Defining mesodiencephalic dopaminergic neurons

Identifying different levels of transcriptional control

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Chapter 1

The emergence of mdDA neurons: different levels of transcriptional control

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OUTLINE

The requirements to form a mesodiencephalic dopaminergic neuron

- Generating a permissive environment: patterning of the midbrain
- Early specification of mdDA neurons
- Terminal differentiation and subset specification
- Lmx1b in mdDA development

A Second level of regulation: Epigenetics

- Epigenetics in mdDA development
- Polycomb protein
- Polycomb protein in neurodevelopment

Aim of this thesis

Outline of this theses

Mesodiencephalic dopaminergic (mdDA) neurons form the largest population of dopaminergic (DA) neurons in the mammalian brain and are involved in the regulation of voluntary movement and emotion-related behavior (Björklund and Dunnett, 2007; Roeper, 2013; Smidt and Burbach, 2007). The mdDA population can be arranged in anatomical distinct nuclei with different functions, including the Ventral tegmental Area (VTA) and the Substantia nigra pars compacta (SNc) (Arenas et al., 2015). The SNc innervates the dorsolateral striatum, forming the nigral-striatal pathway, which is integrated in a complex network that controls voluntary movement and body posture, and the degeneration of this pathway is the hallmark of Parkinson's disease (PD) (Braak et al., 2003). The VTA has projections to the ventral striatum, the amygdala and the prefrontalcortex, which are involved in regulating emotion-related behavior and are linked to addiction, depression and schizophrenia (Prakash and Wurst, 2006; Smidt and Burbach, 2007). The selective vulnerability of the SNc to degeneration has led to the hypothesize that different subsets of mdDA neurons are not only anatomically distinct, but also rely on different molecular programming for their formation and survival. Extensive study into the molecular profile of adult and developing mdDA neurons has led to the identification of several factors that are differentially expressed in the VTA and the SNc (Chung et al., 2005; German et al., 1992; Veenvliet et al., 2013). After the induction of general DA markers like *Tyrosine hydroxylase* (*Th*), the *dopamine transporter* (*Dat*) and the *vesicle monoamine transporter 2* (*Vmat2*) by the orphan nuclear receptor NURR1, complex interplays between differentially expressed transcription factors induce the expression of subset specific genes, like *aldehyde dehydrogenase 2* (*Ahd2*) in the SNc and *cholecytoskin* (*Cck*) in the VTA (Di Salvio et al., 2010; Panman et al., 2014; Smits et al., 2003a; Veenvliet et al., 2013). Here, we review the development of mdDA neurons, from the patterning of the midbrain region until the specification of the different mdDA subsets. In addition, we describe a second level of regulation of neuro-development, epigenetics and more specifically the role of Polycomb (PcG) protein in neuronal specification and differentiation.

THE REQUIREMENTS TO FORM A MESODIENCEPHALIC DOPAMINERGIC NEURON

Generating a permissive environment: patterning of the midbrain

A critical early event in the specification of the permissive mdDA region is the regionalization of the neural tube (Altmann and Brivanlou, 2001; Lumsden and Krumlauf, 1996). The initial AP pattern is established under the influence of neural-inducing factors and modifiers produced by the mesoderm (Lumsden and Krumlauf, 1996) and subdivides the neural plate into four distinct territories comprising the

prosencephalon, the mesencephalon, the rhombencephalon and the spinal cord (Prakash and Wurst, 2004). The regionalization along the dorso-ventral (DV) axis is accomplished after the closure of the neural tube, by signals secreted by the floorplate, the notochord and non-neural ectoderm (Hynes et al., 1995; Prakash and Wurst, 2004). The region in which mdDA neurons develop is designated by the interaction of diffusible factors, including sonic hedgehog (SHH), wingless-type MMTV integration site 1 (WNT1) and fibroblast growth factor 8 (FGF8) (Crossley et al., 1996; Danielian and McMahon, 1996; Lee et al., 1997; Mesman et al., 2014; Roelink et al., 1995). SHH is secreted from the floorplate (FP) and is required for the patterning along the DV axis (Joksimovic et al., 2009; Mesman et al., 2014; Rowitch et al., 1999). While SHH determines the DV axis, FGF8 and WNT1 mediate the patterning abilities of the Isthmic organizer (IsO) along the AP axis (Crossley et al., 1996; Joyner et al., 2000; Lee et al., 1997; Martinez et al., 1999). The IsO is located at the border between the mid- and hindbrain and is required for the specification of both the midbrain and the anterior hindbrain (Brodski et al., 2003; Crossley et al., 1996; Joyner et al., 2000). The position of the IsO is determined by the mutual repression of two opposing factors, *Otx2* and *Gbx2* (**Figure 1A**) (Brodski et al., 2003; Wurst and Bally-Cuif, 2001). Loss- and gain-of-function showed that when the IsO is moved over the anterior-posterior axis the size of the two main neurotransmitter systems formed in this region, the DA and serotonergic (5-HT) system, is affected (**Figure 1B, C**) (Brodski et al., 2003). The over-expression of *Otx2* or *Wnt1*, or loss of *Gbx2* leads to a displacement of the IsO to a more posterior position, extending the permissive midbrain domain caudally and inducing ectopic DA neurons (**Figure 1B**) (Brodski et al., 2003; Joyner et al., 2000; Prakash et al., 2006; Puelles et al., 2003; Sherf et al., 2015). In contrast, genetic approaches in which *Otx2* and *Wnt1* are removed or repressed, or *Gbx2* is over-expressed, causes an anterior shift of the IsO, expanding the hindbrain region rostrally and leading to the appearance of ectopic 5-HT neurons (**Figure 1C**) (Brodski et al., 2003; McMahon et al., 1992; Prakash et al., 2006). Coincident with the formation of the IsO is the refinement of the expression patterns of *Wnt1, En1* and *Lmx1b* to areas adjacent and within the IsO (Adams et al., 2000; Danielian and McMahon, 1996; Guo et al., 2006). These genes form regulatory feedback loops, required to maintain each others expression and preserve the inductive activity of the IsO (Adams et al., 2000; Danielian and McMahon, 1996; Guo et al., 2006). When the formation of the IsO is complete, it remains active during neurogenesis. The signals expressed within and adjacent to the IsO together with the ventralizing factors expressed by the floor plate define the domain in which mdDA progenitors will further develop and are necessary for the maintenance and formation of these progenitor-populations.

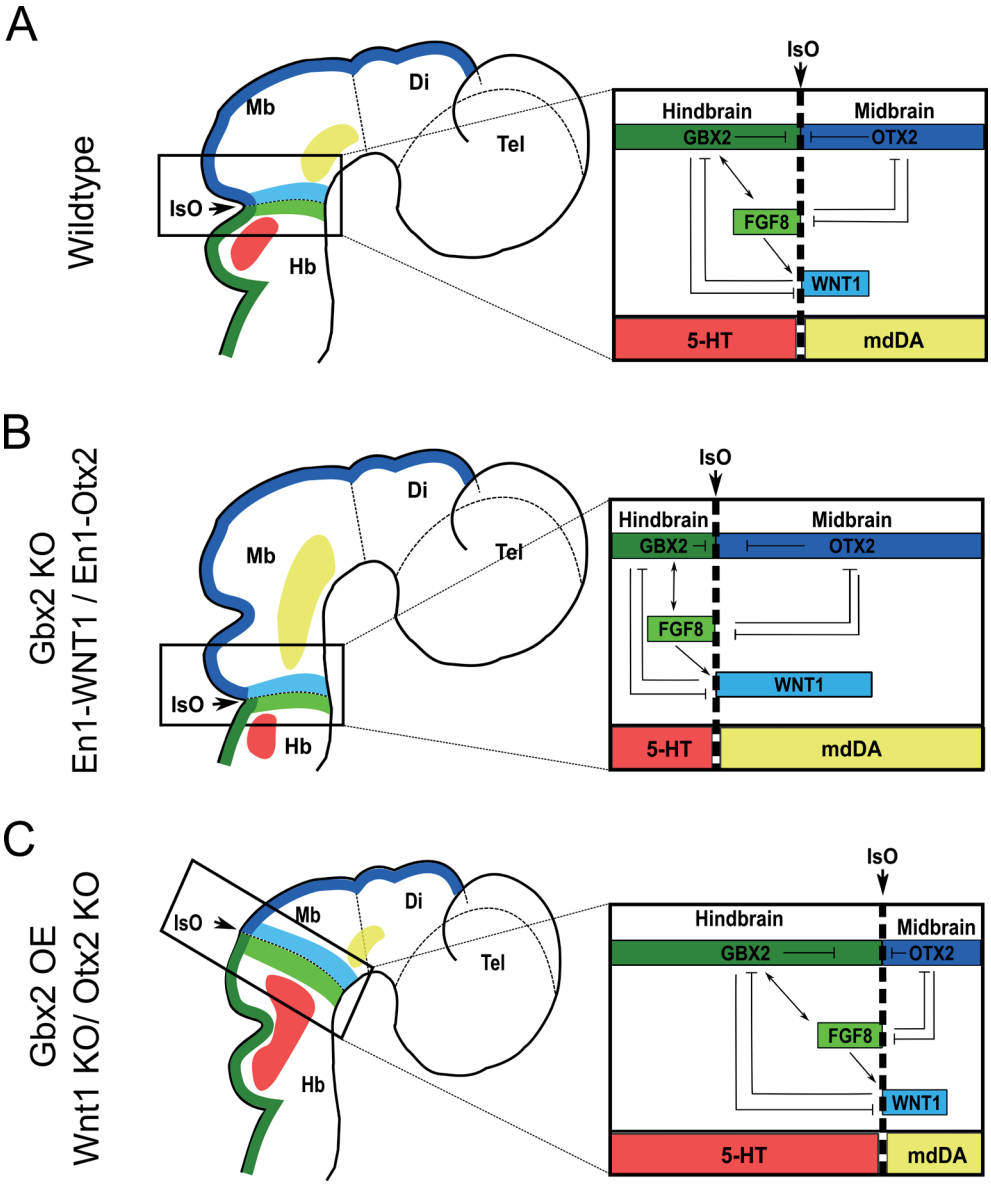

Figure 1: Schematic representation of the mid- hindbrain region in wildtype and several different genetically altered embryos. (A) In wildtype the IsO is formed at the juxtaposition of *Otx2* and *Gbx2* expression and requires both *Fgf8* and *Wnt1* for its proper functioning and maintenance. The 5-HT permissive region is located caudal to the IsO in the hindbrain, while mdDA neurons will develop rostral to the IsO in the midbrain. (B, C) Genetic alterations of *Otx2, Wnt1* and *Gbx2* influence the location of the IsO and the size of the 5-HT and mdDA permissive region. (B) Repression of *Gbx2* and over-expression of either *Otx2* or *Wnt1* re-localizes the to a more caudal position, leading to a larger mdDA domain at the expanse of the 5-HT system. (C) An opposite affect is observed when *Otx2* or *Wnt1* are repressed or *Gbx2* is ectopically expressed in the midbrain. The IsO shifts to a more rostral position, limiting the mdDA permissive region and expanding the 5-HT population rostrally

Early specification of mdDA neurons

MdDa progenitors are thought to originate from floor plate cells that develop at the ventricular zone (VZ) of the ventral mesencephalon. Although floor plate cells are characterized as non-neurogenic cells, previous studies have shown that mesencephalic floor plate cells can acquire mdDA progenitor identity under the influence of OTX2 and WNT1 (**Figure 2A, D** dark blue to light blue cells) (Chung et al., 2009; Joksimovic et al., 2009; Mesman et al., 2014; Ono et al., 2007). The conferment of neurogenic potential on mesencephalic floor plate cells is probably mediated by the induction of *Lmx1a* expression by OTX2 and WNT1. LMX1A is required for the stimulation of neurogenesis by activating *Msx1*, followed by the proneural transcription factor *Neurogenin2* (*Ngn2*), in cooperation with MSX1 (**Figure 2D,** light blue to dark green cell) (Andersson et al., 2006; Joksimovic et al., 2009; Omodei et al., 2008; Ono et al., 2007; Vernay et al., 2005). In addition to the control of neurogenesis, the *Wnt1/Otx2/Lmx1a* pathway is also involved in the developmental programming of mdDA progenitors by suppressing alternative cell fates and maintaining the expression of transcription factors involved in the induction of the DA neurotransmitter phenotype (Andersson et al., 2006; Chung et al., 2009; Omodei et al., 2008; Ono et al., 2007; Vernay et al., 2005). During neurogenesis mdDA progenitors in the VZ enter the G_0 phase of the cell cycle and give rise to post-mitotic mdDA neurons that start migrating to their specific ventral position first via radial migration (**Figure 2B, 1)**, followed by tangential migration (**Figure 2B,2**) (Kawano et al., 1995; Shults et al., 1990). During the migratory phase mdDA neurons start expressing the DA synthesizing enzyme *Aadc* and the orphan nuclear receptor *Nurr1* (Smidt and Burbach, 2007). *Nurr1* is essential for the induction of neurotransmitter-phenotype related genes, including *Th, Vmat2* and *Dat*, in mdDA progenitors and loss of *Nurr1* leads to a developmental arrest of mdDA progenitors and cell death (Saucedo-Cardenas et al., 1998; Smits et al., 2003b; Zetterström et al., 1997). For the establishment of the proper neurotransmitter phenotype NURR1 requires the presence of the paired-like homeobox gene *Pitx3*, which acts as an activator of the NURR1 transcriptional complex (**Figure 2D,** green cell) (Hwang et al., 2003; Jacobs et al., 2009; Munckhof et al., 2003; Nunes et al., 2003; Saucedo-Cardenas et al., 1998; Smidt et al., 2004; Smits et al., 2003b). Most post-mitotic DA neurons are born between E10.5 and E13.5, with a peak in neurogenesis around E12.5 (Bayer et al., 1995; Bye et al., 2012) and by E14.5 most genes that define a mature mdDA neuron are expressed (Arenas et al., 2015; Iversen, 2010).

Terminal differentiation and subset specification

As described above, the mdDA neuronal population consist of different subsets with distinct function and selective vulnerability for neuronal degeneration. The different mdDA subsets can already be distinguished during embryonic development based on

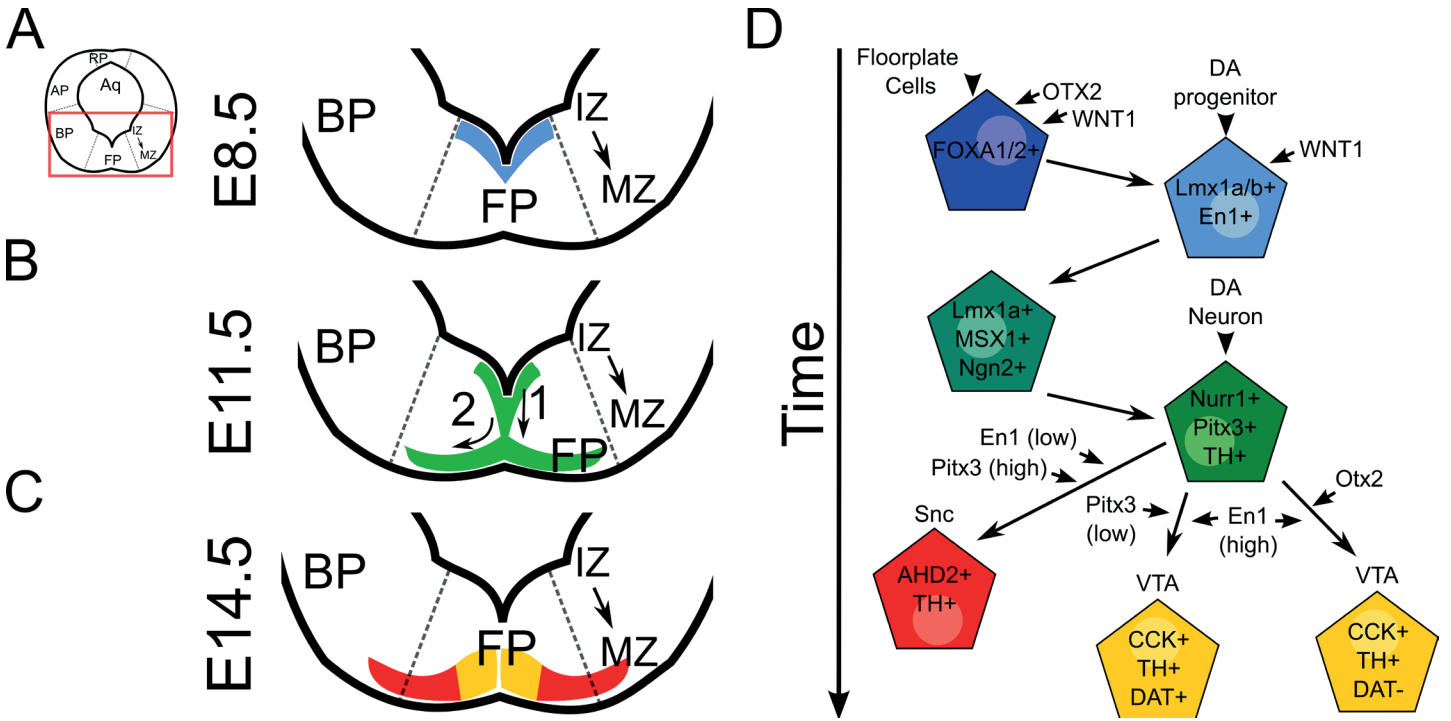

Figure 2: Schematic overview of the differentiation of mdDA neurons. (A) mdDA neurons originate from the floorplate of the midbrain around E8.5. (B) During neurogenesis cells exit the cell-cycle and start migrating towards their final location in the MZ. Post-mitotic cells first migration down to their proper ventral-basal position via radial glial (1), then lateral via tangential migration (2) to reach their final location. (C) By E14.5 most mdDA neurons have reached their final location and form distinct anatomical subsets (red and yellow), that eventually give rise to the different subsets found in adult. (D) Specification of a floorplate to a subset specific neuron. Under the influence of *Otx2* and *Wnt1*, floorplate cells of the midbrain acquire a DA progenitor phenotype, which will give rise to a full NURR+/TH+ DA neurons. Specification of SNc and VTA neurons is dependent on the interplay of *Pitx3* and low levels of *En1* for the SNc and high levels of *En1* in a combination with either low levels of *Pitx3* or *Otx2* for the VTA.

their anatomical position and molecular profile (**Figure 2C, D** red and yellow cells) (Smits et al., 2013). It has been shown that the rostrolateral population, closely marked by the expression of *Ahd2*, will eventually form the largest part of the SNc, while the caudomedial population, characterized by the expression of *Cck*, is destined to become the VTA (Ho¨kfelt et al., 1980; Veenvliet et al., 2013). The proper anatomical location is reached via migration. As described above, migration is a multi-phase process, in which mdDA neurons initially migrate downward from the ventricular zone to the mantle layer along radial glial cells (**Figure 2B**, 1) (Shults et al., 1990). This form of migration is driven by the *Cxcr4* receptor and its ligand *Cxcl12* and genetic ablation of either one of these genes affects the migration of all mdDA neurons (Bodea et al., 2014; Yang et al., 2013). After arriving at their proper ventral/basal position, mdDA neurons start migrating laterally towards their proper location in the VTA or SNc alongside tangential fibers (**Figure 2B**, 2) (Bodea et al., 2014; Kang et al., 2010; Kawano et al., 1995; Xu et al., 2010). In contrast to radial migration, defective tangential migration mostly affects the formation of the SNc (Demyanenko et al., 2001; Kang et al., 2010). Tangential fibers express the neural cell adhesion molecule L1 and even though the total of number of TH+ neurons in L1 KO is not changed, the amount of cell found in the SNc is reduced, while more cells are located in the VTA (Demyanenko et al., 2001). In addition, the formation of tangential fibers has been found to be dependent of the extracellular matrix glycoprotein Reelin. Disruption of the *Reelin* gene lead to the loss of tangential fibers, which negatively affects the number of TH+ neurons in the SNc in p7 *Reeler* mutant mice, while the amount of TH+ cells in the VTA is increased (Kang et al., 2010). Next to accurate migration, recent studies have revealed that each subset is also dependent on different transcriptional programs for their development and survival (Jacobs et al., 2011; Kouwenhoven et al., 2017; Panman et al., 2014; Smits et al., 2006; Veenvliet et al., 2013). The rostrolateral population shows a specific dependency on *Pitx3* for it survival, as *Pitx3* deficiency leads to reduced levels of *Th* in the lateral midbrain at E12.5 and eventually to a selective loss of the mdDA neurons of the SNc in the adult midbrain (Hwang et al., 2003; Maxwell et al., 2005; Smidt et al., 2004). The induction of the rostrolateral fate is dependent on a complex interplay between *Pitx3* and *En1* (**Figure 2D**, red cell). *En1* is initially required for the initiation of the expression of general DA marks and modulation of *Pitx3* activity (Veenvliet et al., 2013). After the generation of a default DA neurons, PITX3 antagonizes the caudomedial phenotype by negatively regulating EN1 activity in the rostrolateral population (Veenvliet et al., 2013), while the remaining DA population will obtain a caudomedial fate under the control of *En1* and *Otx2* (**Figure 2D**, yellow cell) (Bye et al., 2012; Di Salvio et al., 2010; Panman et al., 2014; Veenvliet et al., 2013). Although not all elements involved in subset specification are described here and great progress has been made in establishing the underlying mechanisms of subset specification (extensively reviewed in (Veenvliet and Smidt, 2014)).

LMX1B in mdDA development

Lmx1b has been shown to be crucial for mdDA development and neuronal survival (Adams et al., 2000; Deng et al., 2011; Doucet-Beaupré et al., 2016; Laguna et al., 2015; Smidt et al., 2000). *Lmx1b* expression is initiated in a broad region in the anterior CNS at E8.0, and becomes progressively more restricted to three major areas, including a domain centered rostral to the IsO, where its expression overlaps with the rostral domain of *Fgf8* and the caudal region of *Otx2* expression surrounding the IsO (Adams et al., 2000; Guo et al., 2006; Smidt et al., 2000). Analysis of *Lmx1b* null mutants revealed that LMX1B is an essential component of a positive feedback loop required to maintain genes associated with the formation and functioning of the IsO, including *Wnt1, En1, En2, Pax2* and *Fgf8* (Adams et al., 2000; Guo et al., 2006). In the absence of *Lmx1b* the initiation, maintenance and inductive activity of the IsO were found to be severely impaired, affecting the development of the midbrain in general (Guo et al., 2006). Further analysis of *Lmx1b -/-* embryonic midbrains demonstrated a reduction in TH-expressing cells in the ventral mesencephalon (Deng et al., 2011; Smidt et al., 2000).Although the loss of *Lmx1b* initially seemed to primarily affect the lateral group of the DA progenitor domain (Deng et al., 2011), the medially located TH-expressing cells failed to induce *Pitx3* and were lost during further development (Smidt et al., 2000). In contrast to the *Lmx1b* null mutant, the conditional deletion of *Lmx1b* in mdDA progenitors, but not in the IsO, resulted in normal development of the mdDA system, suggesting that the mdDA phenotype observed in the *Lmx1b* null mutant is most likely a consequence of a defective IsO (Yan et al., 2011). However, *Lmx1b* has also been suggested to be functional redundant with *Lmx1a* in mdDA development, which might also explain the apparent absence of a phenotype. Several studies into the functional redundancy between the two protein showed that both LMX1A and LMX1B can regulate *Msx1* and *Wnt1* expression and influence the expression of *Nurr1* and *Pitx3* (Chung et al., 2009; Nakatani et al., 2010). In addition, a study into double mutants showed that in the absence of both *Lmx1a* and *Lmx1b* the proliferation of mdDA progenitors was affected and alternative cell fates were not suppressed properly (Yan et al., 2011). Next to being able to compensate for each others loss during mdDA development, *Lmx1a* and *Lmx1b* were also found to be able to compensate for each others function during neuronal maintenance and survival (Deng et al., 2011; Doucet-Beaupré et al., 2016; Nakatani et al., 2010; Yan et al., 2011). However, a recent study by Laguna *et al.* identified a critical role for *Lmx1b* in the maintenance of mdDA neurons. They showed that *DatCre* driven deletion of *Lmx1b* reduces the levels of DAT and TH in the nerve terminals of mdDA neurons in the dorsal and ventral Striatum of 18 months old mutant mice (Laguna et al., 2015). In addition, a progressive loss of DA neurons was observed in a *Lmx1a/Lmx1b* double mutant, which was attributed to *Lmx1b*, as *Lmx1a* seemed dispensable (Laguna et al., 2015)

A SECOND LEVEL OF REGULATION: EPIGENETICS

Epigenetics in mdDA development

In recent years it has become apparent that next to transcription factors, transcriptional regulation via epigenetics also plays an important part in the development of a multicellular organism (Bernstein et al., 2006; Mikkelsen et al., 2007; Mohn et al., 2008). Epigenetics can be defined as the biological processes that alter transcriptional activity by influencing the accessibility of the DNA by reorganizing the chromatin structure. Adaptation of a chromosomal region can rely on three distinct processes: (1) Modification of histones; (2) DNA methylation; and (3) non-coding RNAs mediated changes (Heesbeen et al., 2013).Whereas histone modifications are highly dynamic and cause readily reversible changes in chromosomal organization, DNA methylation is associated with stable long-term repression (Cedar and Bergman, 2009). For this reason modifications of histones are usually involved in the regulation of early developmental genes, while DNA methylation is associated with lineage commitment and maintenance (Podobinska et al., 2017). Genes involved in the development of the mdDA system have also been shown to be regulated by different types of epigenetic mechanisms (Heesbeen et al., 2013). During early stages of development the specification of the mdDA region is dependent on the interaction of several developmental genes (Danielian and McMahon, 1996; Joyner et al., 2000; Puelles et al., 2003; Sasaki et al., 1997). Genome-wide mapping of an activating histone mark and repressing marks in ESC and lineage-committed cells showed that the transcription start sites of most developmental genes, including genes involved in midbrain patterning, contain both types histone marks. These so-called bivalent chromatin domains silence the expression of these genes during one stage of development, but keeps them poised for activation during later stages of development (Bernstein et al., 2006; Mohn et al., 2008). In addition, histone acetyltransferases, CBP/P300, have been demonstrated to be recruited to regulatory sites of precursors-type genes, including *Lmx1a/b* and *Foxa2,* leading to transcriptional activation (Heesbeen et al., 2013; Ramos et al., 2010). An important step in the formation of mdDA neurons is the induction of the genes associated with the DA phenotype by *Nurr1*. Several studies into the function of *Nurr1* demonstrated that initially NURR1 is found in complexes that contain Sin3a and SMRT (Jacobs et al., 2009; Veenvliet et al., 2013). SMRT keeps the NURR1 transcriptional complex in a repressed state by recruiting a set of class I and class II histone deacetylases (HDAC), which leads to de-acetylated transcription start sites and inhibition of gene expression (Jacobs et al., 2009). Recruitment of PITX3 and /or EN1, to the NURR1 transcriptional complex induces the release of SMRT/HDAC mediated repression from NURR1 targets and consequently the initiation of expression of genes associated with the DA neurotransmitter phenotype (Jacobs et al., 2009; Veenvliet et al., 2013).

Polycomb repressive protein

Important epigenetic regulators are the Polycomb group (PcG) proteins. PcG proteins were initially identified as genes required for the appropriate expression of homeotic (*Hox*) genes in *Drosophila* (Buchenau et al., 1998; Lewis, 1978), but have been found to control the expression of hundreds of genes in insect and mammals (Bracken et al., 2006). PcG proteins are found in two different multi-protein complexes, Polycomb repressive complex (PRC) 1 and PRC2 (**Figure 3)**. Polycomb repressive complex (PRC) 1 is a highly heterogeneous complex responsible for the ubiquitination of Lysine 119 of Histone 2A (H2AK119Ub), a histone mark associated with gene silencing (Cao et al., 2005; Leeb and Wutz, 2007). All PRC1 type complexes contain an E3 ubiquitin ligase RING1A/B and a type of PCGF protein, associated by either CBX (**Figure 3A,1**) or RYBP subunits (**Figure 3A,2**) (Gao et al., 2012). CBX-containing PRC1 complexes are recruited to PcG target sites via the binding of tri-methylated Lysine 27 of Histone 3 (H3K27me3) by a CBX protein (Bernstein et al., 2006). In contrast, RYBP-containing PRC1 complexes mediates H2AK119Ub in a H3K27me3 independent manner (Tavares et al., 2012). H3K27me3 is mediated by PRC2 and is also associated with repression of transcriptional activity, similar to H2AK119Ub (Mohn et al., 2008; Shen et al., 2008). The mammalian PRC2 consist of three core subunits; Enhancer of zeste homolog 1 or 2 (EZH1/2), Embryonic ectoderm development (EED) and suppressor of zeste 12 (SUZ12), that are all crucial for the catalytic activity of the complex (**Figure 3B**) (Corley and Kroll, 2015).

In *Drosophila* PRC2 is recruited to Polycomb response elements (PREs), that contain clusters of DNA binding motifs recognized by a fixed set of DNA binding factors, including *Pho, Zeste* and *Gaga*, that recruit PRC2 to the DNA (Simon et al., 1993; Tolhuis et al., 2006). In mammals, a DNA sequence homologous to the *Drosophila* PREs seems to be absent and the exact mechanisms via which PRC2 is recruited towards it targets are yet to be determined. Recent studies into the recruitment of PRC2 to target genes in mammals demonstrated that CG-rich regions and CpG islands were shown to be essential for the initial localization of PRC2 Mendenhall et al., 2010). In addition, transcription factors and ncRNA's have been proposed as additional methods of PRC2 recruitment (Hosokawa et al., 2006; Khalil et al., 2009; Zhao et al., 2008).

The role of Polycomb repressive protein in neurodevelopment

Studies into the function of PcG protein in development showed that members of both PRC1 and PRC2 are involved in the regulation of several developmental transitions and that the function of PcG protein vary per developmental stage (Hirabayashi et al., 2009; Morimoto-Suzki et al., 2014; Pereira et al., 2010; Zemke et al., 2015). Loss-of-function studies showed that conditional removal of *Ezh2* (PRC2) or *Bmi-1* (PRC1) in cortical progenitors before the onset of neurogenesis affected the balance between self-renewal

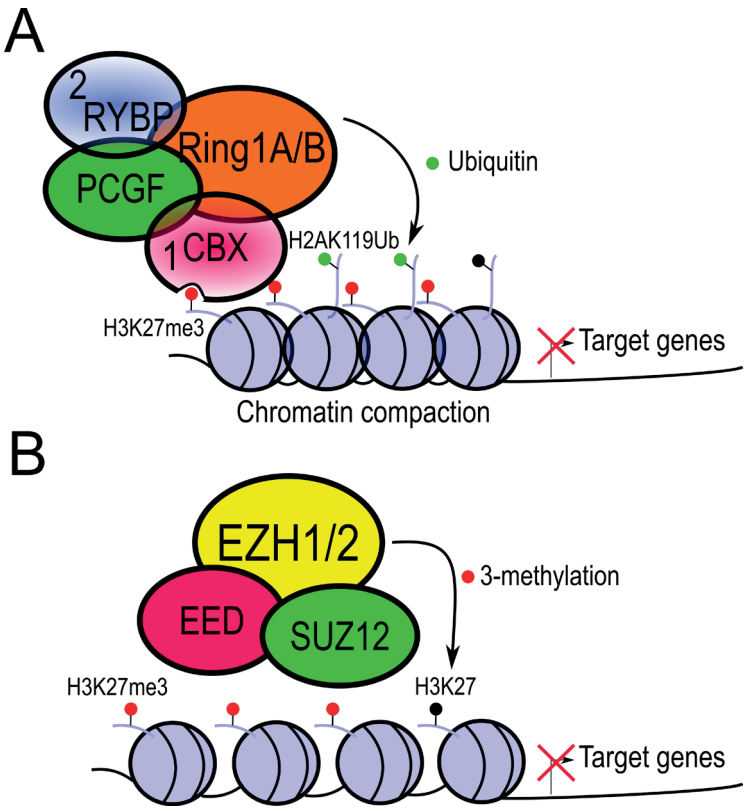

Figure 3: Overview of organization of PcG protein in multiprotein complexes. (A) The PRC1 has two major compositional forms; CBX protein containing (1) or RYBP protein containing (2). Further components of the PRC1 complex are a E3 ligase Ring1 (A or B) and a PCGF protein. CBX protein are capable of binding H3K27me3, which leads to the recruitment of PRC1 to PRC2 target genes. After binding the PRC1 complex mediates the mono-ubiquitination of H2AK119, causing chromatin compaction and gene silencing. (B) PRC2 exist of three main components; EED, SUZ12 and EZH (1 or 2). EZH functions as the methyltransferase of the complex and the complex as a whole mediates the methylation of H3K27, leading to the repression of gene expression.

and differentiation in favor of differentiation (Molofsky et al., 2003; Pereira et al., 2010). Furthermore, neurogenesis was accelerated and onset of gliogenesis was earlier (Pereira et al., 2010; Zencak et al., 2005) However, when *Ezh2* (PRC2) or *Ring1b* (PRC1) were removed during neurogenesis, the neurogenic phase was prolonged at the expense of the onset of astrogenesis (Hirabayashi et al., 2009). In addition, genetic ablation of *Ring1B* at E13 in the cortex prolonged the production period of CTIP2+ subcerebral projection neurons, increasing their numbers in the neocortex (Morimoto-Suzki et al., 2014). Similar results were obtained for other brain regions. *Wnt1Cre* driven deletion of *Ezh2* led reduced number of NPs in the dorsal midbrain, due to elevated cell cycle

exit and differentiation (Zemke et al., 2015). In addition, analysis of the *Bmi-1* null mutant showed reduced thickness of the cerebellar cortex and decreased cell-density in the cerebellum (Lugt et al., 1994). Next to controlling the duration of developmental phases, PRC2 has also been associated with neuronal migration. Genetic ablation of *Ezh2* in cortical progenitors affected neuronal tangential migration by influencing *Reelin* expression (Zhao et al., 2015). In addition EZH2 was found to be required to maintain the tangential migratory program of pontine neurons (Di Meglio et al., 2013), suggesting that PRC2 influences tangential migration of several different neuronal subtypes. An additional function of PcG protein is the maintenance of regional and neuronal identity. *Wnt1Cre* driven deletion of *Ezh2* also led to the up-regulation of forebrain traits in the midbrain, suggesting that PRC2-mediated repression is required for the perseverance of the proper regional identity (Zemke et al., 2015).
Besides a role in neurodevelopment, malfunctioning of several PcG protein have been associated with neurodegenerative diseases, including Huntington's and Parkinson's disease (Li et al., 2013; von Schimmelmann et al., 2016; Södersten et al., 2014). Loss of PRC2 mediated repression in post-mitotic cells led to the up-regulation of several key transcriptional regulators and genes involved in the maintenance of cellular homeostasis, eventually causing degeneration of these cells (Li et al., 2013; von Schimmelmann et al., 2016; Södersten et al., 2014).

AIM OF THIS THESIS

The aim of this thesis is to gain a better insight into the role of transcription factors and epigenetic mechanisms in the development and maintenance of the mdDA system. We concentrate on the role of the transcription factor *Lmx1b* during terminal differentiation and neuronal survival and investigate whether PcG protein are involved in the development and survival of mdDA neurons by studying mouse models in which *Ezh2* (PRC2) or *Cbx8* (PRC1) are conditionally deleted.

OUTLINE THESIS

Lmx1b is has been shown to be essential for the development of the mdDA system. Analysis of the *Lmx1b* null mutant showed that loss of *Lmx1b* leads to loss of TH-expressing cells in the ventral midbrain. However the effect observed on the mdDA population is probably caused by the effect of *Lmx1b* on the formation and functioning of the IsO. In **Chapter 2** we assessed the role of *Lmx1b* in the differentiation of mdDA neurons specifically, by generating a conditional model in which *Lmx1b* was conditionally

removed from mdDA neurons via a *Pitx3* driven *Cre*. We analyzed the presence of several DA marks at E14.5 and in adult stages and also examined the specification of the SNc and the VTA, by examining subset specific genes.

In **Chapter 3** we focus on the role of EZH2/ PRC2 in early midbrain patterning and the formation of the IsO. PRC2 mediates the tri-methylation of lysine 27 of Histone 3 and exist of three main subunits; Enhancer of zeste homolog 2 (Ezh2), Embryonic ectoderm development (EED) and suppressor of zeste 12 (SUZ12), that are all crucial for the catalytic activity of the complex. PRC2 has been shown to be important for the repression of forebrain traits in the dorsal midbrain. In addition PRC2 has been shown to be essential for the regulation of developmental transitions. In order to study PRC2 functioning in early mdDA development we made use of the CRE-Lox system and crossed *En1Cre* animals with *Ezh2*-flox animals.

In addition to a role in neurodevelopment, PRC2 functioning has also been linked to several neurodegenerative disorders and cancer. In **Chapter 4** we therefore studied the role of *Ezh2* in the neurogenesis of DA neurons, the specification of the different mdDA subsets and also in the survival of mdDA neurons. To study the impact of the loss of *Ezh2* on mdDA neurogenesis we crossed *Ezh2*-flox animals with *En1Cre* animals, deleting *Ezh2* in mdDA progenitors right before the start of neurogenesis. To determine whether *Ezh2* also plays a part in the terminal differentiation and survival of mdDA neurons we deleted *Ezh2* in post-mitotic mdDA neurons by making use of *a Pitx3* driven *Cre.*

PcG protein can be found in two major multi-protein complexes, PRC1 and PRC2. In **Chapter 5** we addressed the function of a PRC1 associated gene; *Cbx8,* in mdDA development. *Cbx8* has been shown to bind to the promoter of a considerable amount of genes involved in key pathways controlling development, neuronal differentiation and cell fate decisions. In addition *Cbx8* has been shown to compete with the histone methyltransferase *Dot1l* for the binding of AF9 or ENL, which are protein normally associated with active gene expression. To study the role of *Cbx8* in mdDA neurogenesis, differentiation, subset specification of maintenance we generated a conditional knock out for *Cbx8,* in which *Cbx8* was deleted by *En1Cre* and *Pitx3Cre* drivers.

In **Chapter 6** we summarize our main findings and place them in the larger context of mdDA development and the regulation of neurodevelopment by epigenetics. We will also discuss possible implementation for the obtained knowledge in the generation of mdDA neurons *in vitro* for the purpose of cell-replacement therapy in Parkinson's Disease.

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