequencing of MBD-enriched DNA fractions isolated from control and Dex-exposed NSCs derived from the embryonic rat cortex. The genome-wide DNA methylation levels were quantified by analyzing mapped sequencing reads as methylation peaks, using the supernatant of each sample as standard background. In total, 170,000 peaks and 95,283 peaks were generated in controls and Dex-exposed cells respectively; 110,000 differentially-methylated regions (DMRs) DMRs unique to controls and 37,005 DMRs were unique to Dex-exposed cells.

To further understand the consequences of Dex-exposure on NSCs at single gene level, we performed Go analysis using GREAT ontology tools to identify hypermethylated genes whose promoters were located within the CGI, in both control and Dex-exposed cells. We then analyzed the DMRs located in promoter regions and identified 1479 genes (from 1736 DMRs) in control NSCs, and 575 genes (from 622 DMRs) in Dex exposed cells. Among them, we identified cohorts of genes known to play a role in the regulation of cell proliferation, cell differentiation, migration, cellular senescence, DNA methylation, ion channels, mitochondrial function, and oxidative stress that were differently methylated in control as compared to Dex-exposed NSCs. We next aimed at validating some of the genes identified as DMR enriched, such as Dkk1, Dkk3, Txnip and Cyba, which are of particular interest in relation to the Dex-induced phenotype of NSCs (Moors et al., 2012). The analysis of gene expression and their promoter methylation suggested that three out of four DMRs, Dkk1, Dkk3, Cyba but not Txnip, matched with the methylation information identified in the MBD library confirming that DMRs acquired in the MBD library is reliable.

We then tested whether the phenotype induced by Dex exposure would be carried over to spontaneously differentiating cells. In agreement with our earlier findings (Moors et al., 2012), Dex exposure decreased neuronal differentiation and increased astrocytic
differentiation. In addition, we found that the expression of Dkk1, a well-characterized inhibitor of Wnt signaling (Moors et al., 2012; Seib et al., 2013), and the related Dkk3, were up-regulated in Dex-exposed differentiating NSCs. Similarly, the expression of Tnixp was up-regulated in the Dex-exposed cells. To further strengthen this observation, we analysed the expression of Dkk1 and Dkk3, together with Tnixp and Cyba in the cortex of PND 3 mouse pups exposed to Dex in utero. We found a significant up-regulation of Dkk3, Tnixp, and Cyba mRNA expression, while no significant changes in the expression of Dkk1 were detected. This is in accordance with the lack of dynamic expression of Dkk1 in cortex at this stage of development (Diep et al., 2004).

Because of the critical role that the Tet family plays in DNA demethylation, we investigated the effects of Dex on the expression of Tet family proteins (Tet1-3) in proliferating and differentiating cortical NSCs as well as in cortex from Dex in utero-exposed pups. We found a significantly increased expression of Tet 3 in P1 and D3 proliferating NSCs. A similar increase was observed in NSCs undergoing spontaneous differentiation with a concomitant increase of the Tet product 5hmC as well as a significant decrease in Dnmt3a expression. Notably, in the cortex of 3 day old mouse pups exposed to Dex in utero, we found a significant down-regulation of Dnmt3a expression, and a modest but significant up-regulation of Tet1, 2 and 3 associated with a significant decrease in DNA methylation (5mC level), as well as 5hmC levels.

To test whether Tet3 exerted a non-redundant role in regulating Dex-induced changes in DNA methylation and gene expression, we applied an RNA knockdown strategy to achieve a significant down-regulation of Tet3 gene expression in proliferating NSCs. Interestingly, we found that after Tet3 RNA knockdown, the Dex-mediated down-regulation of Dnmt3a mRNA was reversed and in the conditions with lowered Tet3 expression levels, Dex mediated an increase in Dnmt3a gene expression. These results indicate a direct interaction in the regulation of Tet3 and Dnmt3a expression, and suggest a feedback model where the expression of factors mediating DNA methylation and demethylation is strictly regulated by an internal balance between their levels and, presumably, activity.

To investigate whether Tet3 could play a direct role in the detrimental effects of Dex on proliferating NSCs, we next investigated whether the Dex-mediated up-regulation of the Wnt inhibitor Dkk1 was affected by Tet3 RNA knockdown. Intriguingly, the Dex-mediated increase in Dkk1 expression was abolished by the Tet3 RNA knockdown in NSCs. In light of the demethylation and increased gene expression of Dkk1 induced by Dex, and the well-documented critical role of Wnt signaling in NSCs and forebrain development, our results suggest that Dex-induced up-regulation of Tet3 expression is a critical event underlying the epigenetic effects on proliferating neural progenitors.
Neural stem cells (NSCs) give rise to the major cell types of the brain (i.e. neurons, astrocytes, and oligodendrocytes). During specific developmental time frames NSC-derived cells establish neural networks (Temple, 2001). Normal proliferation and differentiation processes during neural development are fundamental for the establishment of complex brain architecture. Alteration of NSC proliferation and differentiation may alter neural development and neuronal functions, as shown in various experimental models for neurodevelopmental and neurodegenerative diseases. In this thesis, we show that early life adverse stimuli (such as exposure to high levels of GC, or to MeHg) have persistent effects which include alterations in NSC proliferation, differentiation, and migration, as well as mitochondrial dysfunction, oxidative stress, and induction of senescence. Our data indicate that epigenetic mechanisms may contribute to these effects.

We first used hNPCs (Paper I) to understand the direct effects of Dex on proliferation and differentiation. Although we used hNPCs from three different stages of gestation (gestational week 16, 16.5, and 19), Dex had consistent effects in all preparations. We found that Dex exposure markedly decreased proliferation and neuronal differentiation of hNPCs, while promoting glial differentiation. Our findings are in agreement with in vitro and in vivo studies showing that GC affect both fetal and adult neurogenesis by interfering with NSC proliferation (Kim et al., 2004; Lemaire et al., 2000; Sundberg et al., 2006).

GC exert their effects via GR, which is a transcription factor that regulates the expression of many genes and signaling pathways, including the canonical Wnt/β-catenin signaling (Michaelis and Lie, 2008; Nusse, 2008). Dkk1 is a negative regulator of canonical Wnt signaling that inhibits the NSC colony formation from single cells (Kalani et al., 2008). We found that Dex up-regulated the expression of Dkk1, and this effect was directly mediated by GR. This is in line with earlier reports on increased neurogenesis induced by deletion of either GR or Dkk1 (Fitzsimons et al., 2013; Seib et al., 2013).

In Paper II, we used primary cultures of rat NSCs as a rodent in vitro model. We found clear morphological alterations in Dex-exposed P1 NSCs. Moreover, the phenotype was heritable being observed in cells never directly exposed to Dex. Up-regulation of senescence markers, such as p16 (Cdkn1a) and p21 (Cdkn2a), were present in Dex-exposed NSCs. Interestingly, GR knock down abolished the Dex-induced up-regulation of both p16 and p21, pointing to a GR dependent mechanism. Moreover, the investigation of additional senescence markers, including Hmga1, Bmi1 and Hp1-gamma supported our hypothesis that Dex-exposed cells stopped dividing because they underwent senescence.

DNA methylation is a major epigenetic mark that is important for genomic stability, and is essential for stem cell proliferation and differentiation. Global DNA methylation estimates the status of methylation in the genome, with no reference to the identity or the location of the methylated sequence. In Paper II we showed that Dex decreased
global DNA methylation in P1 NSCs, which could result in genomic instability (Robertson and Jones, 2000). We also found that global DNA hypomethylation was associated with oxidative stress and premature senescence, and consequently with a higher incidence of cell death. Moreover, these features were retained in daughter NSCs. The long-lasting effects were probably due to epigenetic modifications that persisted after passaging the cells.

Previously we showed that nanomolar (2.5–10nM) concentrations of MeHg can affect neuronal differentiation of rodent embryonic NSCs (Tamm et al., 2008). Here we investigated whether the effects of low-dose MeHg exposure on NSC proliferation and differentiation were long-lasting (Paper III). We used a primary NSC culture paradigm that we established to study the heritable effects in vitro (Paper II). We found that MeHg decreased NSC proliferation and neuronal differentiation, but had no influence on astrocyte differentiation. Interestingly, we found that MeHg also altered the expression of genes involved in cell cycle regulation, induced cellular senescence, altered mitochondrial functions, and concomitantly decreased global DNA methylation. These results indicate that MeHg and Dex effects may be mediated by shared internal regulatory mechanisms. Ahir and colleagues have shown that GR signaling may be a key mediator of developmental disorders induced by heavy metals (including Hg) (Ahir et al., 2013). Further studies are required to find out the role of GR in MeHg-induced effects on NSCs. In line with our in vitro data, we found a trend for decreased cell proliferation in the subgranular layer of hippocampi from adult mice exposed to low doses of MeHg during the perinatal period. This had a measurable impact on the total number of neurons in the dentate gyrus. Importantly, this effect could be reversed by chronic antidepressant treatment. This is in agreement with our previous report on chronic antidepressant treatment being able to reverse the long-lasting behavioral deficits induced by perinatal exposure to MeHg (Onishchenko et al., 2008).

In Paper IV, which is a continuation of Papers I and II, we specifically focused on the epigenetic mechanisms of Dex-induced alterations in NSCs. We found that Dex induces genome-wide DNA hypomethylation, and mapped the gene specific changes in DNA methylation in the rat genome. Interestingly, the epigenetically altered genes were found to be associated with fundamental cellular events such as proliferation, senescence, differentiation and migration. We could validate, by bisulfite conversion, a number of DMR enriched genes identified in the MBD library, thereby confirming the reliability of the library. We next analyzed DNA modifying enzymes (Dnmts for methylation and Tet1-3 for hydroxymethylation) and their products in proliferating and differentiating NSCs, as well as in mouse pup cerebral cortex. These experiments demonstrate the consistency of the in vitro results with the in vivo data.

In Paper I we have shown that Dkk1 mediates the direct effects of Dex on neuronal differentiation, while in Paper IV we further demonstrated that Dkk1 up-regulation is also mediated by promoter hypomethylation. Importantly, the latter appears to be responsible for the persistent up-regulation of Dkk1 in daughter NSCs. In addition, in Paper IV we found that exposure to Dex down-regulated Dnmt3a, which itself had been shown to be sufficient for promoting the astrocytic differentiation of NSCs (Feng et al., 2005). Therefore, the increased astrocyte differentiation and the reduced neuronal
differentiation reported in Paper IV may be explained by both Dnmt3a down-regulation, and canonical Wnt signaling inhibition by Dkk1.

In conclusion, the main finding of these studies is that epigenetic modifications play a critical role in the reprogramming effects exerted by neurodevelopmental insults, such as exposure to excess GC or MeHg.
6 CONCLUSIONS

Glucocorticoids decrease proliferation and interfere with differentiation in human neural progenitor cells by inhibiting canonical Wnt signaling (Paper I).

Glucocorticoids induce long lasting alterations in rat cortical neural stem cell fate that are associated with increased ROS formation and susceptibility to oxidative stress, expression of senescence markers, and global DNA hypomethylation (Paper II).

Exposure to low dose methylmercury induces long lasting alterations in rat cortical neural stem cells that are associated with down-regulation of mitochondrial genes, up-regulation of senescence markers, and global DNA hypomethylation (Paper III).

Epigenetic alterations play a key role in the long-lasting effects of glucocorticoids on rat cortical neural stem cells. The genome-wide DNA hypomethylation is mediated by alteration in both methylation and demethylation mechanisms (Paper IV).
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