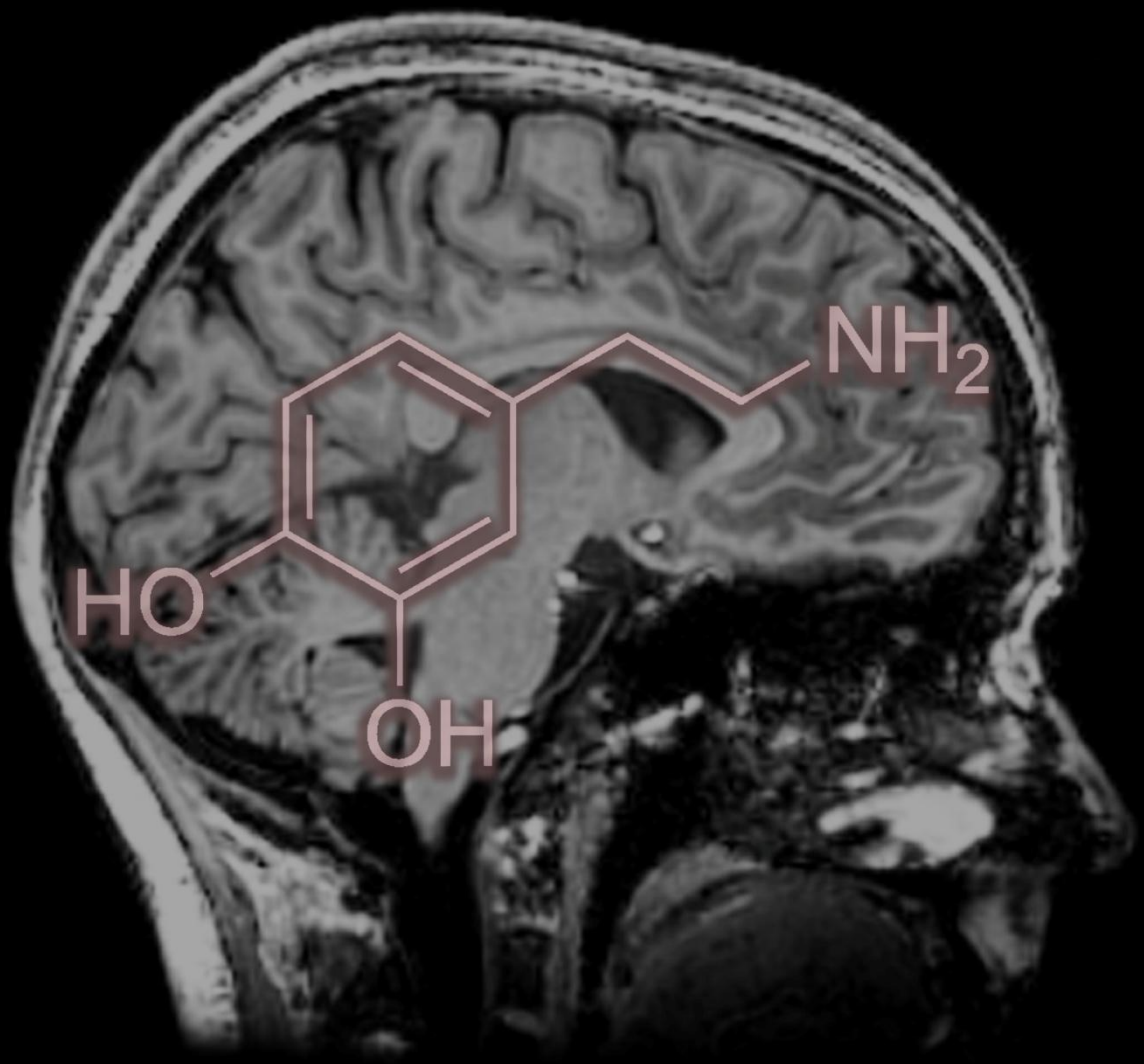
The development of the dopaminergic midbrain:

The importance in health and disease



Master Thesis

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Image on front page:

T1 weighted MRI image of my own brain with on top the chemical structure of dopamine.

Reference chemical structure: h*ttp://it.wikipedia.org/wiki/File:Dopamine_chemical_structure.png*

Content

Abstract

The dopaminergic midbrain consists of the substantia nigra, ventral tegmental area (VTA)and retrorubral field. These structures project to other brain areas via three dopaminergic pathways. These three pathways have different functions concerning voluntary movements, emotions and motivation. Several diseases are known to involve the dopaminergic system. These are Parkinson's disease (PD), schizophrenia, attention deficit hyperactivity disorder , autism spectrum disorders, substance use disorders and Lesch-Nyhan disease. In this thesis it is researched if these diseases have a origin in the development of the dopaminergic midbrain. Therefore the normal development of the dopaminergic midbrain is discussed. This development is highly regulated in a spatiotemporal manner by a high number of genes. When the molecular borders are formed the dopaminergic neurons are born in the ventricular zone, anterior from the isthmus. After that they start migrating towards the ventral midbrain, during this migration they also start to differentiate from precursor cells to immature dopaminergic neurons. When these cells reach the ventral midbrain they start to make connections to other brain parts, making them mature dopaminergic neurons. For all six diseases or disorders there is a relationship with the dopaminergic system. However it is not clear for all of these diseases or disorders if the origin lies in the developmental phase. Especially for PD and schizophrenia, who arise later in life, more research should be done about the development and maintenance of the brain after birth, during life. From genetic studies there are susceptibility genes found for a few diseases. But it is known that also environmental factors play a role in some diseases. It could be that they have an effect on dopaminergic development if exposed during pregnancy or childhood. Or maybe it alters the dopaminergic midbrain later in life. In conclusion, more research must be done to find more genes and mechanisms who are involved in de development of the dopaminergic midbrain. Moreover research about the developing brain must be expanded till adolescence. When more is known about the development, diseases involved in the dopaminergic midbrain will be better understood so the origination can be found. Moreover, potential new therapies can be developed.

Abstract for laymen

Dopamine is an important signaling molecule in the brain, used by some specific brain parts in the midbrain. These brain parts are involved in making voluntary movements, emotions and motivation. There are several diseases involved in the dopaminergic system, these are Parkinson's disease (PD), schizophrenia, attention deficit hyperactivity disorde , autism spectrum disorders, substance use disorders and Lesch-Nyhan disease. In this thesis it is researched if these diseases have a origin during the development of the dopaminergic midbrain. Therefore first the normal development of the dopaminergic midbrain is discussed. This development is a highly regulated process in which cells are born, change in appearance and move to a different place in the brain. During this movement the progenitor cells also start making connections to other brain parts to make them fully functional. For all six diseases or disorders there is a relationship with the dopaminergic system. However it is not clear for all of these diseases or disorders if the origin lies in the developmental phase. Especially for PD and schizophrenia, who arise later in life, more research should be done about the development and maintenance of the brain after birth, during adult life. Also environmental factors play a role, it could be that they have an effect on dopaminergic development if exposed during pregnancy or childhood. Or maybe it alters the dopaminergic midbrain later in life. In conclusion, more knowledge should be obtained about the normal development of the dopaminergic midbrain so it will be easier to understand how diseases originate and develop. Moreover, the development of the brain after birth, till adolescence should be studied more since also a lot of things happen in the brain then. When more is clear about the dopaminergic development and how diseases arise, hopefully a therapy can be found in the future.

Abbreviations

AADC	Amino Acid Decarboxylase
ADHD	Attention Deficit-Hyperactivity Disorder
ALDH	Aldehyde Dehydrogenase
ApoER2	APoE receptor 2
ASD	Autism Spectrum Disorders
BDNF	Brain-derived Neurotrophic Factor
CNS	Central Nervous System
CNV	Copy Number Variations
COMT	Catechol-O-Methyl Transferase
CRHR1	Corticotrophin-releasing hormone receptor 1
DAT	Dopamine Transporter
DCC	Deleted in Colorectal Cancer
DISC1	Disrupted In Schizophrenia 1
DR	Dopamine Receptor
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders edition four
DTI	Diffusion Tensor Imaging
EN1/2	Engrailed 1 or 2
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
Fz	Frizzled
GBX2	Gastrulation Brain Homeobox 2
GDNF	Glia cell-derived Neurotrophic Factor
GWAS	Genome Wide Association Study
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
HPA	Hypothalamic-pituitary-adrenal
HPRT	Hypoxanthine-guanine Phosphoribosyl transferase
LMX1a	LIM Homeobox Transcription Factor
LND	Lesch-Nyhan Disease
mdDA	Mesodiencephalic Dopaminergic neurons
MFB	Medial Forebrain Bundle
Nac	Nucleus Accumbens
NGN2	Neurogenin 2
NLGN	Neuroligin
NPN	Neuropilins
NRG1	Neuregulin 1
OTX2	Orthodenticle Homologue 2
PD	Parkinsons Disease
PDD-NOS	Pervasive Developmental Disorder - Not Otherwise Specified
PFC	Prefrontal Cortex
Ptc	Patched
RA	Retinoic Acid
RBF	Retrorubral Field
Sema	Semaphorin
SHH	Sonic Hedgehog
Smo	Smoothened
SNAP25	Synaptosomal-associated Protein of 25 kDa
SNc	Substantia Nigra Pars compacta and pars reticulata
TGFβ	Transforming Growth Factor β
TH	Tyrosine Hydroxylase
VLDLR	Very-low Density Lipoprotein Receptor
Vmat2	Vesicular Monoamine Transporter 2
VTA	Ventral Tegmental Area
WNT5a	Combination of wingless in *Drosophila* and int1 (integration 1, which is activated by WNT)

1. Introduction

The dopaminergic midbrain consists of multiple structures, including the substantia nigra pars compacta and pars reticulata (SNc), ventral tegmental area (VTA) and retrorubral field (RBF). These structures contain anatomically and functionally distinct subgroups of mesodiencephalic dopaminergic (mdDA) neurons (Van den Heuvel & Pasterkamp, 2008). These neurons project to multiple other brain area's via three different dopaminergic pathways. First, the nigrostriatal or mesostriatal pathway which connects the lateral neurons in the SNc with the dorsal striatum and caudate putamen. This pathway is involved in control of voluntary movements (Alves dos Santos & Smidt, 2011; Van den Heuvel & Pasterkamp, 2008). Second, the mesolimbic pathway connecting the VTA to the nucleus accumbens (NAc), amygdala, the bed nucleus of the stria terminalis, the lateral septal area and the lateral hypothalamus (Dichter, Damiano, & Allen, 2012). The third pathway is established between the VTA and prefrontal cortex (PFC) and is called the mesocortical pathway, these last two pathways are involved in emotions and motivation, see figure 1 (Dichter et al., 2012).

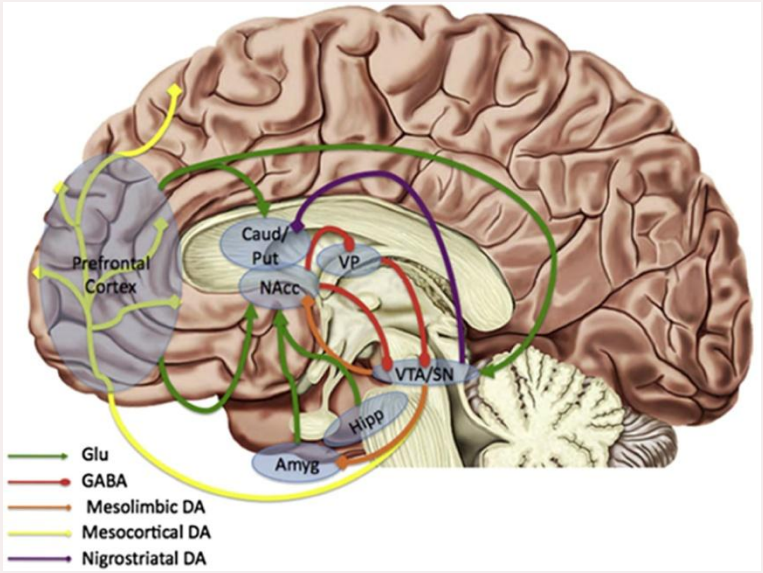

Figure 1: The dopaminergic system. The dopaminergic neurons in the VTA and SN project via three pathways, the mesolimbic pathway (orange) to the NAc, the mesocortical pathway (yellow) to the cortex and the nigrostriatal pathway (purple) to the caudate putamen. Next to the dopaminergic system there are excitatory glutaminergic projections (green) from the prefrontal cortex, amygdala and hippocampus to the NAc and putamen. The NAc in its turn projects inhibitory GABAergic signals (red) to the ventral pallidum so inhibition of the VTA is suppressed. Hereby phasic burst firing of VTA dopaminergic neurons is facilitated. Note: placement of structures is only approximate. Adapted from (Dichter et al., 2012)

Multiple diseases or syndromes exist involving the dopaminergic midbrain. Like Parkinson's disease (PD) whereby the DA neurons in the SNc degenerate, giving rise to motor problems (Bossers et al., 2009). Also psychiatric disorders like schizophrenia, autism spectrum disorders (ASD) and attention deficit-hyperactivity disorder (ADHD) are associated with the dopaminergic midbrain.

In this thesis I will first discuss what is known so far about the development of the dopaminergic midbrain whereby the formation of the molecular borders, migration of the progenitor cells and differentiation of the mdDA neurons will be discussed, as well as how the above mentioned

connections are made by axon guidance. In the second part of this thesis six different diseases or disorders involving the dopaminergic midbrain, will be discussed and whether they could have their origin in the development of the dopaminergic midbrain.

2. Development of the dopaminergic midbrain

2.1 Formation of molecular borders

Early during embryonic development the central nervous system (CNS) is divided along the anterior-posterior axis in four major divisions, the fore- mid- and hindbrain and the spinal cord (Altmann & Brivanlou, 2001). For mdDA neuronal development the midbrain-hindbrain border (MHB), also called isthmus, is important. For the establishment of the correct position of the isthmus several factors like orthodenticle homologue 2 (Otx2) and gastrulation brain homeobox 2 (Gbx2), transforming growth factor-β (Tgfβ), retinoic acid (RA) and Wnt5A are necessary (Altmann & Brivanlou, 2001; Smidt & Burbach, 2007).

The isthmus is a source of inductive signals which are essential for the formation of the midbrain. Examples of these signals are fibroblast growth factor 8 (Fgf8), Wnt1 and Hes1 (Baek, Hatakeyama, Sakamoto, Ohtsuka, & Kageyama, 2006; Kameda, Saitoh, & Fujimura, 2011; Smidt & Burbach, 2007). Fgf8 is expressed together with other factors like sonic hedgehog (Shh), LIM homeobox transcription factor (Lmx)1a/b, OTX1/2 and Nkx6.1. These factors from the ventricular zone determine the fate of newborn neurons at specific locations along the mesodiencephalic axis and give the first signals for differentiation to dopaminergic neurons (Simeone et al., 2011; Smidt & Burbach, 2007).

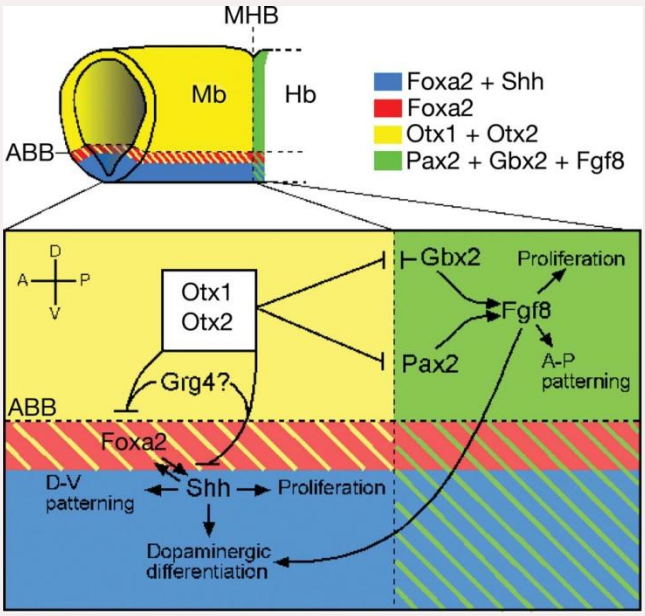

Figure 2: Overview (not complete) of several genes patterning the anterior-posterior and the dorso-ventral axes. Otx1 and 2 are responsible for the proper positioning of signaling molecules such as Fgf8 at the MHB and Shh in the floor plate in the midbrain (Mb). By this dose-dependent antagonism, Otx1 and 2 contribute to the establishment of the identity, fate and extent of midbrain progenitor cells. In this process positioning of Shh and Fgf8 could need the Otx-dependent repression of Gbx2 and Pax2 at the MHB. Moreover, maybe Foxa2 is needed in the proximity of the alar-basal boundary (ABB) for this establishment. Hereby Otx1 and 2 might need co-repressing activity of Grg4. Adapted from (Simeone et al., 2011).

Wnt1 is expressed continuous to Fgf8 rostrally of the isthmus and together these signaling molecules are required for the maintenance of the mid-hindbrain border (Canning, Lee, Irving, Mason, & Jones, 2007; Kameda et al., 2011; Partanen, 2007). Fgf8 and Wnt1 are dependent on each other by a regulatory loop whereby the Fgf8 expression is dependent on the Wnt signaling pathway, stimulated by Fgf itself (Canning et al., 2007). Moreover, *Hes* genes are involved in the development of cells in the isthmus, hereby regulating the formation of the midbrain and anterior hindbrain (Kameda et al., 2011).

Next to the anterior-posterior axis, also the dorsoventral axis is formed. Here, the expression of Shh in the floor plate and bone morphogenetic protein (BMP) from the roof plate are the most important (Alves dos Santos & Smidt, 2011). Shh and other genes specific for the floor plate or ventrolateral sides are regulated by Foxa2. Foxa2 works as a feedback regulator to modulate the level and duration of Shh signaling by repressing multiple downstream molecules like Gli1-3, transcription factors Nkx2.2 and Nkx2.9 and the Shh receptor Ptch1. Shh in its turn can activate Foxa2 via Gli1 and Gli2. This negative feedback loop is important for the floor plate identity, since a prolonged Shh expression leads to a more dorsal midbrain identity (Metzakopian et al., 2012). For a partial overview see figure 2.

By the determination of these molecular borders a specific environment is created with the expression of, or presence of above mentioned molecules. In this environment in the neuroepithelium along the mesencephalic flexure the mdDA precursors are born(Smidt, Smits, & Burbach, 2003, 2004). This happens between E11 and E12 in rats, corresponding with day E9-E10 in mice (Ratzka, Baron, Stachowiak, & Grothe, 2012; Simon, Bhatt, Gherbassi, Sgadó, & Alberï, 2003; Wallén & Perlmann, 2003). These cells do not express dopaminergic markers yet, first they become post mitotic where after differentiation and migration can start (Smidt et al., 2003, 2004).

2.2 Migration of mdDA neurons

When the dopaminergic precursors enter the post mitotic phase in the ventricular zone, they start differentiating during migrating towards the ventral midbrain. In the ventral midbrain the terminal differentiation will take place (Smidt et al., 2004). Here first the migration will be discussed, in the next chapter the differentiation although these processes take place at the same time.

So far, only little research is done concerning the migration of young mdDA neurons from the ventricular zone towards the rostral diencephalon. Migration takes place in two stages (Ohyama et al., 1998; Vasudevan et al., 2012). First, the young dopaminergic neurons migrate ventrally to the ventromedial mesencephalon, thereafter they move on perpendicularly in the basal part of the ventral mesencephalon, hereby giving rise to the distinction of the VTA and SNc (Ohyama et al., 1998; Vasudevan et al., 2012). See figure 3.
By comparing wild type and Pitx3 knockout mice (the Aphakia mouse), it is found that by the absence of Pitx3, the migration of neurons is stuck in the middle of the route, where the dopaminergic neurons are abnormally distributed in the red nucleus. Moreover, these cells showed impaired differentiation demonstrated by the expression of dopaminergic progenitor markers like Otx2, Lmx1b and Foxa2. But not for TH, a marker for mature dopaminergic neurons. This migration defect gives

rise to severe loss of DA neurons in the SNc (Vasudevan et al., 2012). How the knockout of Pitx3 exactly leads to the defect is not mentioned. Pitx3 is important in the differentiation of dopaminergic neurons (Smidt & Burbach, 2007). So the hypothesis could be that because the neurons are not differentiated completely, migration cannot proceed. Although it could also be the other way around that because of the hampered migration, cells miss the specific signaling from the right environment, so differentiation is not completed. But what this research made clear is that perpendicular migration is therefore essential to set up the proper anatomical architecture of ventral mesencephalic structures.

Next to signaling molecules, other cells like glial cells are used by the mdDA neurons to find their way (Ohyama et al., 1998; Smidt & Burbach, 2007). Hereby cell adhesion molecules (CAMs) are used. Ohyama *et al.* (1998) found the expression of L1CAM on the migrating DA neurons and neurepithelial cells making a hemophilic binding to allow tangential migration of the neurons. The laterally migrating neurons do not express L1CAM, but do express a L1CAM receptor, 6B4 PG, which binds L1CAM in a heterophilic way. By these expression differences, a distinction can be made between the two groups of neurons migration laterally or tangentially (Ohyama et al., 1998).

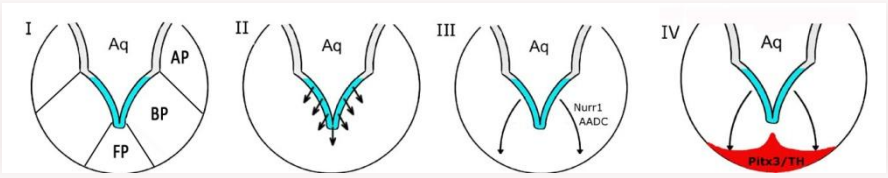

Figure 3: Graph showing the migration of dopaminergic precursor cells. I. A molecular code within the mes-diencephalic neuroepithelium (blue) defines areas that produce dopaminergic progenitors. II. The post-mitotic cells start to differentiate and migrate ventrally via radial-glial processes. III. The first dopaminergic markers (Nurr1 and AADC) are being expressed by the young neurons. IV. When the young neurons arrive at the ventral mes-diencephalon differentiation is completed and neurons express the complete dopaminergic phenotype (red) with i.e. TH and Pitx3. AP Alar Plate, BP Basal Plate, FP floor plate. Adjusted (used original figure partly and added AP, BP and FP)from (Smits et al., 2006).

Reelin, an extracellular matrix glycoprotein is also involved in neuronal migration of mdDA progenitor cells. Reelin is secreted by specific neurons and binds its receptor on other neurons. These receptors are very-low density lipoprotein receptor (VLDLR) and the ApoE receptor 2 (ApoER2)(W.-Y. Kang et al., 2010). When bound to one of these receptors, the cytoplasmic Dab1 is phosphorylated which leads to modifications of the cytoskeleton and altered gene expression of the target neurons (W.-Y. Kang et al., 2010). It was shown by investigating the Reelin knock-out mouse (the *Reeler* mouse) that mainly the lateral migration in the ventral mesencephalon was affected. The underlying mechanism how the lack of Reelin leads to a default in lateral migration is not understood, although it is hypothesized that the mdDA neurons fail to loosen from the radial glial cells (W.-Y. Kang et al., 2010). That knock-out mice are valuable is also shown in the deleted in colorectal cancer (Dcc) mutant, who show a migration deficiency in the first step, such that the mdDA precursors were not able to reach the ventral mesencephalon (Xu et al., 2010).

2.3 Differentiation of mdDA neurons

During the migration of the precursor cells they start to differentiate to mature dopaminergic neurons. For the differentiation of mdDA neurons multiple signaling cascades are necessary to get a correct number of differentiated mdDA neurons which are maintained throughout life.
In mice of E10, when migration starts, the precursor cells express Engrailed 1 and 2 (En1/2), Lmx1a/b and Foxa1/2 (Alves dos Santos & Smidt, 2011).En1 is one of the first transcription factors expressed at E8 in all mdDA progenitors, followed by En2 half a day later which is only expressed in a small subset of progenitors (including cells which become the SNc later). Both genes are involved in multiple mechanisms throughout development en during adulthood, like the organization of the isthmus, morphogenesis of the midbrain around E9-10, maintenance of the precursors around E10-12 and late mdDA differentiation around E14. During adulthood En1/2 is involved in the maintenance of the mdDA neurons (Alves dos Santos & Smidt, 2011).
Lmx1a and Lmx1b have a role in the differentiation of the progenitor cells to immature dopaminergic neurons and the exit from the cell cycle. Lmx1a is especially concerned in differentiation of the medial precursors (the future VTA), whereas Lmx1b is more involved in the differentiation of the lateral precursors which will later give rise to the SNc (Deng et al., 2011). Lmx1a/b expression is regulated by Shh directly or via Otx2 (Simeone et al., 2011). Followed by the activation of Msx1 by Lmx1a/b, which in turn activates Neurogenin 2 (Ngn2) (Simeone et al., 2011; Yan, Levesque, Claxton, Johnson, & Ang, 2011). Ngn2 finally lets the progenitors differentiate into post-mitotic immature mdDA neurons expressing Nurr1 at mouse E10.5 (Alves dos Santos & Smidt, 2011; Simeone et al., 2011). Ngn2 has also regulatory properties during earlier stages, like activating NeuroD1, hereby giving the progenitor cells in the intermediate zone their neuronal fate (Andersson, Jensen, Parmar, Guillemot, & Björklund, 2006).
The earlier mentioned homeobox protein Otx2 has multiple functions during mdDA development. As indicated, it controls Lmx1a/b expression, regulating differentiation, especially for a specific subset of future VTA neurons (Ellisor, Rieser, Voelcker, Machan, & Zervas, 2012; Simeone et al., 2011). A second function of Otx2 is the control on proliferation of the mdDA neurons by repressing Gbx2, preventing activation of Nkx2.2. By this action the progenitor cells will not mature into serotogenic neurons (Giovannantonio et al., 2012; Simeone et al., 2011). Furthermore, Foxa1/2 play a role in early differentiation of the progenitor cells in the floor plate.

Both genes cooperate in a dose-dependent manner to regulate the expression of multiple regulatory genes, like Shh. Moreover, Foxa2 regulates Lmx1a/b and Ferd31 which inhibits Hes1, a member of

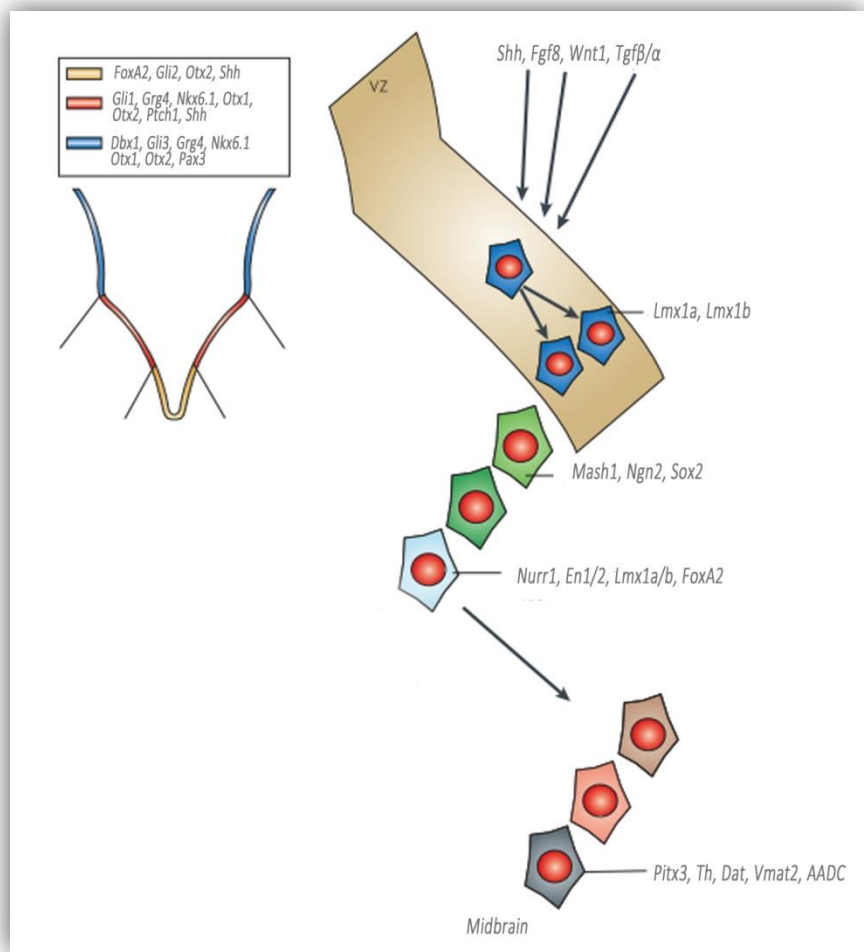

Figure 4: Gene expression during migration and differentiation of dopaminergic precursor cells. A schematic overview of a coronal sextion of mouse E12.5 where all developmental stages are present. In the VZ the cells divide and the start of differentiation starts here and ends at the ventral midbrain where there is also axon outgrowth. The generation of mdDA neurons is dependent on the molecular patterning in the dorsoventral and rostrocaudal axis. In the box the genes that are important for the dorsoventral instructive signaling are indicated. During all stages (at the VZ, migration path and site of terminal differentiation in the VM) other important genes are indicated next to it. Adjusted from: (Smidt & Burbach, 2007)

the family of *Hes* genes which are responsible for the right number of neurons and glia cells and the correct timing of differentiation of mdDA neurons by suppressing the differentiation till the appropriate moment. Hes1 functions by suppressing basic helix-loop-helix (BHLH) transcription factors like Mash1 and Neurogenin 2 (Ngn2) and Neurog2 (Kameda et al., 2011; Metzakopian et al., 2012).Another signaling molecule inhibiting Hes1 (next to neurogenic transcription factor Hes5 and inhibitory transcription factor Id3) is fibroblast growth factor 15 (Fgf15), which is the murine homologue of FGF19 in human). Fgf15 is important for controlling the cell cycle exit of the progenitors and regulate their differentiation into dopaminergic neurons. By repressing these neurogenic and inhibiting transcription factors the progenitors stay in their undifferentiated state till they reach the right spatiotemporal point. At the same time proneural genes like Mash1, Neurog and Ngns are activated to promote cell cycle exit and initiate neurogenesis (Fischer et al., 2011).

From around E11.5 the cells start to express Pitx3, which is essential for the terminal differentiation of the immature dopaminergic neurons. This homeobox protein is activated by glia cell-derived neurotrophic factor (GDNF), which is activated by TGFβ (Peng et al., 2011; Smidt & Burbach, 2007). At the same time the cells start to express TH, an enzyme involved in the production of dopamine (Kameda et al., 2011; Smidt et al., 2004). Also other proteins involved in the synthesis, uptake and transport of dopamine are expressed, like the dopamine transporter (DAT), vesicular monoamine transporter2 (Vmat2), amino acid decarboxylase (AADC) and brain-derived neurotrophic factor (BDNF) which are responsible for the maintenance of the mdDA neurons during the entire life. The expression of these proteins are regulated by the cooperation of Pitx3 and Nurr1 (Alves dos Santos & Smidt, 2011; Baron et al., 2012; Park et al., 2006; Peng et al., 2011; Volpicelli et al., 2012). It should be mentioned that BNDF is only expressed in a subpopulation of the SNc, which makes it an interesting candidate gene involved in Parkinson's disease (Peng et al., 2011).
So, a central key player in this web of cascades is Nurr1, a transcription factor expressed from E10.5 in mice and persisting during adulthood (Volpicelli et al., 2012). In the presence of Nurr1, Mash1 facilitates neuronal differentiation, in contrast to Ngn1/2 and neuroD (NeuroD1), which inhibit Nurr1-mediated induction of dopaminergic differentiation (Fischer et al., 2011; Park et al., 2006). Another gene involved in the terminal differentiation and maintenance of the mdDA neurons is Fgf receptor 1 (Fgfr1). This receptor has two working mechanisms. First it is expressed at the membrane of the progenitor cells and can bind Fgf, hereby stimulating proliferation of the cells. Secondly, a nuclear version of Fgfr1 can activate transcriptional activators, like via Nurr1, so TH expression is promoted (Baron et al., 2012).One of the Fgf's is Fgf2, which is expressed in the ventral midbrain during development and in the SNc in adulthood. Its function is to increase the survival of the differentiated mdDA neurons (Ratzka et al., 2012).

The last key player is Wnt signaling, several proteins from this family of signaling molecules are involved in multiple aspects of the development of dopaminergic neurons where they are expressed in a tightly regulated spatiotemporal way. So is Wnt1 involved in the terminal differentiation at a later stage by regulating Lmx1a, Otx2 and Nurr1. Its especially critical for the development of the VTA (Alves dos Santos & Smidt, 2011; Ellisor et al., 2012). In contrast, Wnt2 regulates the proliferation of the dopaminergic progenitor cells during early differentiation. For an overview see figure 4.

2.4 Connectivity of mdDA neurons

After the migration of the mdDA progenitors, these neurons start to extend their axonal projections to complete the differentiation to mature dopaminergic neurons. As you can see in figures 5 and 6 the axonal processes first grow for a short distance in a dorsal direction, where they make a sharp turn ventrally. This direction is followed by a dorsorostral growth through the diencephalon and towards their final destinations, the PFC and striatum (Van den Heuvel & Pasterkamp, 2008). During this process the axons grow in a bundle, the medial forebrain bundle (MFB). To steer this bundle in the right direction the brain uses multiple kinds of axon guidance molecules like neurotrophic factors such as GDNF, morphogens such as Wnts and Shh, transcriptionfactors, proteoglycans, leucine-rich

repeat proteins and neurotransmitters (Van den Heuvel & Pasterkamp, 2008). The most decisive molecules involved in axon guidance will be discussed below.

First the semaphorins, this family of secreted or transmembrane proteins consists of 7 classes, of which classes 1 and 2 are expressed in invertebrates and classes 3-7 in vertebrate species. Semaphorins bind their receptors, Plexins with or without Neuropilins (Npn) as co-receptor. Both Plexins and Neuropilins are transmembrane proteins (Van den Heuvel & Pasterkamp, 2008). Sema3A is strongly expressed around E13-14 in the midbrain of mice, by this time the mdDA axons are growing towards the striatum. The expression increases from dorsal to ventral. The idea that Sema3A is involved in mdDA axon targeting is strengthened by the finding that the receptors PlexinA1 and A3 are expressed by the mdDA axons in the MFB.

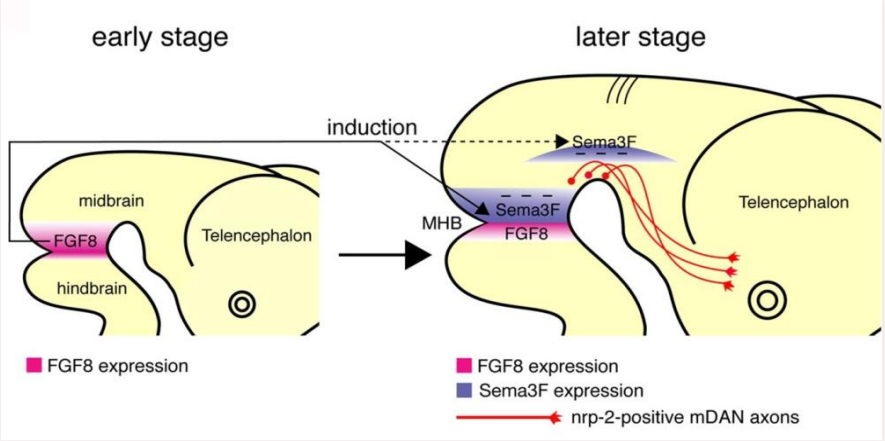

Figure 5: Model diagram of the regulation of axonal growth polarity of mDA axons by FGF8 and Sema3F signaling. In early stages, MHB-derived FGF8 induces the *Sema3F* expression at the MHB. Later, the axons of differentiated mdDA neurons are guided rostrally by sema3F. Sema3F also provides the dorsal nonpermissive/repulsive territory to mdDA axons, defining the dorsal border of these axons. Adapted from (Yamauchi et al., 2009).

However, an almost normal phenotype was seen in the Nrp1 or Nrp2 knockout mice. Therefore it is suggested that Sema3A has a more subtle role as in organizing the termination of the axons in the striatum (Torre, Gutekunst, & Gross, 2010). Semaphorin 3F (Sema3F) is one of the best studied semaphorin regarding to dopaminergic connectivity. It is shown that there is an expression gradient, decreasing from caudal to rostral (Torre et al., 2010; Van den Heuvel & Pasterkamp, 2008). And that the Npn2 and Plexins A1 and A3 are expressed on the mdDA neurons (Torre et al., 2010; Van den Heuvel & Pasterkamp, 2008; Yamauchi et al., 2009).Sema3F can give rise to repulsive or attractive signaling, which depends on the timing and placing during development. It is proposed by Kolk *et al*. (2009) that in proximity of the MFB trajectory Sema3F is expressed, repulsing the axons to keep them in the right track in a narrow bundle (Kolk et al., 2009; Torre et al., 2010; Yamauchi et al., 2009). Hereby Sema3F is induced by Fgf8, see figure 5 (Yamauchi et al., 2009). The subsequent part is not dependent on Npn2 signaling. The temporal gradient pushes the bundle into a rostral direction to the forebrain. Sema3F is also involved in innervating the prefrontal cortex by the mesoprefrontal mdDA axons, where they arrive in the subplate and wait for about 2 days. Hereafter the axons are attracted by Sema3F via Npn2 and are directed towards the pial surface. During the same time another subset of mdDA axons cross the striatum and developing external capsule to innervate the mPFC. Also this

process is mediated by Npn2, next to a repulsive ligand, although it is not known which ligand this is (Kolk et al., 2009).

Of course Sema3F is not the only molecule involved. Torre *et al.* (2010) states that Sema3F works together with Slit1 and Slit2, which are expressed in the ventricular zone and hypothalamus to guide the MFB to the striatum (Torre et al., 2010). In mammals three Slit genes (Slit1-3) and 3 receptors (Robo1-3) are identified (Dugan, Stratton, Riley, Farmer, & Mastick, 2011). That Slits signaling via Robo's is used for axon guidance is confirmed by the finding of the expression patterns of Robo1, which is expressed in the SNc and VTA and Robo2, which is more abundant in the SNc (Lin, Rao, & Isacson, 2005). An important function of Slit/Robo signaling is to prevent the axons from crossing the midline from the left to right hemisphere and vice versa (Dugan et al., 2011; Lin et al., 2005). Hereby Slits repel the axons to maintain the ipsilateral trajectory, this is compensated by a attractive signaling from Netrin1 and Shh (Dugan et al., 2011; Xu et al., 2010). More researchers showed that Slits are important for axon guidance whereby Slit/Robo signaling determine the dorsoventral topology and preclude that axons grow into the floor plate. Hereby Slit2 plays a major role which is expressed in the adjacent areas to the striatum (Dugan et al., 2011; Lin et al., 2005). Slit3 is only expressed from postnatal day 5 in rat, so does not play a role in development (Lin et al., 2005). *In vitro* it was shown that Slit2 has a repellent effect on dopaminergic neurite outgrowth, which is mediated by Robo receptors (Lin et al., 2005). So as said earlier, Slit2/Robo signaling is involved in limiting the dorsal side of the MFB together with Sema3F/Nrp2 signaling during the growth towards the rostral brain. Moreover, Slits and Robos also set a ventral boundary for the axons.

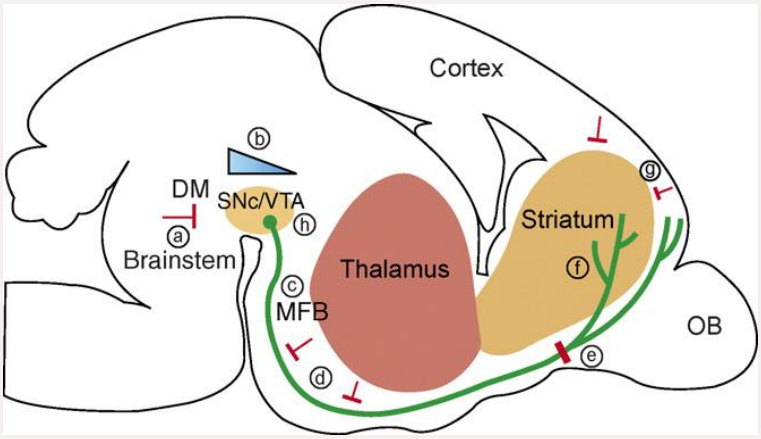

Figure 6: Overview of signals guiding the mdDA axons to their targets. a. Wnt5a is expressed in the rostral VM which around E11.5 inhibits outgrowth of the MFB out of the VM. Also Netrin and Dcc are expressed in the VM, but their role is not clear yet. b. Wnt5a expression changes to a caudal to rostral decreasing gradient around E14.5. Next to this there is a high caudal to low rostral gradient of Sema3F guiding the axons out of the VM and deflect rostrally. c. Slits in the VM and hypothalamus guide the MFB along a rostral trajectory and set the dorsal and ventral boundaries of the bundle. d. The thalamus cannot be entered by the axons through expression of repellent factors. One of these factors are probably Slits who prevent the axons from crossing the midline to the opposite hemisphere, facilitated by attractive signaling from Shh and Netrin1. Moreover, a Shh medial to lateral decreasing gradient makes a separation between the future nigrostriatal pathway and mesolimbic and –cortical pathways. e. Between E14 and E17 the mdDA axons accumulate ventral to the striatum at the boundary of the GE where they wait for 2 days. A role for Wnt5a is suggested as a repressing factor at the GE boundary. f. After the waiting period the GE and striatum start to attract the axons. Possible involved molecules are Slits, who are expressed adjacent to the striatum or Netrin and Dcc who are expressed in the striatum. g. To prevent inappropriate innervations of cortex areas there are repulsive signals expressed although it is not exactly clear which. VM ventral midbrain, MFB medial forebrain bundle, GE ganglionic eminence. Adapted from (Van den Heuvel & Pasterkamp, 2008).

This mechanism works by attracting and repelling, giving rise to a push/pull mechanism whereby the exact path of the axons is determined (Dugan et al., 2011).

Shh is a chemoattractant for mdDA neurons *in vitro* (Dugan et al., 2011). *In vivo* it is shown that Shh acts as a local guidance cue in the ventral hypothalamus, hereby guiding the medially projecting neurons (Hammond, Blaess, & Abeliovich, 2009). Shh acts via its receptor Smoothened (Smo) and co-receptor Patched (Ptc). There is a decreasing medial to lateral gradient of Shh expression, whereby the medial axons (later giving rise to the mesocortical and mesolimbic pathways) are distinguished from the lateral axons (which will form the nigrostriatal pathway) (Hammond et al., 2009).

Netrin1 is also an attractive molecule, interacting with DCC. However when Netrin1 interacts with the UNC5 receptor there is chemorepellent activity. Also the amount of cAMP in growth cones in the axons determine the responsiveness to Netrin1 (Lin et al., 2005; Xu et al., 2010).
Netrin1 and DCC are expressed in the ventral midbrain and striatum during development and adulthood, UNC5 is more expressed during adulthood (Xu et al., 2010).

The last to be discussed is the Wnt signaling. As described earlier, different Wnt molecules are involved during the whole development of dopaminergic neurons. During the period where the axonal connections are made, Wnt5a seems to be involved. Wnt5a signals via its receptor, Frizzled (Fz) and co-receptors, which are atypical tyrosine kinases, Ryk and Ror2 (Blakely et al., 2011). Research of Blakely *et al.* (2011) showed that Wnt5a is expressed during the development of the axonal pathways whereby it works as a chemorepellent at E11.5 in mice. This chemorepulsion is achieved via Fz and the non-canonical pathway. The mode of action of Wnt5a is time-dependent, shown in mouse cultures of ventral midbrain. At E11.5 Wnt5a promotes the extension of axons, but in contrast lets axons retract around E14.5 (Blakely et al., 2011). *In vivo* this might work in the following manner: the high expression of Wnt5a in the ventral zone and rostral ventral midbrain prevents the prematurely growth of axons anteriorly so the axons can elongate within the ventral midbrain. Around E14.5, the rostral expression decreases but expression increases more caudally, repelling the axons from the hindbrain towards the forebrain. Since *in vitro* Wnt5a can promote axon retraction, it is proposed Wnt5a has a function in the pausing of extension when the axons reach the ganglionic eminence (Blakely et al., 2011).At E18 there is still a low expression level of Wnt5a in the ventral midbrain for maintenance of the axons (Blakely et al., 2011). For an overview see figure 6.

3. Diseases involved in the dopaminergic midbrain

3.1 Parkinson's Disease

Parkinson's disease (PD) is the second neurodegenerative disorder with the highest prevalence after Alzheimer's disease, about 1% of the population above 65 years of age suffers from this disease (Bossers et al., 2009). PD is characterized by movement disorders, like tremors, rigidity, slowness of voluntary movements an postural instability (Bossers et al., 2009; Lewis & Cookson, 2012). These symptoms are caused by a loss of dopaminergic neurons in the substantia nigra pars compacta and by deposition of the protein α-synuclein as intracellular inclusions called Lewy bodies in the brain (Bossers et al., 2009; Cooper-Knock et al., 2012; Lewis & Cookson, 2012). Although the disease is known for centuries, and lot of research is done the last decades, the exact cause of the specific cell death is not found.
In the last decade many gene expression profiling studies are done to investigate differences in gene expression of Parkinson's patients compared with healthy controls. Multiple approaches are used like microarray based gene-by-gene expression, exon microarray analysis and transcriptome RNA sequencing (Lewis & Cookson, 2012). Moreover, different kinds of tissues are used like whole SNc, or only the medial/lateral part of the SNc or other brain parts. Furthermore, it is possible to study the gene expression of a single cell by laser capture microdissection (Lewis & Cookson, 2012).
With these gene expression profiling experiments multiple susceptibility genes are discovered. Common pathways involve cellular processes, metabolism pathways, environmental information processing, pathways involved in diseases and signaling pathways involved in the immune system (Zhang, Xia, Lin, & Huang, 2012). But also pathways involved in energy metabolism and mitochondrial function are mentioned, just as impairments in the proteasome (Cooper-Knock et al., 2012; Zheng et al., 2010). However, pathways or genes involved in development of the dopaminergic midbrain are rarely mentioned. Below the few results concerning genes involved in the dopaminergic midbrain development will be discussed.

Bossers *et al.* (2009) shows that transcription of *NURR1* is down regulated in the SNc, caudate nucleus and putamen of patients suffering from Parkinson's disease (Bossers et al., 2009). NURR1 is a key transcription factor involved in the differentiation and maintenance of dopaminergic neurons in adulthood (Volpicelli et al., 2012). Although transcription of *NURR1* is down regulated in adulthood of Parkinson patients, this does not implicate that *NURR1* was down regulated during development.

In PD patients neurothropic signaling via GDNF is also affected, shown by downregulation of *DOK6* and *DLK1*, which are regulated by GDNF (Bossers et al., 2009). Moreover, BDNF signaling is decreased due to the truncation of the BDNF receptor, TRKB-T1, of which the intracellular kinase domain is lacking so making it impossible to pass on the signaling (Bossers et al., 2009). GDNF and BDNF are neurothropic factors, involved in the maintenance of the mdDA neurons during development and adult life and expression of BDNF is regulated by PITX3 and NURR1, PITX3 in turn is regulated by GDNF (Alves dos Santos & Smidt, 2011; Baron et al., 2012; Park et al., 2006; Peng et al., 2011; Volpicelli et al., 2012). So it could be due to the down regulation of *NURR1* and/or *PITX3* that BDNF is also affected. It is proven several times that levels of BDNF are lowered in PD patients in a specific subset of SNc neurons. Moreover, one polymorphism in the promoter region of the *BDNF* gene was

associated with familial PD (Peng et al., 2011). So *GDNF* and *BDNF* seem very interesting candidate genes involved in PD.

From work on animals, mainly mice, it came forward that *En1* is potential gene involving PD. By knocking down *En1* expression, mice showed a phenotype corresponding to PD symptoms like motor deficits, anhedonia and depression-like behavior. This theory is even strengthened by the fact that these animals had a decreased number of dopaminergic neurons in the SNc and a lower level of dopamine in the striatum (Alves dos Santos & Smidt, 2011). Moreover, En1 seems to be regulating α-synuclein, which is implicated to be stacked in Lewybodies (Alves dos Santos & Smidt, 2011).

Another interesting gene affected is *ROBO2*, an important axon guidance molecule together with its ligand SLIT1-3 (Bossers et al., 2009; Dugan et al., 2011; Lin et al., 2005). In the SNc, caudate nucleus and putamen *ROBO2* transcription is decreased, which could lead to an altered connectivity of dopaminergic neurons. Lastly, the researchers found an increase in extra cellular matrix proteins in the SNc (Bossers et al., 2009). Since neuronal migration is dependent on surrounding cells via extracellular matrix proteins this could be involved in the origin of PD.

Lu *et al.* (2006) showed that NRG1 is highly expressed in the whole CNS, including the SNc (Lu et al., 2006). It is expressed at synapses and is involved in activation of several receptors, i.e. of acetylcholine and glutamate. It is said it has a role in neurogenesis, neuronal migration and neurite extension, although more research is necessary to investigate if this is also true for dopaminergic neurons (Lu et al., 2006).

Last, Cantuti-Castelvetri *et al.* (2007) state that expression of several genes from the Wnt pathway are increased in female PD patients compared to male patients. This could be interesting since males are more susceptible for PD than females (Cantuti-castelvetri, Keller-mcgandy, & Bouzou, 2007). However it is not specified which specific genes or pathways are affected.

So, gene expression profiling is done with samples of adult PD patients and age-matched controls. Therefore it is impossible to see if there was a discrepancy of expression levels of developmental genes during the development. Now only developmental genes that are also present during adulthood are studied. It would be nice to perform gene expression profiling during development of the dopaminergic midbrain to get more insight in differences in development between controls and future-patients, but for obvious reasons this is not possible in practice. Since animals don't get PD spontaneously is it not possible to study the idiopathic version of PD in animals, although En1/2 and Pitx3 knock-out models are giving more information about a possible developmental cause of PD.

3.2 Schizophrenia

About 1% of the world population suffers from schizophrenia, a complex and heterogeneous disease which is characterized by positive and negative symptoms. The most common negative symptoms are cognitive deficits, social avoidance and emotional flattening. The core positive symptoms are paranoia, hyperactivity and psychosis. The disease initiates usually between the ages of 15 till 25 years, and males develop schizophrenia about 5 years earlier than females. Males also have a higher

risk of getting schizophrenic. How schizophrenia is developed is not clear, although there is a high influence of the genetics, but also environmental effects (like season of birth, cannabis use, urban birth, immigrant status) contribute to the disease (Autry & Monteggia, 2012; Mulle, 2012). There are multiple pharmacotherapies for schizophrenia, and although it is still not clear how they exactly work, the main effect is that they inhibit the dopamine system. Next to dopamine, also the neurotransmitters GABA and glutamate are implicated in being associated with schizophrenia (Autry & Monteggia, 2012).

However a lot of genetic studies are done, like linkage studies, copy number variation (CNV) studies and genome wide association studies (GWAS), it is proven to be hard to get a high significant association between certain genes and schizophrenia. This means there are no common loci having a big effect on the disease, but probably a lot of loci together increase the risk on getting the disease. In this chapter I will mention some genes which are or could be involved in the development of the dopaminergic midbrain and are nominated as candidate genes involved in the risk of getting schizophrenia.

First BDNF will be discussed, a well studied neurothropic factor which seems to be involved in schizophrenia. It is hypothesized that alterations in BDNF expression lead to the cognitive and emotional dysfunction as well as developmental abnormalities, seen in schizophrenia patients. Although studies about the expression level of BDNF contradict each other. Some say that BDNF is up regulated in the cortex of schizophrenia patients compared to healthy controls, while others claim BDNF expression is decreased in the cortex of patients. These contradicting results could be due to the different farmacotherapies patients receive, or they indicate an imbalanced level of BDNF due to a changed developmental path for BDNF expression or a secondary revelation of pathological condition (Autry & Monteggia, 2012). Moreover, the cortex is a big heterogeneous part of the brain, with different expression patterns for different parts of the cortex. An argument why BDNF could be involved comes from multiple MRI studies that show that schizophrenia patients have enlarged ventricles and reduced gray matter, especially in the cortex (although not specified where in the cortex) and hippocampus (Autry & Monteggia, 2012). Since BDNF is involved in cell proliferation and maintenance it could be that BDNF had a causative role for these structural alterations.
A second argument is that a polymorphism is found in the *BDNF* gene in schizophrenia patients. Due to this polymorphism, V66M, BDNF expression is decreased. This polymorphism is strongly correlated to cognitive defects like impaired learning and memory in humans, which are also seen in patients suffering from schizophrenia (Autry & Monteggia, 2012; Craddock, O'Donovan, & Owen, 2006).

Another candidate gene which seems to be strongly involved in schizophrenia is *neuregulin1 (NRG1*). NRG1 has multiple functions in the brain and other organs during development and adulthood. It is suggested that during development it has functions in neurogenesis, neuronal migration, neurite extension and neuronal specification, although the exact role is not clear (Harrison & Law, 2006; Lu et al., 2006). During development its receptor, ERBB4 is located in the dopaminergic midbrain in humans. The expression is increasing over time till neonatal stage (Kato et al., 2011). This gives a good clue NRG1 is involved during development. There are several reasons to support this association. First, the *NRG1* gene is located nearby the 8plocus. This locus on chromosome 8 (around 8p21.1-22) contains multiple genes implicated with schizophrenia. Secondly, the functions of NRG1 are implicated to the development of the disease; moreover the Nrg1 mutant mouse shows a

schizophrenia-like phenotype. Lastly, some studies show an altered expression of *NRG1* in schizophrenia patients. Besides, there are polymorphisms found in the *NRG1* gene which increase the risk on developing schizophrenia (Craddock et al., 2006; Girard, Dion, & Rouleau, 2012; Harrison & Law, 2006). Such a polymorphism was published by Fleck *et al.* (2012), whereby it was shown that a Val-to-Leu mutation in the transmembrane domain of the *NRG1* gene is associated with an increased risk on developing schizophrenia (Fleck, Garratt, Haass, & Willem, 2012). Furthermore they stated that when NRG1 signaling is blocked, synaptic AMPA receptors are destabilized and NMDA currents are lost. This blockage of NMDA signaling is an important mechanism in schizophrenia (Fleck et al., 2012). Kato *et al.* (2011) investigated the effect of neonatal treatment of *NRG1β1* (an artificial NRG1 variant containing all splice variants) on protein expression, molecular signaling and behavior (Kato et al., 2011). They saw an increase TH activity with increased level of dopamine in the frontal cortex and striatum, which was sustained during adulthood. Moreover, the treated mice showed behavior as seen in schizophrenia patients like abnormalities in the prepulse inhibition test, social interactions and latent inhibition (Kato et al., 2011).

Disrupted In Schizophrenia 1 (DISC1) is a gene expressed in the brain and involved in neurite outgrowth, neuronal migration, synaptogenesis and glutamatergic neurotransmission and has been mentioned as a strong candidate gene involved in schizophrenia. The involvement of the gene was first seen in a Scottish family of which a lot of members suffered from schizophrenia or bipolar disorder. In this family a rare structural variant in the genome was discovered whereby chromosome 1 and 11 are translocated. This translocation disrupted the *DISC1* gene. Although this disruption is rare, in the general population several SNPs in *DISC1* are associated with schizophrenia (Craddock et al., 2006; Girard et al., 2012; Mulle, 2012).

Another gene mentioned often is *Catechol-O-methyltransferase (COMT)*, an enzyme involved in the degradation of catecholamines like dopamine and adrenalin. This gene lies on chromosome 22q11, a locus which is deleted in the Velo-cardio-facial syndrome, by which patients often suffer from schizophrenia. Mainly one SNP is studied, V158M in the membrane-bound form of COMT and V108M in the soluble form, although results on an association with schizophrenia are mixed (Craddock et al., 2006). Till now there is no clear evidence COMT is directly involved in schizophrenia.

A very interesting model, described by Grant *et al.* (2012) to investigate the involvement of DCC in schizophrenia, is the heterozygous *Dcc* knock-out mouse. In this knock-out mouse the expression of *Dcc* is reduced (but still present) and these mice show behavior which corresponds to schizophrenia patients. Intriguingly, these behaviors occurred after puberty, during adolescence (Grant, Fathalli, Rouleau, Joober, & Flores, 2012). What makes DCC even more interesting is the finding of a SNP in the 3'UTR of the gene. The 3'UTR contains regulatory sequences which determine stability, cellular localization and translation of the mRNA. Schizophrenia patients are less likely to be heterozygous for this SNP compared with healthy controls (Grant et al., 2012). Overall, DCC is a very strong candidate gene which is involved during development of the dopaminergic midbrain with migration of precursor cells and during the connectivity of dopaminergic axons. Since it is concerned with midline guidance, a decrease in DCC could lead to abnormal lateralization of the brain. Intriguingly this abnormal lateralization is described in schizophrenia patients and related disorders (Grant et al., 2012).

So, there are not a lot of genes involved in development emerged from genetic studies associated with schizophrenia. Here the same problem arises as with Parkinson's disease. It is impossible to investigate the gene expression during development of humans. There are many different animal models for schizophrenia, like pharmacological models, lesion models, genetic and developmental models. However, all these models are not 'complete', meaning they do not show all positive and negative symptoms like schizophrenia patients. Besides, they do not all respond to medication (Jones, Watson, & Fone, 2011). Therefore it is hard to interpreted these models to investigate the gene expression of developmental genes. The only way to study involvement of developmental genes is by knocking these genes out, although it could be hard to draw conclusions from the effects since genes often have widespread functions throughout the body. Moreover, the focus of this thesis is on the early development of the dopaminergic midbrain, but developments continues till adolescence. Especially during puberty there are some dramatic changes occurring. Since the first symptoms of schizophrenia start during puberty it seems logic that around that time development is altered in patients. Unfortunately this goes beyond the scope of this thesis but would be very interesting to study in the future.

3.3 Attention Deficit Hyperactivity Disorder

Attention Deficit Hyperactivity Disorder (ADHD) is a heterogeneous disorder characterized by inattention, hyperactivity and impulsivity which arises during childhood and most often persists throughout life (Caylak, 2012; Liston, Malter Cohen, Teslovich, Levenson, & Casey, 2011; Roessner et al., 2010). About 8-12% of the children are diagnosed with ADHD and occurs about 8 times more in boys than in girls (Caylak, 2012).It is thought that the symptoms are caused by a deficiency of the main neurotransmitters dopamine, noradrenalin, serotonin and GABA and abnormalities in these systems (Caylak, 2012; Roessner et al., 2010). But how these deficiencies arise must be investigated more. However, there is a strong correlation between environmental factors during pregnancy and the development of ADHD. Examples of these environmental factors are the use of nicotine (smoking) and alcohol by the mother or by exposure for example to lead, methyl mercury, insecticides, arsenic, flame retardants, or by malnutrition and dietary deficiencies (Caylak, 2012; Landrigan, Lambertini, & Birnbaum, 2012). It could be hypothesized that due to these environmental factors, the development of the dopamine system, next to the other neurotransmitter systems, is affected.
That the dopamine system is involved in the symptoms of ADHD is clear, it has been shown that dopamine transmission is impaired, giving rise to inattention, hyperactivity and impulsivity. Moreover, it has been suggested that the dopamine turnover is reduced in ADHD patients, causing lowered levels of intra-synaptic dopamine (Caylak, 2012).

From imaging studies it was proven that connectivity of mesocortical and mesolimbic pathways are altered (Liston et al., 2011). Connections between the basal ganglia and prefrontal cortex where significantly decreased in children with ADHD compared with controls. Also other investigators who used Diffusion Tensor Imaging (DTI) saw structural deficits in frontrostriatal projections in ADHD patients compared to control subjects (Liston et al., 2011). It is also thought these deficits persist into

adulthood since the same deficits where found in adult patients (Liston et al., 2011). It could be that these alterations are due to environmental factors mentioned earlier or due to altered genetics. Next to the frontrostriatal connections, also the nigrostriatal projections are harmed in young ADHD patients. It was shown that the volume of the caudate nucleus, part of the nigrostriatal pathway and thalamo-cortical pathway is reduced in children with ADHD compared with control children (Shook et al., 2011).

Via genetic studies also several candidate genes are found to be implicated in ADHD, which are involved in above mentioned neurotransmitter pathways. The genes which are interesting for their involvement in the development of the dopaminergic midbrain are *COMT*, where the same polymorphism is investigated as with schizophrenia (rs4680, V158M). Multiple studies have investigated an association between the polymorphism and ADHD with contradicting results. Some found an association between the valine allele and ADHD, while others did not find an association. A different study stated they found that the methionine allele accorded to the risk on developing ADHD in boys and the valine allele was associated with ADHD in girls. But since results are very divergent, more research will be needed to study a possible association between COMT and ADHD (Caylak, 2012). Secondly, *Synaptosomal-associated protein of 25 kDa (SNAP25)* was investigated. SNAP25 is i.a. involved in axonal growth. In these studies 7 different polymorphisms were found. However for only one polymorphism in the 3'UTR a significant association could be made between the polymorphism and ADHD (Caylak, 2012). Third, *BDNF*, which has been mentioned earlier to be involved in schizophrenia, has been studied to see if the same polymorphism (V66M) is involved in ADHD. So far no clear association is seen between polymorphisms in the *BDNF* gene and ADHD (Caylak, 2012).

So in sum, there is strong evidence that during formation of dopaminergic connections alterations occur , giving rise to ADHD. The symptoms like hyperactivity, inattention and impulsivity come from a misbalanced dopamine transmission. Several genes are mentioned with polymorphisms in ADHD patients, but more research will be needed to examine the direct consequences of these alterations.

3.4 Autism Spectrum Disorder

Autism spectrum disorder (ASD) is a greatly varying syndrome, individuals show abnormalities in communication and social functioning, furthermore they have repetitive and stereotypic behaviors and restricted interests (Devlin & Sherer, 2012; Li, Zou, & Brown, 2012). Moreover, language delays are seen and cognition is often harmed, although this also varies greatly with individuals functioning above average and others having serious intellectual disabilities. The onset of the disease is before the age of three years (Devlin & Sherer, 2012). According to the Diagnostic and Statistical Manual of Mental Disorders edition four (DSM-IV) there are four subgroups in ASD, first classic autism, next childhood disintegrative disorder which gives a bigger decrease in skills and often occurs on a later age. Third is pervasive developmental disorder - not otherwise specified (PDD-NOS) which often occurs at a later age and Asperger syndrome whereby language delays and cognitive deficits are often less present (Devlin & Sherer, 2012; Li et al., 2012). The prevalence of ASD (including all types) is estimated at 6/1000 to 10/1000 whereby boys are four times more likely to have ASD than girls (Devlin & Sherer, 2012; Li et al., 2012).

Through genetic studies, like twin studies it was shown that genetics play a role in the origin of ASD, but there is strong evidence the genetics of ASD is arranged in a complex manner. There is a strong gene-gene and gene-environment interaction in ASD (Devlin & Sherer, 2012; Li et al., 2012).For about 10% of all individuals suffering from ASD a Mendelian condition or genetic syndrome underlies the symptoms. Examples of the most common syndromes are Fragile X syndrome, tuberous sclerosis, Rett syndrome and neurofibromatosis. Moreover, multiple syndromes are known where patients also suffer from ASD where microdeletions or single gene effects are the cause (Devlin & Sherer, 2012).
Also chromosome rearrangements or single gene disorders are seen in about 5-15% of all ASD patients. On every chromosome there is at least one structural alteration found, although most of them are rare and it is hard to prove there is a causal relationship with ASD. But there are some common chromosome rearrangements related to ASD, these are the 15q11-q13 duplication of the Prader-Willi/Angelman syndrome, trisomy 21, X Turner syndrome, XYY and XXY (Devlin & Sherer, 2012).
Of the idiopathic ASD cases 5-10% had a rare *de novo* mutation or a inherited copy number variation, especially genes involved in synaptic function were affected by these mutations. It are mainly the *de novo* mutations who have an important role in developing ASD, this is logic, since individuals with ASD are less successful in producing offspring (Devlin & Sherer, 2012).How these *de novo* mutations arise is not answered by Devlin & Sherer (2012), although a relation between parental age and germline or gonodal mosaicism is mentioned (Devlin & Sherer, 2012). Landrigan *et al.* (2012) reviewed which environmental factors contribute to the risk on getting ASD. There seems to be a linkage between the use of thalidomide, misoprostol and valproic acid by the mother during the first trimester of the pregnancy and ASD. Also a rubella infection during this trimester gives an increased risk. Furthermore exposure to the insecticide chlorpyrifos and phthalates during pregnancy is linked to ASD (Landrigan et al., 2012).

Although genetics of ASD is complex, few candidate genes are found so far who have a role in brain development (Li et al., 2012). The most strongly implicated genes for neurodevelopmental disorders (including ASD, Dyslexia, ADHD and mental retardation) are involved in the neurotransmitter synthesis (mainly GABA and serotonin), cation trafficking, oxytocin receptors and synaptogenesis (Landrigan et al., 2012).
First, Reelin, an extracellular matrix glycoprotein that guides the migration of several neural cell types and is responsible for neural connections. In ASD patients significantly reduced levels of Reelin mRNA and protein where found in the cortex, cerebellum and peripheral blood. Although results are not uniformly, a relation was found between ASD and a GGC repeat just before the ATG initiator codon of the *Reelin* gene. Moreover, larger alleles (of more than 11 repeats) were transmitted more often than chance level to ASD patients (Li et al., 2012). Next, the *Neuroligin (NLGN)* genes, this family of genes are cell-adhesion molecules involved in the formation of functional synapses. Splice variants of *NLGN3* and -4 genes are reported, next to a missense variant and two single substitutions giving rise to defect synaptogenesis. This could lead to ASD (Li et al., 2012).Third are the *MET* genes, these genes encode for transmembrane receptor tyrosone kinase of the hepatocyte growthfactor/scatter factor (HGF/SF). These genes have an important role during development of the brain. If signaling via this receptor is reduced, abnormal interneuron migration and neural growth in the cortex appears. Moreover, there is decreased proliferation of granule cells. These features are

also often seen in the brains of autistic individuals (Li et al., 2012).Lastly, a polymorphism in the *dopamine-3-receptor (DRD3)* gene showed a significant association with ASD. *DRD3* is highly expressed in the basal ganglia, mainly the caudate nucleus. This is interesting since an enlargement of the caudate nucleus has a strong relationship with behavioral rigidity in ASD. Furthermore, the circuitry between the basal ganglia and frontal lobe, which is partially regulated by dopamine, plays an important role in repetitive and stereotype behavior (Staal, De Krom, & De Jonge, 2012).

So much research is already done about genetics involved in ADS, however due to the complex genetics of the disorder, al lot is still to discover. ASD are often called neurodevelopmental disorders, although more proof is needed.

Such proof comes from imaging studies from where the underconnectivity theory is devised. This theory states in individuals who suffer from ASD there is less anatomical and functional connectivity between the frontal and more posterior cortical systems (Just, Keller, Malave, Kana, & Varma, 2012). Just *et al.* (2012) reviewed different kinds of imaging studies to proof this theory. They showed that fMRI studies demonstrate a consistent decreased frontal-posterior synchronized activity during many different kinds of thinking. Moreover, in structural imaging like DTI, white matter abnormalities are seen in ASD patients, implicating that there are changes in the cortico-cortical connection in people with ASD. These changes are an increase in white matter volume in some regions, like the frontal radiate and a decrease in other regions like the corpus callosum. This last decrease in the corpus callosum has a strong correlation with the lower degree of functional connectivity in ASD during thinking tasks and resting state (Just et al., 2012).

So it is most clear from these connection studies that the origin of ASD lays in the development of the dopaminergic midbrain. So far there are only a few genetic studies that show some relation with developmental genes, but much more research will be needed. Moreover, there is evidence that due to environmental factors the development of the dopaminergic midbrain is altered. Probably a combination of genetic vulnerability and exposure to environmental factors contribute to the development of ASD.

3.5 Substance Use Disorders

Substance use disorders (SUDs) are a collection of addiction for several kinds of substances, like nicotine, cocaine, heroin and alcohol. It is estimated that between the 3.4 and 6.6% of the world population in 2010 was using any kind of drugs including cannabis, opioids, opiates cocaine, amphetamine-type stimulants and ecstasy (United Nations Office on Drugs and Crime, 2012). Addiction is seen as a multifaceted psychiatric disorder, caused by long-term use of drugs, and abuse and dependence on the drug is seen. This is characterized by compulsive seeking and taking of the drug, even if this had negative consequences. Moreover, there is sensitization to the effect of the drug. When the drug is absent due to withdrawal there is craving and enhanced susceptibility to relapse (Autry & Monteggia, 2012; Sutherland, McHugh, Pariyadath, & Stein, 2012).The etiology of SUDs is still not completely understood, although it is thought that there are neurobiological, pharmacological, environmental and genetic components (Chen et al., 2011; Sutherland et al., 2012).

There is a lot of evidence the dopaminergic system is involved in addiction since the dopamine level is directly or indirectly altered by drug use. Like cocaine that works as a stimulant by inhibiting dopamine reuptake from the synaptic cleft, thereby increasing the synaptic dopamine level in the VTA and NAc and subsequently, activating all three dopaminergic pathways (Autry & Monteggia, 2012; Chen et al., 2011; Kreek et al., 2012). But mainly the mesolimbic and mesocortical pathways, also called the reward pathway are stimulated by drug use (Autry & Monteggia, 2012). Next to these short-term effects there are also long-term effects where there are regulatory changes on mRNA and protein level in the dopamine system (Kreek et al., 2012). These long-term changes even prolong long after withdrawal, causing the chronic relapsing nature of addiction (Kreek et al., 2012). The dopaminergic system is modulated by the opioid system, dependent on which receptor, dopamine release can be stimulated (via MOP receptor) or inhibited (via KOP receptor). Cocaine and heroin act as a MOP receptor agonists on interneurons in the VTA and SNc, relieving the GABAergic inhibition of the dopaminergic neurons. In rodent models of cocaine addiction, an increase of number of MOP receptors was seen in the NAc and caudate putamen. But also KOP receptor expression was elevated in the caudate putamen and VTA, who project to the NAc. These changes are still seen long after withdrawal (Kreek et al., 2012).

Next to the dopamine system, also the serotonin system is involved, playing an important role in the rewarding effect of drugs (Kreek et al., 2012). Serotonin also regulates synaptic activity of the reward pathway (mesoaccumbens pathway). The serotonin 2C-receptor (5-HT_{2C} receptor) plays a role in the sensitization of drugs and the side effects of cocaine like anxiety, hypolocomotion and disturbed motor function (Yoshimoto, Watanabe, Tanaka, & Kimura, 2012).

The vulnerability to develop an addiction is partly due to genetic factors. There are polymorphisms known in i.a. opioid receptors and their ligands associated with drug addiction (Kreek et al., 2012). Other associated genes are *dopaminergic receptors 2* and 4 *(DRD2* and *DRD4*), where certain polymorphisms increase the risk on abusing opioids (Chen et al., 2011). For alcohol abuse it has been found via a GWAS that *aldehyde dehydrogenase 2 (ALDH), ADH1B* and *autism susceptibility candidate 2 (AUTS2)* are related to alcohol consumption (Sullivan, Daly, & O'Donovan, 2012). Moreover, the extra-hypothalamic *corticotrophin-releasing hormone receptor 1(CRHR1)* contributes to the susceptibility of relapses. The function of CRH signaling is to activate stress-induced behaviors by mediating the hypothalamic-pituitary-adrenal (HPA) axis (Molander et al., 2012; Refojo et al., 2011). That stress is related to substance use disorders is confirmed in the review of Rodriques *et al.* (2011), who state that glucocorticoids (the main stress hormone) has an effect on the dopaminergic system by up regulating TH synthesis and so dopamine production (Rodrigues, Leão, Carvalho, Almeida, & Sousa, 2011). It has been studied in human and animal models that in utero exposure to stress or glucocorticoids have a long-lasting effect on cognition, mood, affective and affiliative behavior and addiction (Rodrigues et al., 2011). Moreover, it was shown that by separating pups from their mothers in rodents during the first week of life, shifted the timing of the development of the brain. There is a strong idea this is due to an altered catecholaminergic transmission in the VTA and raphe nuclei (Rodrigues et al., 2011).

So far, only few genes seem to be involved in the risk on developing an addiction, and these do not seem to be directly involved in the development of the dopaminergic midbrain. The only gene mentioned in the last years is *BDNF. BDNF*, like mentioned before, is involved in development and maintenance of the dopaminergic midbrain. Moreover, it seems to be involved in the reward

pathway. When cocaine is used, BDNF levels rise in the NAc shell, even long after withdrawal. Furthermore this can have effect on the plasticity of the reward pathway which is altered when cocaine is used for longer time. Also there is an increased number of spines and a lower threshold for LTP (Autry & Monteggia, 2012). On the contrary, morphine or heroin addicts have a decreased BDNF level in the VTA compared with healthy controls. This decrease is immediately lifted when the users withdrawal, though the level decreases again later. The effect of decreased BDNF expression is a higher TH level in VTA neurons, who are also become bigger in size (Autry & Monteggia, 2012). Polymorphisms in the *BDNF* gene as well as its receptor TrkB are associated with smoking of cigarettes. It was investigated that BDNF expression in the cortex, hippocampus and striatum, but lowered in the VTA when nicotine is given to rodents for a long time (Autry & Monteggia, 2012).

In sum, the ontology of SUDs is complex. A few polymorphisms have been found in several genes, although these only explain a small part of the phenotype. Moreover, an altered expression of genes can be caused by drug use, instead of the other way around in which an altered expression could lead to a higher chance of using drugs. Alterations in the dopaminergic system have been found in drug users and in animal models of SUDs. But here the same idea counts, probably the drugs cause these alterations, not the other way around. But still there is a chance the development of the dopaminergic midbrain can have an effect on drug use. It could be that genetic variations (i.a. in genes involved in dopaminergic development) determine together with environmental factors the vulnerability of getting involved in SUDs.

3.6 Lesch-Nyhan Disease

Lesh-Nyhan Disease (LND) is a rare X-linked recessive disorder caused by a deficiency in the *hypoxanthine-guanine phosphoribosyltransferase (HPRT)* gene. This gene is a housekeeping enzyme, recycling purines hypoxanthine and guanine into utilizable purine nucleotides. Due to the *HPRT* deficiency it is not or scarcely expressed, giving rise to an overproduction of uric acid. The stacking of uric acid leads to gut and renal dysfunction, moreover, patients suffer from neurological symptoms. These symptoms are generalized dystonia, spasticity and self-mutilation (Ceballos-Picot et al., 2009; Cristini et al., 2010; Deon, Kalichman, Booth, Slavin, & Gaebler-Spira, 2012; García, Puig, & Torres, 2012; Guibinga, Hrustanovic, Bouic, Jinnah, & Friedmann, 2012; T. H. Kang, Guibinga, Jinnah, & Friedmann, 2011). These neurological symptoms arise from dysfunction of the dopaminergic neurons in the basal ganglia (Air, Ostrem, Sanger, & Starr, 2011; Deon et al., 2012; T. H. Kang et al., 2011). This is shown by deep brain stimulation in a LND patient in the globus pallidus, which relieved much of the dystonia and self-mutilating behavior (Air et al., 2011; Deon et al., 2012).

Next to dopamine, also serotonin and adenosine in the basal ganglia are implicated in LND. All of these three neurotransmitters bind G-protein coupled receptors and are involved in motor function and behavior (García et al., 2012). García *et al.*(2012) studied the expression of the dopamine-, serotonin- and adenosine receptors in peripheral blood lymphocytes (PBL) of LND patients. These cells can be easily obtained, since it is hard to get brain tissue from living patients, and show all characteristics of HPRT deficient cells. There is for instance increased purine synthesis, enhanced production and excretion of hypoxanthine and adenine nucleotides and express all receptors of the three mentioned neurotransmitters (García et al., 2012). This study showed an increase of serotonin,

dopamine and adenosine (5-HTR1A, DRD5 and ADORA2A respectively) receptors in the PBL of LND patients compared with controls on both RNA and protein level. The researchers hypothesize that the hypoxanthine excess causes a lowered adenosine transport since there is competition of transporters. Hereby the synaptic adenosine levels are increased, causing a decreased expression of adenosine receptors. Since adenosine, dopamine and serotonin receptors seem to interact with each other, alterations take place in the serotonin and dopamine receptors (see figure 7) (García et al., 2012).

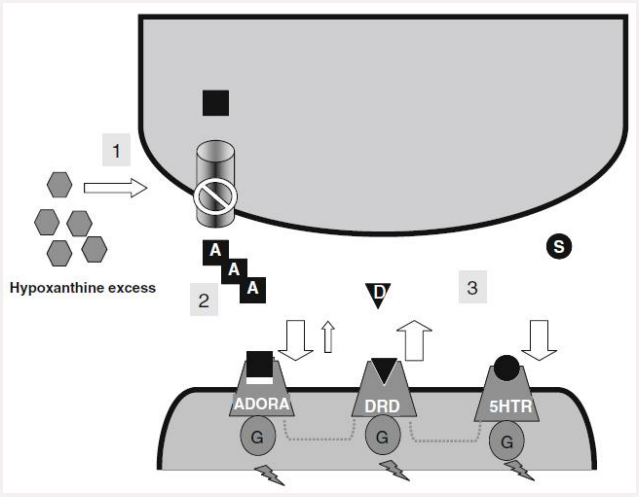

Figure 7: The hypothesis to link HPRT deficiency to neurotransmitter dysfunction. 1) The Hypoxanthine excess competes with adenosine transporters, leading to an increase of adenosine in the synaptic cleft. 2) The increase of adenosine leads to a decreased adenosine receptor expression. 3) Since adenosine, serotonin and dopamine receptors interact with each other, the decrease of adenosine receptors disturbs expression of the serotonin and dopamine receptors. Adapted from Guibinga et al. (2012)

There are several studies investigating gene expression of cell lines where *Hprt* is down-regulated. In MN9D cells, which are dopaminergic mouse neuroblastoma cells the spontaneous *Hprt* deficient cells were selected. In this study, gene expression was compared with the other cells expressing *Hprt.* The investigators showed a highly significant over expression of *En1* and *En2* in the *Hprt* deficient cells compared with the controls (Ceballos-Picot et al., 2009). However, other researchers showed in human neuroblastoma cells (SH-SY5Y) where *HPRT* is knockdown by retrovirus expressing shRNA targeting to *HPRT*, that *EN1* and *EN2* expression is decreased compared to controls. Next to the engrailed genes, also the transcription factors *Lmx1a* and *Brn2* are down-regulated (Guibinga et al., 2012). The authors state this is due to over expression of a microRNA, miR181a. MiR181a is known to regulate the engrailed genes by binding the 3'UTR of the gene, causing inhibition of translation or degradation of the mRNA. It is hypothesized that *Lmx1a* and *Brn2* are also targets of miR181a, whereby the targets of these two genes (*Wnt1, Nurr1, Pitx3* and *Mash1*) are also dysregulated, see figure 8 (Guibinga et al., 2012).

In the same sort of cells an increased level of cytosolic phosphorylated β-catenin was observed. Together with some other aberrations the major downstream transcription factor effectors of Wnt signaling are down regulated, including *En1* and *Lmx1a* (T. H. Kang et al., 2011).

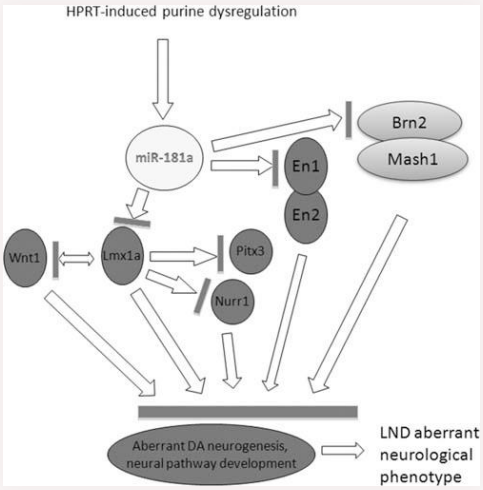

Figure 8: Schematic representation of a hypothesis showing how a HPRT deficiency can lead to dopaminergic dysfunction. Due to the HPRT deficiency there is an aberration in purines, which has an effect on the expression of miRNAs (in this case miR181a). The overexpression of miR181a down-regulates expression of En1, En2, Lmx1a and Brn2, which in turn down regulate their targets Nurr1, Pitx3, Wnt1 and Mash1. This cascade leads to an abnormal development of the dopaminergic midbrain, giving rise to the neurological symptoms of LND. Adapted from Guibinga et al. (2012)

Cristinini *et al.* (2010) performed an expression profiling assay in human neural stem cells from LND fetuses (Cristini et al., 2010). They demonstrated 5 genes that had a decreased expression, *CD44, NCAM1, NEUROG2, TUBB3* and *ALDH1*. And 5 up-regulated genes, *PPARD, BMP2, NOTCH1, MYST1* and *GCN5L2* (Cristini et al., 2010). In the context of dopaminergic development *ALDH1, BMP2* and *NOTCH1* are interesting. ALDH1 is involved in the synthesis of RA, which is thought to be important in dopaminergic development. It precedes the TH expression in DA neurons and is expressed in the SNc and targets of the SNc (Cristini et al., 2010).

So although the cause of the LND is clear, due to HPRT deficiency still a lot of research is needed to determine the precise cause of the neurologic symptoms. There is strong evidence there are alterations in genes involved in the development of the dopaminergic midbrain. Multiple researchers found independently of each other aberrations in engrailed expression, one of the main transcription factors involved in the dopaminergic system.

4. Conclusion

The aim of this thesis was to investigate if the above mentioned diseases or disorders have an origin in the development of the dopaminergic midbrain. For this reason, first the latest literature was searched to describe the normal development of the dopaminergic midbrain. A lot is already known about the main mechanisms of how the dopaminergic neurons arise, migrate and make connections to other brain areas in rodents. It is assumed in humans the same strategy is used, although the timing will be somewhat different. The development of the brain is regulated by a strict spatiotemporal organization of gene expression. Already a lot of genes, their expression patterns and functions are known, but still more must be discovered to understand the whole system.

Parkinson's disease is caused by specific cell death of the dopaminergic neurons in the substantia nigra, causing a decrease of the dopamine level, giving rise to difficulties in motor functions. So it is clear the dopaminergic system is involved in PD, but how only this specific subset of cells dye is still a mystery. Although the disease manifest mostly above the age of 65 years, the idea is that an alteration in the development of the dopaminergic midbrain underlies the disease. There are multiple gene expression differences found between PD patients and healthy controls, but how these together results in the phenotype, is not clear yet.
It is hard to investigate if development is altered, since there is a big gap in time between development and the moment cell death of dopaminergic neurons starts.
In animal models an almost identical phenotype can only be created by disruption of the nigrostriatal pathway, making it impossible to study the idiopathic version of PD in animals. By knocking out candidate genes, more insight can be gained about the function of the gene in development and in PD. Like it has been done for the En1 knock out, which showed very promising results. This insinuates that the differentiation and maturation of dopaminergic precursors could be affected. Moreover, it seems to be that there are also genes involved in PD who have a function in axon guidance, so maybe the connectivity of the dopaminergic neurons could be affected, giving rise to PD later in life.
So to answer the main question of this thesis: is PD a developmental disorder? So far, there is not enough proof found to answer with 'yes'. Although it is clear the dopaminergic midbrain is involved in the disease, it is not clear if the cause can be found during the development.

Schizophrenia is a mental disorder with as main symptoms paranoia and psychosis. It is known that the mesolimbic and mesocortical pathways are involved in this disease, giving rise to the question if a cause could be found during development of the dopaminergic midbrain. There are a few genes that are involved in dopaminergic development which are candidate genes involved in schizophrenia. These genes are involved in every mechanism of development. Like BDNF is involved in cell proliferation and cell maintenance, there is a SNP in the BDNF gene with a strong correlation to behavior also seen in schizophrenia. Another interesting gene is NRG1, this gene is involved in neurogenesis, migration and specification. There are several reasons why this gene is involved in schizophrenia. Next mutations are seen of DISC1 and COMT in schizophrenic patients, but how this leads to the phenotype is not clear yet. Lastly, the DCC gene looks very promising, by knocking down this gene, mice show schizophrenia-like behavior. Most intriguingly is that these appear after puberty of the mice, just as in humans. So there are a few candidate genes who seem to be involved although these genes alone cannot cause a complicated disease like schizophrenia. That's why it is thought that environmental factors seem to play a strong role in the origination of schizophrenia. If a fetus or

child is exposed to certain factors, this could alter development, maybe by influencing dopaminergic factors, leading to schizophrenia later in life. Maybe this is not visible (anymore) as gene expression alterations during adulthood. The last decade a lot of genetic studies are done to find candidate genes involved in this disease. However, genetics are not involved in a simple manner. And instead of trying to find small correlations between genes and schizophrenia, in my opinion it would be more wise to study environmental factors influencing the disease and if these factors can influences genes involved in development. Also more research should be done on the development of the dopaminergic midbrain till adolescence since the disease appears most of the times during puberty. So although a lot of research is still needed, with the knowledge we have at this moment, there are clues that the origin of schizophrenia lies in the development of the dopaminergic midbrain. Since there are correlations between the disease and some genes involved in dopaminergic development. Moreover it has been shown that connectivity of neurons is altered in patients, giving a hint alterations take place during development.

ADHD is a heterogeneous disorder characterized by hyperactivity, inattention and impulsivity. In patients abnormalities are found in the multiple neurotransmitter systems like dopamine, noradrenalin, serotonin and GABA. With imaging studies it was proven that the connectivity of frontrostriatal and nigrostriatal pathways is altered. This could be due to genetic polymorphisms, which are seen in a few genes involved in dopaminergic development like *COMT, SNAP25* and *BDNF*, however correlations are not convincing. There is more evidence that environmental factors play a role in the altered brain development of ADHD patient. There are several substances that have been proven to increase the risk on developing ADHD. So, ADHD seems to be originating due to altered connectivity of the dopaminergic system. This could be due to genetic polymorphisms or due to exposure to environmental factors. How these factors influence the development of the dopaminergic midbrain must be studied further.

ASD is a great varying syndrome with abnormalities in communication and social functioning. Often a cognitive deficit is seen, but there are also variants where cognition is normal. In about 10% of all patients suffer from a genetic syndrome or Mendelian condition where autism is one of the problems. Also chromosome rearrangements are seen as a cause of ASD in about 5-15% of the patients. So there is strong evidence genetics is involved in the origin of ASD. But genetics does not explain all cases of ASD, also environmental factors have an effect. It is known that exposure of some substances increases the risk on developing ASD, but how these influences the dopaminergic system is still unknown. Genes who seem to be involved in ASD are *Reelin, Neuroligin, MET* genes and dopamine transporters, but ASD is a genetically complex disorder, so much more research will be needed. First more responsible genes should be found and secondly functional connections between genotype and phenotype has to be studied. That ASD is a neurodevelopmental disorder is confirmed by the underconnectivity theory. This theory states that the symptoms are caused by a decreased connectivity between the frontal en more posterior cortical systems. Moreover, alterations in white matter volume are shown. These connectivity alterations can be caused by genetic polymorphisms and/or by environmental factors. How this mechanism exactly works remains to be elucidated.

SUDs are a collection of different disorders where individuals are addicted to one or more substances like alcohol, nicotine or drugs. It is clear the dopaminergic system is involved, since i.a. cocaine and heroin directly seize on this system by increasing the level of dopamine in the synaptic cleft. The

vulnerability to develop an addiction is partly due to genetic factors that determine the susceptibility to relapse and the effect of stress on the individual. A few alterations in genes are found that are involved in dopaminergic development, giving rise to alterations in the system. But it is not clear if these are the cause or effect of the drugs. So, for substance use disorders it is not clear if the origin lies in the development of the dopaminergic midbrain, although there is some evidence that genetic and environmental factors together determine the vulnerability to develop an addiction.

The origin of Lesch-Nyhan disease is very clear. A deficiency in the HPRT gene gives rise to a stacking of uric acid, causing gout and renal dysfunction. But there are also neurological symptoms in LND patients like dystonia, spasticity and self-mutilating behavior which cannot be explained. It is known that dopaminergic neurons of the basal ganglia give rise to the motor deficits in LND. In multiple genetic studies it is shown that expression of genes involved in the dopaminergic development is altered in LND patients compared with controls. There are multiple theories how HPRT deficiency leads to these alterations. The first hypothesis is that the excess of hypoxanthine leads to an increase of adenosine in the synaptic cleft. Therefore the expression of adenosine receptors is decreased, just as the dopaminergic and serotinergic receptors, since these are functionally connected. Secondly, it is thought that microRNAs play a role. In HPRT deficient cells, miR181a is up regulated, hereby inhibiting *Lmx1a* and *Brn2*, that in turn inhibit other dopaminergic genes like *Wnt1, Nurr1, Pitx3* and *Mash1*.It is clear the dopaminergic system is affected in LND, since there is strong evidence multiple genes involved in dopaminergic development are altered.

The research question of the thesis was if the six diseases or disorders mentioned have their origin in the development of the dopaminergic midbrain. For all these six diseases and disorders there is a relationship with the dopaminergic system, all be it in different ways for each disease. The development of the dopaminergic midbrain is a highly structured in a spatiotemporal manner with a lot of genes involved. From research from the last decades a lot of knowledge is gained about the mechanisms and genes involved in the dopaminergic development, but there is still more to discover. By gaining more insight in involved genes and mechanisms, hopefully a direct relationship can be found between dopaminergic development and the diseases. Therefore better animal models have to be developed, most likely by knocking-out candidate genes. Moreover, more attention should be given to the development of the brain after birth. Since development of the brain continues till adolescence, the events that happen from birth till adolescence should not be underestimated. This is the most important concerning PD and schizophrenia since these diseases emerge later in life. When more is known about the development, diseases involved in the dopaminergic midbrain will be better understood so the origination can be found. Moreover, potential new therapies can be developed.

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