The Polycomb complex:
a key regulator in altered neurogenesis resulting from early-life stress?

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Abstract

Exposure to stress during a critical period in childhood is associated with cognitive and emotional deficits in adulthood, underlied by structural and functional changes in the hippocampus. A disturbed process of adult neurogenesis in the hippocampus, which is under tight regulation of epigenetic mechanisms, seems to cause these disorders. Polycomb proteins are important epigenetic mediators in early development and play a critical role in neuronal differentiation as well. Polycomb complexes silence their target genes via methylating Lys27 on histone H3. In cortical development, Polycomb acts as spatiotemporal switch between proliferation and differentiation. In adult neurogenesis, the exact function of Polycomb proteins is not clarified yet, although it is clear that interactions with other epigenetic mechanisms are crucial for their executive roles. On the effect of early-life stress on Polycomb proteins, just a few studies have appeared yet. Most promising features for future studies to focus on would be: the downregulation of Polycomb protein BMI1 as a result of early-life stress; Polycomb complexes regulating the genes encoding the glucocorticoid receptor and corticotropin releasing hormone; and interaction of glucocorticoids with the mitogen- and stress-activated kinases 1 and 2 which replace Polycomb proteins from the DNA. An interaction between the epigenetic mechanisms of Polycomb regulation and DNA methylation seems plausible, although the level of complexity of Polycomb interference remains an evident question.

Chapter 1 Early-life stress and adult neurogenesis

Exposure to stress during perinatal life seems to be correlated to cognitive deficits and psychopathologies occurring later in adult life (McEwen 2003, Mehta, Schmauss 2011, Meyer, Chrousos et al. 2001).The quality of the embryonic environment and chronic stress during the infant's life, which can be caused by extreme poverty, loss of parent, drug or alcohol abusive mother, social deprivation or abuse, are found to correlate with cognitive impairment in adult life (McClelland, Korosi et al. 2011, Korosi, Naninck et al. 2011). In institutionalized Romanian orphans, neuropsychological assessment showed mild cognitive impairment, impulsivity, attention and social deficits and an impaired glucose metabolism in the hippocampus, when compared to healthy adults (Chugani, Behen et al. 2001). When compared to never-institutionalized children or children who were moved from an institution to a foster family, institutionalized children showed a lower cognitive outcome as well (Nelson, Zeanah et al. 2007). Next to cognitive impairments, psychiatric disorders such as depression, anxiety, schizophrenia and autism are linked to exposure to early-life stress (Koenig, Kirkpatrick et al. 2002, Baier, Katunar et al. 2012, Kinney, Munir et al. 2008). Placing institutionalized children in a foster family within two years substantially reduces the stereotypies, which are associated with lower outcome measures on language and cognition (Bos, Zeanah et al. 2010). This indicates that the detrimental effects of infant stress can be restored when intervention takes place within a certain time span. Apparently, there is a critical time window in infant life wherein life-lasting effects can be established (Korosi, Naninck et al. 2011).

Early-life stress influences hippocampal structure and function

Exposure to stress during this critical time window can modify the structure of the hippocampus which can result in psychopathologies and disturbed learning and memory processes later in life (Baier, Katunar et al. 2012, Ivy, Rex et al. 2010). However, vulnerability to psychiatric and cognitive disorders is, besides early-life stress, determined by numerous genetic susceptibility factors and multiple environmental influences (Korosi, Naninck et al. 2011). This makes it hard to identify causal relations between early-life stress and structural and functional hippocampal changes. To properly study this causal relation, animal models for early-life stress were developed (McClelland, Korosi et al. 2011). Mice are often used as model and early-life stress can be mimicked by daily maternal separation or by a stressed mother due to cage impoverishment (McClelland, Korosi et al. 2011, Korosi, Naninck et al. 2011). Learning and memory impairments can be assessed in mice with the widely used Morris water maze test (Morris, Garrud et al. 1982).

Learning and memory processes occur principally in the hippocampus, which is part of the limbic system. Since reduced learning and memory capabilities as well as social disorders are associated with perinatal stress, it is relevant to study the hippocampus (Ivy, Rex et al. 2010). Importantly, the hippocampus is one of the two brain regions were neurogenesis, which is a form of brain plasticity, occurs throughout life. Because the process of adult neurogenesis is disturbed by early-life stress leading to structural and functional changes in the hippocampus, adult neurogenesis is used as readout parameter representing the cognitive impairments caused by early-life stress (Korosi, Naninck et al. 2011). Hippocampal neurogenesis takes place in the subgranular zone of the dentate gyrus (DG). New neurons, astrocytes, and oligodendrocytes are derived of neural stem cells, a process which occurs during embryonic development as well as throughout adult life. Adult stem cells undergo proliferation, cell fate determination, maturation, migration and will subsequently be included in the already existing neural circuitry (Ma, Marchetto et al. 2010).

Adult neurogenesis is regulated by extrinsic and intrinsic signals

During adult life, hippocampal neurogenesis is regulated by life experiences and hormonal and environmental conditions (Korosi, Naninck et al. 2011). In response to early-life stress exposure, the hypothalamic-pituitary-adrenal (HPA) stress response will be activated in particular (Gudsnuk, Champagne 2011). External or internal signals which are threatening to an individual induce the release of the peptide corticotropin releasing hormone (CRH) from the hypothalamus, hippocampus, and amygdala (McClelland, Korosi et al. 2011). Hypothalamic CRH and vasopressin induce the release of adrenocorticotropic hormone (ACTH) in the pituitary gland which will subsequently stimulate secretion of glucocorticoids from the adrenal gland (Gudsnuk, Champagne 2011). Augmented glucocorticoid levels in institutionalized children resulted in an impaired hippocampal glucose metabolism (Chugani, Behen et al. 2001). Glucocorticoids prepare the body to fight or flight and, more importantly, move through the blood-brain-barrier and bind to their cognate receptors on principal cells in the hippocampus (de Kloet, Reul et al. 1990). Glucocorticoids are thought to impair hippocampal neurogenesis (Schoenfeld, Gould 2011), but the relation is complex and dependent of age, mouse strain, exposure-time, and concentration (Korosi, Naninck et al. 2011). Enhanced levels of the stress hormone corticosterone can sometimes lead to increased neurogenesis (Schoenfeld, Gould 2011). Since expression of the glucocorticoid receptor is regulated by perinatal stress as well, understanding how glucocorticoids influence neurogenesis is even more complex (Korosi, Naninck et al. 2011). Hippocampal neurogenesis is thus influenced by these glucocorticoids but can be reversible when applying recovery periods or drug treatments (Heine, Maslam et al. 2004, Oomen, Mayer et al. 2007). However, stress during the critical time window early in life can permanently influence

neurogenesis in adult life, possibly due to the fact that development of the DG occurs in the same time period as early-life stress (Korosi, Naninck et al. 2011).

Adult neurogenesis can be influenced by extrinsic signals, but is intrinsically regulated via epigenetic mechanisms (Ma, Marchetto et al. 2010). Epigenetic changes are responsible for the temporal and spatial control of gene activity, making many essential processes possible, including cell differentiation during development (Ma, Marchetto et al. 2010). How severe stress early in life can establish changes in the hippocampus which influence neurogenesis throughout adult life remains largely unknown. It is suggested that epigenetic regulators play an important role in this process since they are evident in managing adult neurogenesis (Ma, Marchetto et al. 2010). Proteins from the Polycomb Group (PcG) play a key role in early development via chromatin remodelling (Margueron, Reinberg 2011) and also function in neuronal differentiation (Hirabayashi, Suzki et al. 2009, Prezioso, Orlando 2011). PcG proteins were recently suggested to be involved in adult neurogenesis (Ma, Marchetto et al. 2010, Covic, Karaca et al. 2010) and could therefore be potential mediators of the persisting changes in adult neurogenesis that are established after early-life stress. This thesis will study in detail what role epigenetic changes, and especially the Polycomb complex, play in altered neurogenesis resulting from early-life stress.

Chapter 2 Epigenetic factors involved in regulation of adult neurogenesis

Epigenetics can be defined as heritable changes in the function of genetic elements without changes in the actual DNA sequence (Bird 2007). Epigenetics are the interface between environmental factors, experience and internal physiological states represented by signalling molecules and regulation of adult neurogenesis (Ma, Marchetto et al. 2010). Epigenetic changes can be categorized in three main mechanisms: DNA methylation, histone modification, and regulation by non-coding RNA. Other epigenetic strategies less well-known are chromatin remodelling, transcriptional feedback loops and hydroxylation of the 5-methyl group (Ma, Marchetto et al. 2010, Kriaucionis, Heintz 2009). This chapter will take a closer look at the epigenetic factors controlling adult neurogenesis and how these factors could be affected by early-life stress.

DNA methylation

DNA methylation is probably the most extensively studied mechanism and is found to play a fundamental role in gene regulation, including tissue-specific gene expression, inactivation of the X chromosome, gene imprinting and cell reprogramming (Sun, Sun et al. 2011). In mammalian

genomes, DNA methylation principally occurs at the cytosine residue of CG dinucleotide sites where 5-methylcytosine is generated on the pyrimidine ring (Covic, Karaca et al. 2010, Sun, Sun et al. 2011). Conventionally, DNA methylation is thought to be a very stable epigenetic mark, but recent research observed loss of DNA methylation and demethylation in many biological processes (Sun, Sun et al. 2011). This suggests a role for DNA methylation in tight regulation of gene expression in response to signalling molecules. Figure 1 shows the main epigenetic mediators of DNA methylation and a few of them will be shortly discussed below.

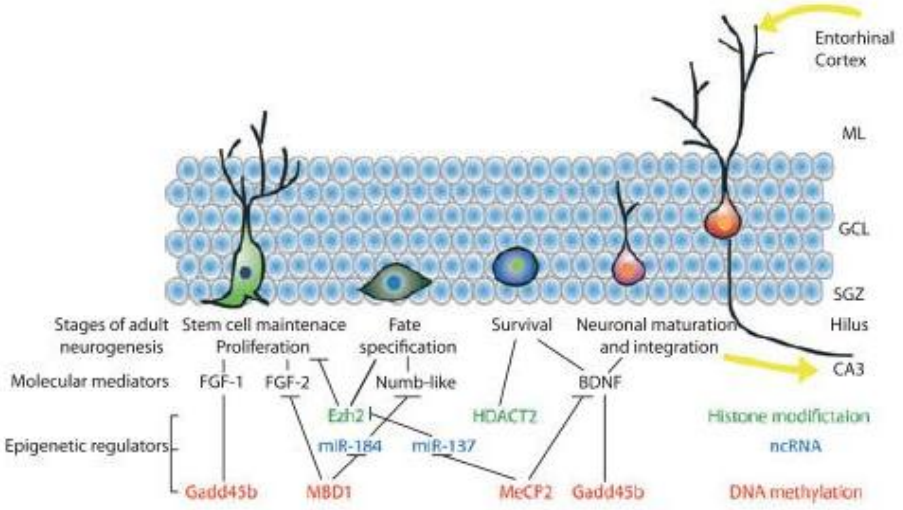

Figure 1 Epigenetic and molecular regulators during adult hippocampal neurogenesis. Epigenetic regulators interfere on different levels at different stages of adult neurogenesis. ncRNA, non-coding RNA; ML, molecular layer; GCL, granule cell layer; SGZ, subgranular zone. Taken from (Sun, Sun et al. 2011).

DNA methyltransferases (Dnmts) are responsible for the epigenetic inheritance (Ma, Marchetto et al. 2010) and catalysis (Sun, Sun et al. 2011) of DNA methylation at the 5-position of the cytosine ring and are crucial for maintenance and fate choice of neural progenitors during embryonic development (Sun, Sun et al. 2011). Their role in adult neurogenesis remains to be elucidated.

Methyl-CpG-binding domain protein 1 (MBD1) is a key mediator of DNA methylation in regulating gene expression. MBD1 interferes in two epigenetic mechanisms of adult neurogenesis and controls the balance between proliferation and differentiation adult neural progenitors (figure 1) (Sun, Sun et al. 2011).

Methyl-CpG-binding protein 2 (MeCP2) is just as MBD1 a major regulator in controlling the balance between proliferation and differentiation in the hippocampus via targeting two epigenetic mechanisms (Sun, Sun et al. 2011). MeCP2 inhibits expression of brain-derived neurotrophic factor (BDNF), which regulates several aspects of adult neurogenesis, via binding on its promoter region (figure 1) (Sun, Sun et al. 2011). MeCP2 is also found to regulate the *Crh* gene, which induces glucocorticoid release at stressful events (McClelland, Korosi et al. 2011) and might subsequently influence hippocampal neurogenesis.

Gadd45b (growth arrest and DNA damage-inducible protein 45 b) was found to establish DNA methylation via a base-excision-repair-like mechanism (Mohamed Ariff, Mitra et al. 2012). Proliferation of neural progenitors in response to activity seems to be perturbed in Gadd45b knockout mice (Ma, Jang et al. 2009). Gadd45b seems to regulate *Fgf1* and *Bdnf* expression and is therefore suggested as a key epigenetic regulator in adult neurogenesis (Mohamed Ariff, Mitra et al. 2012).

Histone modifications

N-terminal tails of histone proteins can be modified post-translationally in multiple ways including acetylation, methylation, ubiquitination, phosphorylation, ribosylation and SUMOylation (Sun, Sun et al. 2011). These modifications influence accessibility of the DNA and provide a binding platform for other molecules (Sun, Sun et al. 2011).

Phosphorylation and acetylation of histone H3 in adult hippocampal neurons are correlated with depressive-like behaviours (Tsankova, Berton et al. 2006) and psychologically stressful events (Chandramohan, Droste et al. 2008). Histone acetylation is catalysed by histone acetyltransferases whereas the reversed mechanism, histone deacetylation, is achieved by histone deacetylases (HDACs) (Sun, Sun et al. 2011). Early-life stress is likely to influence *HDAC* expression and hereby alter adult neurogenesis (Levine, Worrell et al. 2012). In Balb/c mice suffering from maternal separation expression of *HDACs* was reduced, although this effect was not observed in C57Bl/6 mice (Levine, Worrell et al. 2012). Thus, stress during early-life can have a prolonged influence on gene expression of histone modifiers and the response to early-life stress is delicate since it differs per mouse strain (Mehta, Schmauss 2011, Levine, Worrell et al. 2012).

Histone methylation on loci H3K27 and H3K4 is regulated by Polycomb (PcG) and Trithorax (TrxG) group proteins (Ma, Marchetto et al. 2010). These complexes are each others antagonists and silence or activate, respectively, their target DNA sequences (Ma, Marchetto et al. 2010). PcG and its role in adult neurogenesis will be elaborately discussed in the next chapter.

MicroRNAs

Another important group of epigenetic regulators involved in adult neurogenesis is that of the microRNAs (miRs). As shown in figure 1, miR-184 and miR-137 are targeted by other epigenetic mediators including DNA methylation and histone modification proteins (Ma, Marchetto et al. 2010). MiR-124 expression is tightly regulated in rodents during early-life and is affected by adverse experiences occurring in this period (Vreugdenhil, Verissimo et al. 2009). MiR-124 is thought to regulate adult neurogenesis spatiotemporally, because expression levels changed during the various stages of this process (Cheng, Pastrana et al. 2009). MiR-124 has several targets, mainly proteins

involved in adult neurogenesis and glucocorticoid and mineralocorticoid receptors (Vreugdenhil, Verissimo et al. 2009), and is therefore a candidate target for establishing life-long effects during early-life stress (Korosi, Naninck et al. 2011). Next, miR-9 and miR-132 are both regulated by stress, in adult life as well as in early-life and could therefore be crucial in altered neurogenesis after early-life stress (Uchida, Hara et al. 2010, Rinaldi, Vincenti et al. 2010, Kawashima, Numakawa et al. 2010). Via targeting other proteins, mi-9 regulates self-renewal and differentiation of the neural progenitors in the hippocampus (Eendebak, Lucassen et al. 2011). Mi-132 moderates morphological changes of neurons and memory formation (Hansen, Sakamoto et al. 2010).

Chapter 3 The Polycomb complex

The Polycomb group (PcG) is defined by a set of genes whose mutations result in improper body segmentation and was initially discovered in *Drosophila* (Margueron, Reinberg 2011). PcG genes are a conserved group found in many species, which illustrates their fundamental role. Human PcG proteins are crucial in embryonic development; deletion of one of these genes can be lethal (Margueron, Reinberg 2011, Prezioso, Orlando 2011). Throughout life, PcG, together with its antagonist group Trithorax (TrxG), maintains the spatially appropriate patterns of homeotic gene expression (Margueron, Reinberg 2011). PcG silences chromatin domains via (di- or tri-) methylation of Lys 27 of histone H3 (H3K27me2/3), where TrxG catalyzes methylation of Lys4 of histone H3 (H3K4) and thereby activates gene expression (Covic, Karaca et al. 2010). Trimethylation of H3K27 is particularly related to PcG function since these two are often colocalized on genome-wide scale, as opposed to (di)methylation which is more widespread (Simon, Kingston 2009). Both H3K27 methylation and H3K4 methylation can be present at the same promoters and hereby facilitate a rapid shift between silenced and activated states (Covic, Karaca et al. 2010). To assess the role of PcG in altered adult neurogenesis resulting from early-life stress, it is important to discuss in detail the general functioning of the individual PcG proteins and moreover their duty in embryonic and neuronal differentiation.

Polycomb repressive complex 1 and 2

In humans, PcG proteins are found in two multiprotein complexes, namely the Polycomb repressive complex 1 (PRC1) and 2 (PRC2) (Margueron, Reinberg 2011). Both complexes have distinct roles in several post-translational histone modifications, but often they are both needed to sustain gene repression (Margueron, Reinberg 2011).

The PRC1 complex has multiple compositions, always consisting of two subunits, the first being RING1A/B and the second being variably BMI1 (B lymphoma Mo-MLV insertion region 1), MEL18 (also known as PCGF2 (Polycomb group ring finger protein 2)) or NSPC1 (nervous system polycomb 1, also known as PCGF1) (Margueron, Reinberg 2011). A key function of PRC1 is histone H2A ubiquitylation on Lys 119 (H2AK119ub), which is thought to be crucial in PcG repression (Prezioso, Orlando 2011, Simon, Kingston 2009). RING1A/B ubiquilates H2A and the second subunit of PRC1 compacts chromatin efficiently (Margueron, Reinberg 2011, Simon, Kingston 2009). Whether the variously composed complexes of PRC1 function similarly and whether they are independently or subsequently targeted remains the question (Simon, Kingston 2009). PRC1 is thought to function downstream of PRC2 and is seen as the direct executor of target sequence silencing (Simon, Kingston 2009). PRC1 binds to the product catalysed by PRC2, which is trimethylated Lys 27 on histone H3 (H3K27me3), and can compact chromatin without using enzymatic activity (Margueron, Reinberg 2011). There are several possible mechanisms by which PRC1 silences genes, including blocking of transcription factor binding; blocking the association of RNA polymerase; blocking transcriptional initiation or elongation; or localize the genes in repressive nuclear compartments (Simon, Kingston 2009). Considering recent experiments on this topic, blocking of transcriptional elongation seems the most plausible option (Simon, Kingston 2009).

PRC2 comprises EZH1/2 (enhancer of Zeste homologue 1/2), SUZ12 (suppressor of Zeste 12), EED (embryonic ectoderm development) and RbAp46/48 (retinoblastoma-binding protein p46/48, also known as RBBP7/4) (figure 2) (Margueron, Reinberg 2011, Simon, Kingston 2009). Recently, three new subunits belonging to PRC2 were discovered: AEBP2 (adipocyte enhancing binding protein 2), PCLs (polycomb-like proteins), and JARID2 (Jumonji, AT rich interactive domain 2) (Margueron, Reinberg 2011). These three proteins interact transiently with other PcG proteins but were found to contribute significantly to the effectiveness of PcG (Margueron, Reinberg 2011). AEBP2 is a zinc-finger protein and enhances the enzymatic activity of other PRC2 components (Cao, Zhang 2004). PCL1/2/3 contains several domains and has various functions such as regulating enzymatic activity (Nekrasov, Klymenko et al. 2007) and gene recruitment of PRC2 (Walker, Chang et al. 2010), which might be explained by their tissue-specificity (Walker, Chang et al. 2010). In addition, PCL proteins are suggested to stimulate the methyltransferase activity of PRC2, methylation from H3K27me2 to H3K27me3 in particular (Sarma, Margueron et al. 2008). JARID2 catalyses the demethylation of histone proteins but is deprived of enzymatic activity itself (Li, Margueron et al. 2010). JARID2 regulates the catalytic potency of PRC2 depending on the biochemical conditions and could be involved in PRC2 recruitment to CG rich DNA sequences (Li, Margueron et al. 2010). The enzymatic subunit EZH1/2 of PRC2 contains a SET domain and is responsible for di- and trimethylation of Lys 27 of histone H3 (H3K27me2/3) (Margueron, Reinberg 2011). EZH1 has less methyltransferase activity

than EZH2 and is capable of compacting polynucleosomes what EZH2 is not (Simon, Kingston 2009, Margueron, Li et al. 2008). EZH1 as subunit of PRC2 is thought to restore H3K27me2/3 after demethylation or when methylation is lost after histone exchange, because it is found in both dividing and differentiated cells (Margueron, Li et al. 2008). EZH2 as part of PRC2 establishes cellular H3K27me2/3 levels through its methyltransferase activity and is only found in actively dividing cells (Margueron, Li et al. 2008). Hence, PRC2 is likely to switch from catalytic to non-catalytic when EZH2 is replaced by EZH1 (Simon, Kingston 2009). Because PRC2 also recruits H3K4 methylation by TrxG proteins it manages the balance between gene silencing and repression (Simon, Kingston 2009).

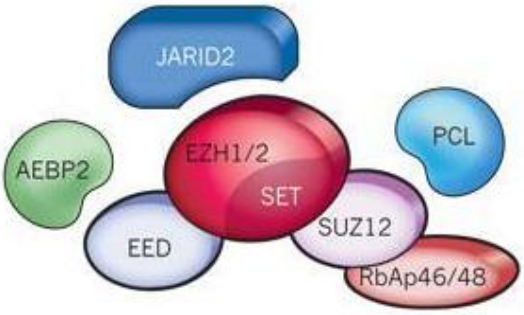

Figure 2 Composition of mammalian PRC2. PRC2 can be seen as holoenzyme which optimally functions when all cofactors contribute. Adopted from (Margueron, Reinberg 2011).

Targets of PcG complexes

Conventionally, PcG was thought to target genes which regulate early developmental processes, the so-called *HOX* genes (Schuettengruber, Cavalli 2009). Recent genome-wide association studies however showed that PcG has impact on a lot more genes, mostly transcription factors engaged in cellular and developmental processes (Schuettengruber, Cavalli 2009).

How PcG complexes are targeted to genes it not elucidated yet. No specific DNA elements which attract PcG, so-called Polycomb response elements (PREs), have been identified in mammals up till now (Prezioso, Orlando 2011, Simon, Kingston 2009). Remarkably, 97% of PRC2-positive targets bind to CG-rich sequences (Ku, Koche et al. 2008), however this is specifically valid for embryonic stem (ES) cells and not for neural stem cells (NSCs) (Mohamed Ariff, Mitra et al. 2012). Some proteins bound to DNA are suggested as potent PcG complex recruiters and in addition their might be a role for non-coding RNAs (figure 3) (Simon, Kingston 2009). A first good candidate recruiter is yin and yang 1 (YY1) (Thomas, Seto 1999), another candidate is the octamer-binding transcription factor 4 (OCT4), which is thought to specifically recruit PcG in ES cells (Simon, Kingston 2009). Recruiters of PcG complexes are probably numerous and specific per cell type and process they regulate.

The dynamics between PRC1 and PRC2 in regulating chromatin is complex and not yet known in detail. In recent studies it is implied that PRC1 targets methylated H3K27 *in vivo*, even when this methylation is not PRC2 dependent (Lee, Villa et al. 2007, Mujtaba, Manzur et al. 2008). Since PRC2 can accumulate at chromatin without PRC1, and PRC1 sporadically accumulates without PRC2, it might however be that methylated H3K27 often promotes but is not always necessary for targeting PRC1 (Simon, Kingston 2009). PRC2 is targeted for H3K4 demethylation by the retinol-binding protein 2 (RBP2) (Simon, Kingston 2009). Since the chromatin landscape is highly dynamic, it is plausible that PRC2 will have more contributions in epigenetic regulation which are not discovered yet (Simon, Kingston 2009).

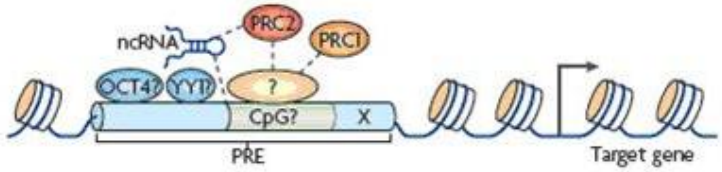

Figure 3 Recruitment of PcG complexes to target sequences. This model shows how PcG complexes could interact at their genomic target regions. The dotted lines indicate plausible interactions in PcG targeting. Adopted from (Simon, Kingston 2009).

The role of Polycomb in embryonic stem cell differentiation

As mentioned before, PcG proteins play an essential role in early development and are found to be crucial in ES cell differentiation. ES cells are able to self-renew and are pluripotent, meaning that they can differentiate into every adult human tissue. The future of an ES cell is under tight regulation of PcG proteins and transcription factors such as *Oct4*, *Sox2*, and *Nanog* (Prezioso, Orlando 2011). PcG regulates the transcription of *Oct4*, *Sox2*, *Nanog*, *Hox* genes and many other genes involved in developmental processes via binding their promoters (Boyer, Plath et al. 2006). In ES cells, PcG complexes are specifically found to silence genes which promote differentiation and not self-renewal (Boyer, Plath et al. 2006). Remarkably, bivalent domains are present in ES cells, which are H3K27 as well as H3K4 methylated, probably facilitating a rapid response to developmental cues (Prezioso, Orlando 2011). After cell-fate determination, bivalent genes who were not induced are inclined to keep the repressive H3K27me3 mark and lose the activating H3K4me3 mark (Schuettengruber, Cavalli 2009). A non-processing form of RNA polymerase II is found to be stalled on several silenced promoters of ES cells to enable quick transcription when the gene becomes activated (Prezioso, Orlando 2011, Schuettengruber, Cavalli 2009). PRC1 subunit RING1A/B has the decisive role in activating these promoters (Prezioso, Orlando 2011). In addition, PRC1-mediated ubiquitylation of H2AK119 seems to be an important regulating factor which silences bivalent genes in ES cells and simultaneously prepares them for activation by protecting them from DNA methylation (Schuettengruber, Cavalli 2009). *Eed*-deficient mice cells show a significant increase in expression of

PcG target genes compared to wild-type mice cells (Boyer, Plath et al. 2006), which implies an indispensable role for EED. PRC2 subunit JARID2, previously described as being involved in PRC2 recruitment towards DNA, was found to regulate RNA polymerase II as well (Prezioso, Orlando 2011) and might remove the H3K4me3 mark from bivalent promoters during differentiation in addition (Schuettengruber, Cavalli 2009). Subunit SUZ12 seems to be crucial in differentiation of ES cells (Schuettengruber, Cavalli 2009). ES cell differentiation is not disturbed when a single PRC subunit is non-functional, which emphasizes the crucial role of PcG proteins and the cleverness of the PcG system (Prezioso, Orlando 2011, Schuettengruber, Cavalli 2009). Interestingly, non-coding RNAs (ncRNAs) are found to interact with PRC2 and are therefore suggested to mediate between DNA and chromatin remodelling activities (Prezioso, Orlando 2011).

The role of Polycomb in neuronal differentiation

NSCs have the ability to self-renew and can differentiate into three major cell types being neurons, astrocytes and oligodendrocytes. The development of NSCs into neural progenitor cells (NPCs) seems to be under tight regulation of transcriptional changes established by PRC2-mediated repression, including the repression of transcription factor *Sox2* (Prezioso, Orlando 2011, Pereira, Sansom et al. 2010). BMI1 is essential in NSC maintenance and needed for neuronal differentiation, but its role in self-renewal is minor (Prezioso, Orlando 2011, Schuettengruber, Cavalli 2009). RING1B promotes NSC self-renewal; when RING1B is non-functional NPCs differentiate into neurons, but not into glial cells, precociously (Prezioso, Orlando 2011, Schuettengruber, Cavalli 2009). Regulating the development of cortical progenitor cells temporally and probably stage-dependent (Hirabayashi, Suzki et al. 2009) is the responsibility of EZH2 (Pereira, Sansom et al. 2010). When *Ezh2* is deleted in mice, the silencing mark H3K27me3 in cortical progenitor cells is lost and also not inherited in progeny cells, resulting in expression of genes promoting differentiation (Pereira, Sansom et al. 2010). This occurs before the onset of neurogenesis, indicating that Ezh2 is crucial for timing in neuronal differentiation (Pereira, Sansom et al. 2010). On the contrary, Hirabayashi and colleagues show that deletion of *Ezh2* at embryonic day 12 lead to an extended neurogenic period and a delayed gliogenesis (Hirabayashi, Suzki et al. 2009). During late developmental stages, PcG proteins are thought to switch NPC fate towards astrogenesis via accumulating trimethylation of H3K27 at the promoter of *Ngn1*, a transcription factor promoting astrogenesis (Hirabayashi, Suzki et al. 2009). These studies indicate that PcG proteins might regulate major developmental transitions in cortical progenitor cells, such as from early neuroepithelial cells to neurogenic glial cells; changing cortical progenitor cells' competence to generate neurons of different laminar fates; switching from neurogenesis to gliogenesis (Pereira, Sansom et al. 2010). In mice, *Ezh2* is highly expressed during NSC proliferation and *Ezh2* expression declines when NSCs differentiate into neurons (Sher, Rossler et al. 2008).

Remarkably, no *Ezh2* expression was found when NSCs differentiated into astrocytes, as opposed to oligodendrocytes where Ezh2 levels were still high (Sher, Rossler et al. 2008). The follow-up study showed that abundant expression of *Ezh2* in cultured astrocytes dedifferentiated them towards proliferating cells (Sher, Boddeke et al. 2011). Interestingly, miR-137 was found to repress EZH2 leading to a global decrease in H3K27me3 in NSC cell cultures (Szulwach, Li et al. 2010). Overexpression of miR-137 promoted cultured NSCs to proliferate instead of differentiate (Szulwach, Li et al. 2010). However, with coexpression of *Ezh2* this effect was restored, suggesting that interaction between miR pathways and PcG proteins play a critical role in neurogenesis (Szulwach, Li et al. 2010). Several studies show contradictory results with regard to the role of the methyltransferase EHZ2 in neuronal differentiation, whether it is promoting self-renewal (Pereira, Sansom et al. 2010, Pereira, Sansom et al. 2010, Sher, Rossler et al. 2008, Sher, Boddeke et al. 2011) or differentiation (Hirabayashi, Suzki et al. 2009, Szulwach, Li et al. 2010). EZH2 might indeed regulate self-renewal as well as differentiation, dependent on the developmental stage. Neuronal differentiation persists throughout life in the olfactory bulb and hippocampus enabling learning and memory processes. PcG proteins have a specific function in fate determination of NSCs (Prezioso, Orlando 2011), although this function is not completely understood yet.

Chapter 4 Does the Polycomb complex play a key role in altered adult neurogenesis after early-life stress?

Unfortunately, this question cannot be fully answered considering our current knowledge. The Polycomb complex is associated in numerous mechanisms and processes, but its complete role remains to be clarified. In addition, the mechanism by which early-life stress accomplishes life-long changes in adult neurogenesis are unknown either. Still, a few studies showed how stress affected neurogenesis and how PcG proteins might play a role in this. This chapter will highlight the most promising studies and will give suggestions for future research.

Effects of stress on Polycomb proteins

Intrauterine administration of the synthetic glucocorticoid dexamethasone in cultured rat embryonic NSCs results in impaired NSC proliferation but does not alter cell viability or differentiation (Bose, Moors et al. 2010). Upregulation of cell-cycle regulating genes *p16* and *p21* resulting in cell cycle arrest was found to underlie the decrease in NSC proliferation (Bose, Moors et al. 2010). Simultaneously, DNA methylation and the expression of Dnmts were globally decreased and PRC1 subunit Bmi1, described as marker for senescence, was downregulated. Daughter cells not exposed

to dexamethasone show similar features which emphasizes the role of epigenetic mechanisms. This study suggests that *in vivo*, abnormal expression of glucocorticoids resulting from early-life stress could negatively affect neurogenesis leading to cognitive deficits in adult life (Bose, Moors et al. 2010). The involvement of Bmi1 in this regulatory process definitely suggests a role for PcG proteins in the persistent changes in adult neurogenesis resulting from early-life stress. Downregulation of *BMI1* in humans will result in less H3K27me3 and loss of gene silencing. It should be examined if activation of PcG target genes will result in a diminished NSC proliferation, which could underlie disturbances in adult neurogenesis. First experiment would be to inactivate BMI1 in a NPC culture, this could be done by RNA interference, and to determine the amount of proliferating cells. Conform the study of Bose and colleagues NPC proliferation is expected to increase (Bose, Moors et al. 2010). Next, the same experiment could be performed using other PcG proteins, including proteins of PRC2 such as EZH2, SUZ12 or EED.

It is suggested that early-life stress could persistently affect the expression of *Glucocorticoid Receptor (GR)* and *CRH* and thus alter adult neurogenesis (McClelland, Korosi et al. 2011). These life-long effects are likely established via epigenetic mechanisms (McClelland, Korosi et al. 2011), but it remains to be elucidated which epigenetic mechanisms will be involved. Since PcG proteins have a major role in epigenetics they could be responsible for the attenuation of GR and CRH after early-life stress by silencing their promoters. It would be interesting to find out if PcG proteins target the *GR* or *CRH* promoter region and whether this is dependent on a stress- or non-stressful situation. First this could be tested in a cultured cell line using chromatin immunoprecipitation (ChIP) followed by sequencing (ChIP-seq), which will possibly reveal the *GR* and *CRH* promoters as PcG targets. Subsequently, this ChIP-seq should be performed in an *in vivo* model due to the complex chromatin remodelling configurations which could be influenced systemically. This *in vivo* model could be elaborated by adding the early-life stress component via maternal separation or via stressing the mother.

Gehani and colleagues showed that phosphorylation of serine 28 of histone H3 (H3S28p) results from a stress response and that this epigenetic mark can displace PcG complexes from chromatin and induce transcription when collocated with the H3K27me3 mark (Gehani, Agrawal-Singh et al. 2010). In response to mitogenic, stress, and neuronal differentiation signals, mitogen- and stress-activated kinases (MSK) 1 and 2 generate the double mark H3K27me3S28p. Importantly, phosphorylation of S28 next to trimethylated K27 might prevent the previously used antibody for H3K27me3 from binding, which might explain the reduction of H3K27me3 observed on various PcG targets genes during differentiation (Gehani, Agrawal-Singh et al. 2010). Upon phosphorylation of H3K27me3 on S28, the PcG proteins EZH2, SUZ12, and EED lose their association with the histone resulting in activation of the target genes (Gehani, Agrawal-Singh et al. 2010). This mechanism seems

to be an important mediator in translating environmental cues in regulation of gene expression (Gehani, Agrawal-Singh et al. 2010). Whether this mechanism applies to early-life stress is unknown but unlikely, since early-life stress is mediated by glucocorticoid signalling and not MSK phosphorylation. Literature on the interaction between glucocorticoids and MSK signalling is rare, so it would be useful to examine this further. A neuronal cell culture overexpressing glucocorticoid hormones could be used here. Glucocorticoids will be abundantly present in the culture medium via excretion and will induce intracellular signalling pathways upon binding the glucocorticoid receptor present on the cell surface. Whether glucocorticoids influence the amount or potency of MSK can be assessed by using respectively fluorescent antibodies against MSK1 and 2 and the antibody against H3K27me3S28p, described by Gehani and colleagues (Gehani, Agrawal-Singh et al. 2010).

The removal of H3K27me3 upon neuronal differentiation, up to now regarded as a key epigenetic mechanism in regulating gene expression, could overshadow the real mechanism regulating gene expression being H3K27me3S28 phosphorylation by MSK kinases (Gehani, Agrawal-Singh et al. 2010). Studies which founded their results on using an antibody against H3K27me3 should be revised and performed again with an antibody against H3K27me3S28p in addition. For understanding the dynamics in epigenetic regulation it is crucial to distinguish between loss of methylation of K27 of H3 or phosphorylation of S28 of H3.

Complexity level of epigenetic interactions

The role of DNA methylation in regulating adult neurogenesis is still indistinct. Recently it became clear that DNA methylation is very reversible, while it was conventionally thought to be a stable epigenetic mark. During NPC fate determination, *de novo* DNA methylation sites occur (Mohn, Weber et al. 2008), which proves the dynamics of DNA methylation. Since DNA methylation occurs on many promoters encoding genes with numerous functions, its purpose in adult neurogenesis is not straightforward but complex. There is accumulating evidence that PcG proteins interact with DNA methyltransferases and that there is interplay between both epigenetic systems (Schuettengruber, Cavalli 2009). One hypothesis is that PcG proteins attract DNA methyltransferases in order to establish a stable epigenetic mark (Schuettengruber, Cavalli 2009). Since evidence that DNA methylation is more dynamic than previously thought is expanding, this hypothesis seems implausible. An interaction between PcG proteins and DNA methylation is however very probable, also shown by Bose and colleagues who found a decrease in DNA methylation and Dnmts as well as in PcG protein Bmi1 after administration of intrauterine glucocorticoid (Bose, Moors et al. 2010).

The possibility of an interaction between PcG complexes and DNA methylation is actually part of a bigger query: how many levels of complexity exist in regulation of adult neurogenesis? Two extensively studied epigenetic mechanisms, DNA methylation and PcG silencing, are both found to be

highly dynamic, while changes in neurogenesis established during childhood remain life-long. These epigenetic changes should thus be able to persist. The complex interactions present between different epigenetic systems suggest that accumulation of these epigenetic regulations eventually controls the fate of a genomic region. This implies an epigenetic settlement of multiple levels. Fitting this theory, PcG proteins were found to be phosphorylated themselves (Gehani, Agrawal-Singh et al. 2010) and additionally there are indications for O-linked beta-N-acetylglucosamine glycosylation in the regulation of PcG proteins (Myers, Panning et al. 2011). Not least, the promoters of the PcG proteins are probably under tight regulation themselves as well, which adds another level to the epigenetic settlement. In *Drosophila*, expression of PcG proteins was indeed found to be under regulation of PcG complexes (Park, Schwartz et al. 2012). Mammalian ChIP studies should show whether PcG proteins target the PcG genes as well.

Conclusion

Epigenetic studies are pointing to many possible regulatory mechanisms for controlling adult neurogenesis. In addition, several epigenetic mechanisms, such as HDACs (Levine, Worrell et al. 2012), DNMTs (Bose, Moors et al. 2010), and miRNAs (Korosi, Naninck et al. 2011) are found to be influenced by (early-life) stress. A role for Polycomb proteins as mediators between early-life stress and altered neurogenesis is certainly not abolished. However, the complete function of PcG proteins is not elucidated yet which makes it hard to draw any conclusions on this mediating role at this very moment. PcG regulation appears to be very subtle, depending on plentiful other regulations which can be signalling molecules or other epigenetic mechanisms or differentiation stage. Results of the suggested future studies will hopefully provide valuable insights leading to a better understanding of the role of Polycomb proteins in altered adult neurogenesis after early-life stress.

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