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CHEMOKINES AND THEIR ROLE IN DOPAMINERGIC DEVELOPMENT

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Till min pappa som skulle ha varit stolt

ABSTRACT

The main pathological hallmark in Parkinson's disease (PD) is the progressive loss of dopaminergic (DA) neurons in the substantia nigra (SN), affecting movements such as walking, talking and writing. Today, there is no cure available for PD and existing treatments only provide symptomatic relief, but the prospect of using stem cell based cell replacement therapies for patients with PD have provided new optimism. In order to improve the efficiency of DA neuron production, engraftment and function, a solid understanding of the molecular mechanisms that govern DA neurogenesis is crucial. In recent years, morphogens and intrinsic DA neuron determinants, that enable stem and progenitor cells to establish proper identity, have been identified. However, the whole story has yet to be uncovered. In order to improve the efficiency of DA neuron production, engraftment and function, a solid understanding of the molecular mechanisms that govern DA neurogenesis and differentiation is crucial. This thesis examines the expression and function of chemokines during DA neurogenesis.

In *paper I* we demonstrate that three α-chemokines, CXCL1, -6 and -8, increase the number of DA neurons in ventral midbrain (VM) precursor and neurosphere cultures by diverse mechanisms. CXCL6 does so by promoting the differentiation of Nurr1+ precursors into DA neurons *in vitro*. Intriguingly, CXCL8, a ligand expressed only in *homo sapiens*, enhanced progenitor cell division, neurogenesis and Tyrosine hydroxylase positive (TH+) cell number in rodent precursor and neurosphere cultures. CXCL1, the murine ortholog of CXCL8, was developmentally regulated in the VM and exhibited similar, but not identical activities to CXCL8.

In *paper II* we identify two β-chemokines, CCL2 and CCL7, as novel regulators of DA neurogenesis. CCL2 and CCL7 were found to promote the development of DA neurons by selectively enhancing the differentiation of Nurr1+ precursors into TH+ DA neurons. Moreover, both CCL2 and CCL7 were found to increase neuritogenesis in TH+ neurons in VM precursor and neurosphere cultures.

In *paper III* we report that CXCL12/CXCR4 signaling regulates migration of A9-A10 DA neurons, neuritogenesis and the initial orientation of their processes.

Taken together, our data provides evidence that chemokines are highly involved in DA neuron development and suggest that they may be useful to enhance DA cell preparations for cell replacement therapy as well as for drug discovery in PD.

LIST OF PUBLICATIONS

I. **Linda C Edman,** Helena Mira, Alejandro Erices, Seth Malmersjö, Emma R Andersson, Per Uhlén and Ernest Arenas.
α-chemokines regulate proliferation, neurogenesis and dopaminergic differentiation of ventral midbrain precursors and neurospheres. (2008) Stem Cells.

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The β-chemokines CCL2 and CCL7 are two novel differentiation factors for midbrain dopaminergic precursors and neurons. (2008) Exp. Cell Res.

III. **Linda C Edman**, Juan Antonio Sánchez-Alcañiz, Nicolas Fritz, Sonia Bonilla, Jonathan Hecht, Per Uhlén, Samuel J. Pleasure, Oscar Marin and Ernest Arenas.
CXCL12/CXCR4 signaling controls the migration and the initial process orientation of A9-A10 dopaminergic neurons. Manuscript.

LIST OF ABBREVIATIONS

ADHD	Attention deficit disorder
AHD2	Aldehyde dehydrogenase 2
ALS	Amyotrophic lateral sclerosis
A-P	Anterior-posterior axes
BBB	Blood brain barrier
BMP	Bone morphogenic protein
BrdU	Bromodeoxyuridine
bHLH	Basic helix-loop-helix
Ca^{2+}	Calcium
CNS	Central Nervous System
CRT	Cell replacement therapy
DA	Dopaminergic
DM	Dorsal Midbrain
DAG	Diacylglycerol
DAT	Dopamine transporter
DBS	Deep brain stimulation
D-V	Dorsal- ventral axes
E	Embryonic
EGF	Epidermal Growth Factor
ESC	Embryonic Stem cell
ERK-1/2	Extracelllar Signal-Regulated Kinase
EGL	External Granule Layer
FP	Floor Plate
FGF8	Fibroblast Growth Factor 8
FACS	Fluorescence-activated cell sorting
Gbx	Gastrulation brain homoebox
GPCR	G protein-coupled receptors
IP3	Inositol Trisphosphate
IZ	Intermediate Zone
ICM	Inner cell mass
JAK/STAT	Janus Kinase-Signal Transducers and Activators of Transcription Pathway
m	Mouse
MZ	Marginal Zone

MAPK	Mitogen-Activated Protein Kinase
mDA	Midbrain dopaminergic
MHB	Mid- Hindbrain barrier
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mES	Mouse embryonic stem
Ngn	Neurogenin
NSC	Neural Stem Cell
Nurr1	Nuclear receptor related 1
Otx	Orthodenticle homologue
PKC	Protein Kinase C
PI3K	Phosphatidyl Inositol-3-OH Kinase
PLC	Phospholipase C
PD	Parkinson’s Disease
Pitx3	Paired-like homeodomain transcription factor 3
PTX	Pertussis toxin
RA	Retinoic acid
RNAi	Ribonucleic acid interference
RP	Roof Plate
Sox	Sex determining region Y box two
Shh	Sonic hedgehog
SVZ	Subventricular Zone
SNpc	Substantia Nigra pars compacta
SN	Substantia Nigra
TH	Tyrosine hydroxylase
TUJ1	β Tubulin-III
7TMR	Seven Transmembrane Receptors
TFs	Transcription factors
VM	Ventral Midbrain
VZ	Venticular Zone
VTA	Ventral Tegmental Area
VMAT	Vesicular monoamine transporter
wt	Wild type

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1 INTRODUCTION

The main symptoms of Parkinson's disease (PD) result from the loss of a specific set of neurons located in the Substantia nigra pars compacta (SNpc), in the adult brain. These neurons are responsible for the production and release of the neurotransmitter dopamine; a crucial signaling agent that transmits messages to specific brain areas that co-ordinate movement. The depletion of dopamine producing cells results in severe dopamine reduction, which in turn gives rise to motor symptoms such as tremor, rigidity of muscles, and slowness of movement.

In this thesis, I will portray the rise and fall of midbrain dopaminergic (mDA) neurons. The aim of my Ph.D. studies has been to examine what role chemokine ligands and receptors play in the development of the DA neuron, and to elucidate whether chemokines can be used to enhance the generation of DA cells in embryonic precursor and/or neural stem cell/progenitor cultures *in vitro*, in order to improve current protocols for cell replacement therapy (CRT) in PD.

1.1 THE MAKING OF THE CNS

The complex formation of the vertebrate central nervous system (CNS) is preceded by a number of spectacular developmental events. Approximately one day after fertilization, the zygote starts to divide into undifferentiated blastula cells giving rise to a blastocyst. At this stage of embryogenesis, the embryo consists of the inner cell mass (ICM), which subsequently gives rise to the embryo as well as the trophoblast, which later forms the placenta. During a process known as gastrulation, the cells of the blastocyst rearrange into three germinal layers: The endoderm, the mesoderm, and the ectoderm. The endoderm gives rise to cells lining the digestive and respiratory tract, the mesoderm gives rise to muscle tissue, and the ectoderm gives rise to epidermis and neural tissue (Gilbert, 2003). The conception of the vertebrate CNS is initiated through a process known as neurulation. This process commences by the thickening of a specialized region of the ectoderm creating the neural plate. The neural plate will subsequently fold and form the neural tube. Gastrulation is completed around mouse embryonic day (mE) 9.0 and the neural tube is, at this time point, organized into specific regions like forebrain (prosencephalon), midbrain (mesencephalon), hindbrain (rhombencephalon), and spinal cord.

1.2 PATTERNING OF THE NEURAL TUBE

The neural tube is regionalized along different polar opposite axes already at an early stage in development (i.e. mE 6-7). The antereo-posterior (A-P, or rostro-caudal) axes reach from head to tail –with the midbrain territory located caudally to the forebrain and rostrally to the hindbrain- and the dorso-ventral (D-V) axes reach from back to belly. The establishment of these axes is dependent on a number of important signaling centers. Since the focus of this thesis is the midbrain, I will describe the establishment of these axes and the signaling centres involved in this region.

1.2.1 The antereo-posterior axes is formed

Patterning of the A-P axes is mainly controlled by one signaling center located at the mid-/hindbrain boundary (MHB), referred to as the isthmus (Wurst and Bally Cuif, 2001; Prakash and Wurst, 2004). The position of the isthmus is controlled by two transcription factors (TFs), orthodenticle homologue 2 (Otx2) and gastrulation brain homoebox 2 (Gbx2). Otx2 expression (positioned rostrally to the isthmus) is restricted to the forebrain and the midbrain between mE8.5 and mE12.5 (Ang, 2006), whereas Gbx2 expression (positioned caudally to the isthmus) is restricted to the hindbrain. These two TFs have the ability to repress each other and it has been shown that ectopic expression of Otx2 and the removal of Gbx2 shift the isthmic markers, creating a new MHB territory (Broccoli *et al*., 1999; Millet *et al*., 1999).

Another MHB derived signal that is crucial for the induction of the isthmus is Fibroblast growth factor 8 (Fgf8) (Ye *et al*., 1998). Fgf8, alone, is sufficient to ectopically induce an isthmus (Crossley *et al.*, 1996; Martinez *et al*., 1999). Also, Fgf8 signaling regulates both proliferation and A-P polarity in the developing midbrain (Crossley *et al.*, 1996; Lee *et al.*, 1997). In concert with Wnt1 (McMahon *et al*., 1990; Thomas *et al*., 1990), Fgf8 maintains the expression pattern of a number of genes, including *Engrailed (En) 1* and *2* (Wurst *et al*., 1994; Danielian and McMahon, 1996), and *Pax2/5* (Schwarz *et al*., 1997). Accordingly, *in vivo* studies have demonstrated that depletion of Wnt1 results in the absence of most of the midbrain and the rostral hindbrain (McMahon *et al.*, 1992).

1.2.2 The dorso-ventral axes is formed

Patterning of the D-V axes is mainly controlled by two signaling centers located along the midline of the neural tube: The floor plate (FP, ventral) and the roof plate (RP, dorsal) (Placzek and Briscoe, 2005). The glycoprotein Sonic hedgehog (Shh) is secreted from the notochord and the FP, and is required for the establishment of the D-V axis. Shh creates a ventralizing signaling gradient and regulates the generation of a number of cell types in a concentration-dependent manner (Yamada *et al*., 1991; Hynes *et al*., 1995a; Ericson *et al.*, 1997; Orentas *et al*., 1999). Importantly, DA neurons are born around E10.5 in the intersection of Shh and Fgf8 expression. Of these components, Shh has proven to be necessary for mDA progenitor induction (Hynes *et al*., 1995a; Hynes *et al*., 1995b; Wang *et al*., 1995; Ye *et al*., 1998; Briscoe *et al*., 1999) and mDA neuron development (Blaess *et al*., 2006). Morevoer, ectopic expression of Shh, as well as its downstream effector molecule Gli1, is sufficient to induce DA neurogenesis in dorsal regions of the mid-/hindbrain (Hynes *et al*., 1997). Furthermore, Shh signaling has been implicated to act as an axonal chemo-attractant for midbrain DA axons both *in vitro* and *in vivo* (Hammond *et al.*, 2009).

Factors secreted from the RP, such as Bone morphogenetic proteins (BMPs), generate a dorsal to ventral signaling gradient and acts in an antagonistic fashion to FP signals. Unfortunately, the dorsal patterning of the midbrain has been poorly studied (for review see (Alexandre and Wassef, 2005). Nevertheless, in a recent publication by Joksimovic et al., it was shown that FP-derived Wnt/beta-catenin signaling is crucial for proper DA neuron generation (Joksimovic *et al*., 2009).

In sum, the signaling concert created by the different signaling-center-derived morphogenic factors directs the overall plan of the CNS and dictates neural cell development based on their temporal and spatial position along these axes.

1.3 DEVELOPMENT OF MIDBRAIN DOPAMINERGIC NEURONS

1.3.1 The progenitor

The generation of mDA progenitor cells starts around E9.0 in mouse, taking place close to the midline of the VM. Factors such as Shh, Fgf8, Wnt1, and TGF-β act collectively to induce the generation of mDA neurons (Hynes and Rosenthal, 1999; Farkas *et al*., 2003). In 2008, Bonilla et al. showed that FP residing radial glia-like cells can undergo neurogeneis *in vivo* and generate mDA neurons (Bonilla *et al*., 2008). Genetic fate mapping experiments have also identified Shh-expressing progenitors in the MHB as mDA progenitors (Zervas *et al.*, 2004). Alternative mDA origins have also been proposed, for example: The isthmus (Marchand and Poirier, 1983); the basal plate/lateral FP (Hynes *et al*., 1995b); and the diencephalon (Marin *et al.*, 2005).

The first DA specific markers to be expressed by proliferating progenitors are Lmx1a and aldehyde dehydrogenase (Aldh1/Raldh1). Lmx1a is homeodomain TF that is induced in response to early signaling in the VM and is expressed in proliferating DA progenitors (Andersson *et al.*, 2006b; Ono *et al*., 2007). Aldh1a1 is involved in the production of Retinoic acid (RA) (Lindahl *et al*., 1984), which is involved in neuronal patterning, differentiation, axonal outgrowth and survival (reviewed by Maden, 2007). At this time point, i.e. mE9-9.5, the cells expand in the ventricular zone (VZ). In the most apical layer of the VZ, the dividing progenitors express proliferative markers such as and Ki67 and as they undergo mitosis they express phosphohistone 3 (PH3). The TF Sex determining region Y box two (Sox2) is also expressed at this time point in VZ progenitors (Kele *et al*., 2006).

1.3.2 The precursor

As mDA progenitors (i.e. radial glia-like progenitor cells) divide, daughter cells exit cell cycle and migrate ventrally from the VZ, following the process of radial glia, giving rise to postmitotic precursor cells (Foster *et al.*, 1988; Bayer *et al*., 1995; Kawano *et al.*, 1995). Once the cells enter the intermediate zone (IZ) they downregulate Sox2 expression (Kele *et al*., 2006) and initiate the expression of the orphan nuclear receptor 1 (Nurr1). Nurr1 has proven to be required for the differentiation of DA precursors into Tyrosine hydroxylase (TH) positive expressing cells (Zetterström *et al., 1997*). Another neuronal marker that is also expressed by

postmitotic DA precursors and neurons is the pan-neuronal marker βIII Tubulin (Tuj1). Together, these markers, in the absence of TH expression, define DA precursors at mE10.5.

1.3.3 The neuron

From mE11 and onwards, the DA precursors migrate radially on radial glial fibers into the marginal zone (MZ), further differentiating into mDA neurons (Hanaway *et al.*, 1971; Kawano *et al.*, 1995). At this point, precursor cells start to express the time-rating enzyme TH. Neurogenesis continues until around E13.5 when the cells develop into a mature phenotype (Di Porzio *et al.*, 1990). DA differentiation terminates when the cells express markers such as TH (Hanaway *et al.*, 1971; Kawano *et al.*, 1995), the midbrain homeodomain TF Paired-like homeodomain transcription factor 3 (Pitx3) (Smidt *et al.*, 2004), c-ret (Wallén *et al.*, 2001), and the dopamine transporter (DAT) (Sacchetti *et al*., 1999). Once the cells have acquired a more mature phenotype, they send long axonal processes and establish complex networks that regulate functions as diverse as control of voluntary movements, reward, emotions and cognition (Ikemoto and Panksepp, 1999). This will be discussed further in paragraph 1.4.

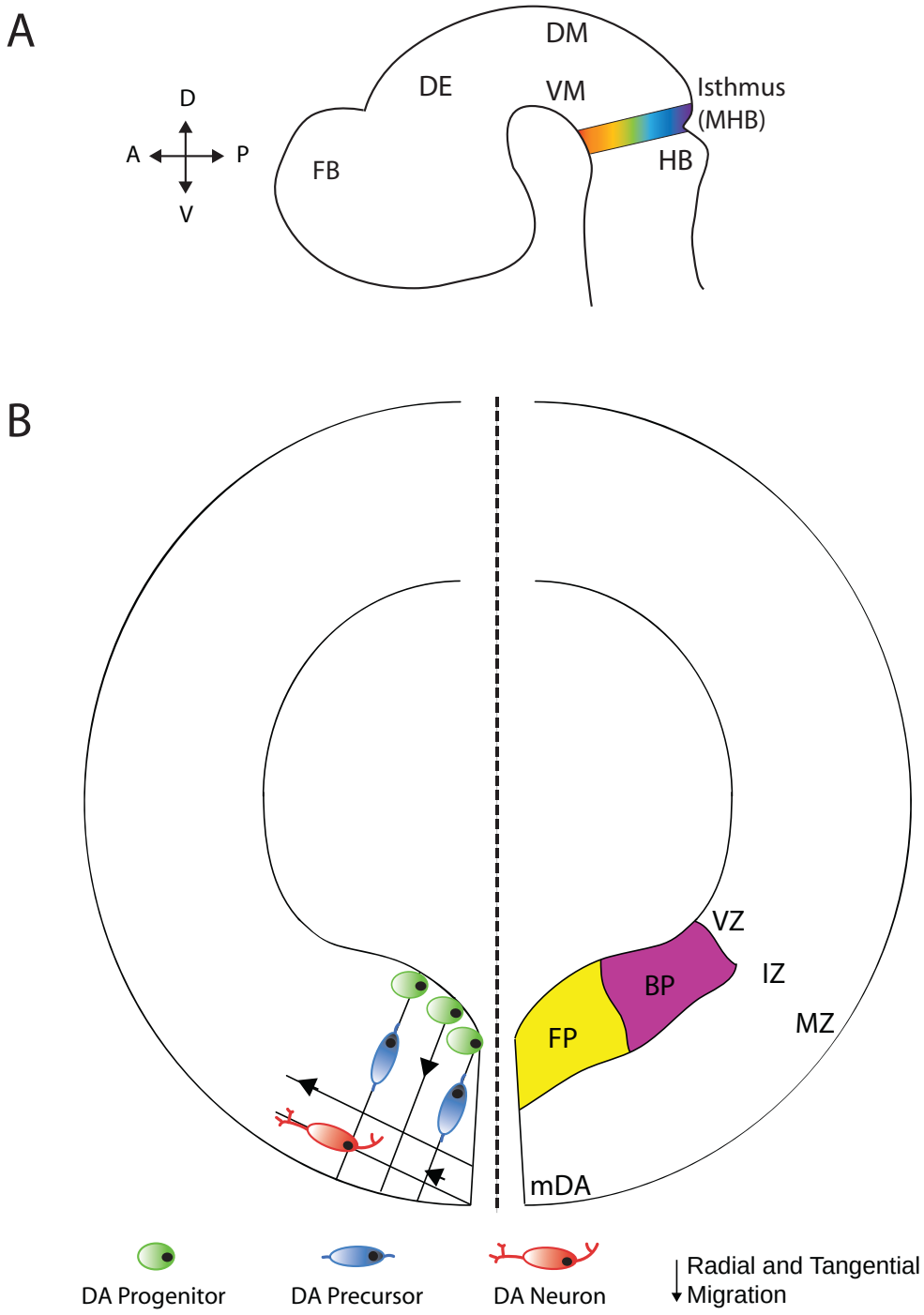


Figure 1. (A) Sagital view of an E10.5 mouse brain. (B) Coronal view of an E10.5-11.5 mouse brain. The illustration shows the three different layers in which the dopaminergic progenitors migrate on their way to become a fully differentiated dopaminergic neuron.
Forebrain (FB), Diencephalon (DE), Dorsal midbrain (DM), Ventral midbrain (VM), Hindbrain (HB), Mid-hindbrian boundary (MHB), Floor plate (FP), Basal plate (BP), Ventricular zone (VZ), Itermediate zone (IZ), Medial zone (MZ). Midbrain dopaminergic (mDA).

1.3.4 Signaling molecules necessary for DA neuron development

A limited number of intrinsic and extrinsic signaling molecules regulate CNS patterning and regionalization in a spatiotemporal manner along the neural tube. Gene expression and mouse mutant studies have implicated several TFs in the maturation and postmitotic differentiation of mDA neurons, some of which are presented below:

Lmx1b

LIM homeobox transctiption factor 1β (Lmx1b) expression in the midbrain starts before neural tube closure (around mE7.5). Lmx1b is co-expressed with Lmx1a and Msx1 in mDA neuronal precursors (Smidt *et al.*, 2000; Andersson *et al.*, 2006b). Intriguingly, the expression of Lmx1b is down-regulated and disappears at around mE11, but reappears in postmitotic TH+ and Pitx3+ neurons in the VM, at mE16 to persist into adulthood. Lmx1b is not able to induce a DA cell fate in the midbrain when ectopically expressed, but instead controls the onset and maintenance of Wnt1 and FGF8 expression in the isthmic organizer (Guo *et al*., 2007) Additionally, Lmx1b null mutants maintain a small number of TH-positive neurons in the VM which do not express Pitx3 and are lost shortly after their birth. (Smidt *et al.*, 2000; Simon *et al.*, 2003).

En1/2

The two homeobox genes *engrailed-1* (*En1*) and *engrailed-2* (*En2*) are initially expressed early in the developing brain, around mE8, in a band rostral and caudal to the isthmus (Davidson *et al.*, 1988; Gardner *et al.*, 1988) where they are essential for the patterning of the MHB (Liu and Joyner, 2001). mDA precursors and neurons start to express both *En1/2* between mE11.5 to mE14 and their expression continues throughout life (Albéri *et al.*, 2004). The two genes are involved in both survival (Albéri *et al.*, 2004) and maintenance (Simon *et al.*, 2001) of mDA neurons. Analyses of En1/2 double mutants reveal that mDA neurons are generated and become postmitotic, but die soon after the time when En1/2 should have been expressed (Simon *et al.*, 2001; Albéri *et al.*, 2004).

Lmx1a

The expression of the LIM homeobox TF 1α (Lmx1a) is first detected at mE9.0 in proliferating progenitors residing in the midbrain FP. Being the first marker of the cellular phenotype of midbrain DA neurons, Lmx1a expression is maintained in postmitotic Nurr1-positive mDA precursors and neurons. Lmx1a is a key determinant of the mDA neuronal cell fate that also activates Msx1 a TF expressed in progenitors that is involved in DA neurogenesis. By using RNAinterference (RNAi), Andersson et al., were able to knockdown the expression of Lmx1a in chick embryos, resulting in the loss of DA neurons in the midbrain. Additional gain-of-function experiments in mouse and human embryonic stem cells (ESC) lead to a robust generation of mDA neurons with the correct molecular identity (Smidt *et al.*, 2000; Andersson *et al.*, 2006b; Friling *et al.*, 2009).

Ngn2

Neurogenin 2 (Ngn2) is a proneural gene belonging to the basic helix-loop–helix (bHLH) family of TFs expressed in progenitors of the VZ and precursors in the IZ of the VM. Ngn2 is initially expressed at mE10.5, and has proven to be partially required for mDA development and neurogenesis (Andersson *et al.*, 2006a; Kele *et al*., 2006). Analyses of Ngn2 null mutants revealed an 80-90% reduction in the number of mDA neurons. However, the mDA population recovered postnatally to about 50-60% compared to wild type (wt) mice (Andersson *et al.*, 2006a). On the other hand, over-expression of Ngn2 enriched overall neurogenesis, but did not specifically promote the generation of mDA neurons (Andersson *et al.*, 2006a).

Nurr1

The orphan receptor Nuclear receptor related 1 (Nurr1/Nr4a2) is a member of the steroid-thyroid hormone receptor family of TFs. Nurr1 is expressed in different areas of the CNS, including the DA neurons of the SNpc and the ventral tagmental area (VTA) (Zetterström *et al.*, 1996), but also the hippocampus, olfactory bulb, cortex, and the cerebellum, amongst others (Saucedo-Cardenas *et al.*, 1998). Cells located in the VM start to express Nurr1 briefly after they become postmitotic, around mE10.5, and the expression is maintained throughout adult stages (Wallén *et al.*, 2001). Nurr1 has been proven essential for the acquisition of a DA phenotype and their survival. mDA neurons lacking Nurr1 do not express key components of the DA phenotype, including c-ret, TH, the vesicular monoamine transporter (VMAT) 2 and DAT. In

agreement with this, Nurr1 overexpression in embryonic neural stem cells is capable of inducing a DA phenotype (Sakurada *et al.*, 1999; Wagner *et al.*, 1999; Kim *et al.*, 2002). During later developmental stages, Nurr1 also serves a role in survival. For example, heterozygous Nurr1 mutants display an age related loss of DA neurons residing in the SN as well as a higher sensitivity to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced toxicity, leading to a decreased DA concentration in the SN, subsequently resulting in motor dysfunctions analogous to PD (Saucedo-Cardenas *et al.*, 1998).

Pitx3

Within the CNS, Paired-like homeodomain transcription factor 3 (Pitx3) is exclusively expressed in mDA neurons of both the SN and the VTA. Pitx3 starts to be expressed in the VM shortly after Nurr1 and continues to be expressed into adulthood (Smidt *et al.*, 1997). Analysis of the Pitx3 null mice revealed a predominant absence of mDA SN neurons and a loss of axonal projection to the dorsal striatum/caudate putamen (Nunes *et al.*, 2003; Smidt *et al.*, 2004; van den Munckhof *et al.*, 2006). The role of Pitx3 in SN DA neurons is also manifested from ES cells studies (Chung *et al.*, 2005). These experiments showed that ES cells expressing Pitx3 up-regulated aldehyde dehydrogenase 2 (AHD2), among others genes, suggesting a role of retinoic acids during the development of Pitx3 regulated DA neurons. Interestingly, a study by Hedlund and co-workers (Hedlund *et al.*, 2008) showed that Pitx3 can also be used as a marker to enhance the mDA population from ES cells by fluorescence-activated cell sorting (FACS).

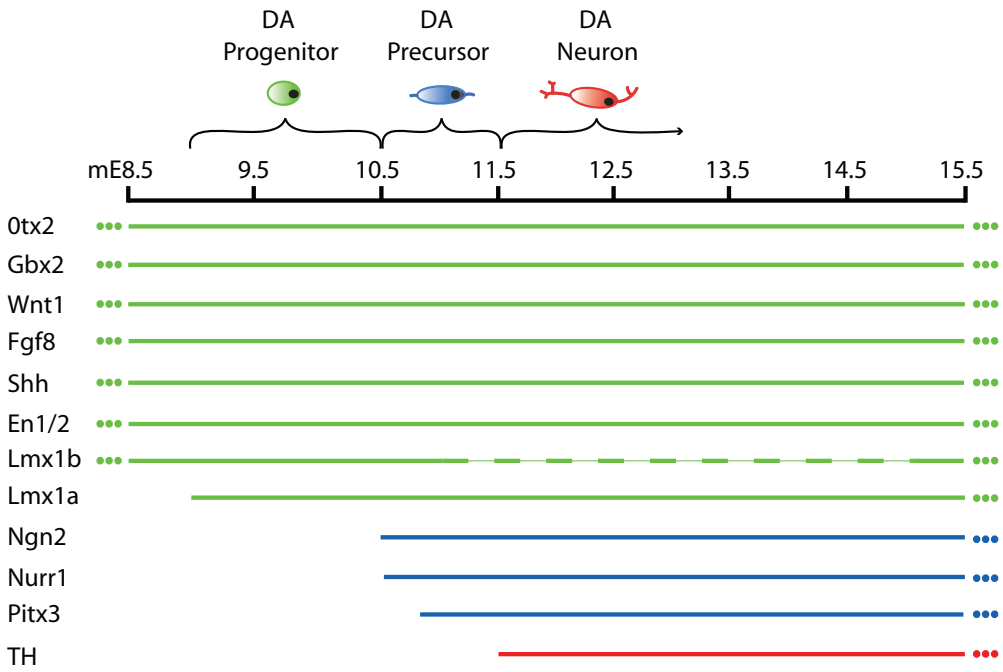


Figure 2. Schematic illustration of factors that are important for proper midbrain development.

1.4 DOPAMINERGIC SYSTEM IN THE ADULT BRAIN

DA is a member of the catecholamine family and was first synthesized in 1910 by George Barger and James Ewans. In 1958, Arvid Carlsson and Nils-Åke Hillarp proved that DA was not only a precursor of other catecholamines but a bona-fide neurotransmitter (Carlsson *et al.*, 1958). This neurotransmitter that is also a precursor of noradrenalin and adrenalin is synthesized from L-tyrosine. L-tyrosine is an essential amino acid that is converted into L-dihydroxy-phenylalanine (L-DOPA) by the time-rating enzyme TH. L-DOPA is subsequently decarboxylated by L-aromatic amino acid decarboxylase (Aadc/Ddc) to produce DA (Cotzias *et al.*, 1967).

In neurons, DA is packaged in vesicles by VMAT to be released into the synapse upon stimulation by a presynaptic action potential. Once released, DA can bind to different subset of dopamine receptors (D1-5) that are coupled to adenylate cyclase. DA is then cleared from the synapses by DAT (Kandel *et al.*, 2000).

DA neurons are found in several areas of the mammalian brain. Within the midbrain region, their cell bodies are present in the SNpc (A9), the VTA (A10), and in the retrorubral field (RrF, A8). DA neurons are also found in the diencephalon (A11-A15) and in the telencephalon (A16, A17). However, the most prominent DA cell group resides in the ventral part of mesencephalon, which contains approximately 90% of the total number of DA cells (for review see Prakash and Wurst, 2006).

The mesencephalic DA system is subdivided into two pathways, the nigrostriatal- and the mesocorticolimbic pathway. The nigrostriatal pathway comprises DA cells from the SN that innervate the striatum. It mainly controls voluntary movement and body posture (Wise, 2004). In contrast, the mesocorticolimbic pathway arises from DA cells present in the VTA and projects into the limbic system and cortex where they modulate cognitive and emotion-based behavior (i.e. motivation and reward).

1.4.1 Dopamine related diseases in human

Malfunctions of the mesencephalic DA system are known to give rise to a number of different disorders. For example, abnormal functionality in the mesocorticolimbic system can give rise to disorders such as schizophrenia, drug addiction, and attention deficit hyperactivity disorder (ADHD) (Björklund and Dunnett, 2007; Björklund and

Dunnett, 2007). Conversely, the loss of mDA neurons of the nigrostriatal pathway lead to PD, the second most common neurodegenerative diseases.

1.4.1.1 Parkinson's disease

James Parkinson first characterized this disease in 1817. Today, PD affects approximately 2% of the population over the age of 65 years. The main pathological hallmark of PD is a progressive loss of DA neurons in the SNpc, projecting to the caudate nucleus and the putamen. This cell loss results in a severe DA reduction in the striatum, which is responsible for motor symptoms typical of this disease, such as hypokinesia, tremor, and rigidity. The DA cell loss is associated with the presence of Lewy bodies, which are inclusion bodies composed of multiple proteins, including α-synuclein, ubiquitin, synphilin-1 and Parkin (Dawson and Dawson, 2003). However, the actual cause of PD has yet to be unraveled. Today, it is still debated wether genetic contributions or environmental factors are the main triggors causing the disease. So far, a few genes have been found to be associated with inherited PD and it is mutations in the genes encoding for the above-mentioned α-synuclein and Parkin that have received the most attention. Regarding enviromental factors, the discovery that the protoxin MPTP causes parkinsonism in both humans and nonhumans support the hypothesis that this disease has an environmental etiology. A number of other environmental toxins, and in particular pesticides have also been implicated as risk factors for PD (for review see Steece-Collier *et al.*, 2002). Additionally, inflammatory processes have also been thought to cause DA cell loss. Activated microglia and astrocytes are found in the area associated with cell loss, and the release of cytokines and/or pro-inflammatory prostaglandins is possibly contributing to the inflammatory process (Candy *et al.*, 1983).

1.4.2 Current treatments and putative approaches

1.4.2.1 L-DOPA

The most commonly prescribed drug for PD is the DA precursor, L-DOPA (Cotzias *et al.*, 1967). Since L-DOPA, and not DA, is capable of crossing the blood brain barrier (BBB), it is used to increase DA concentrations in patients suffering from PD. However, while L-DOPA reduces the severity of PD symptoms it is only symptomatic and does not change the progressive degeneration of neurons or the course of disease. Additionally, long-term treatment with L-DOPA leads to DA

receptor desensitization and periods of non-responsiveness to medication, as well as unwanted side effects such as dyskinesias, hypotension, and nausea. More recently, L-DOPA has started to be used in combination with DA agonists (Turle-Lorenzo *et al.*, 2006).

1.4.2.2 Deep Brain Stimulation

Deep brain stimulation (DBS) is the most commonly used surgical approach in the treatment of PD. In DBS, a pacemaker that is surgically placed in the brain sends electric impulses to the subthalamic nucleus or the internal part of the globus pallidus, where it is thought to inhibit excitatory neurons and compensate for the lack of dopamine (Gildenberg, 2005; Kringelbach ML *et al.*, 2007). However, the exact function behind DBS is not known and it only provides symptomatic relief. Needles to say, there exists an obvious need for a long-term treatment.

1.4.2.3 Cell Replacement Therapy

Today, one of the most promising long-term treatments for PD is CRT, a surgical approach that has been experimentally used since the 1980s (Lindvall *et al.*, 1988). Several cell types have been used in the past for DA CRT. However, only human fetal mesencephalic tissue has been found to efficiently reinnervate the striatum of patients suffering from PD. In few successful cases, cells have been seen to survive, integrate and induce functional improvements for as long as 10 years, despite the progression of the disease (Piccini *et al.*, 1999; Lindvall and Hagell, 2000; Dunnett *et al.*, 2001; Winkler *et al.*, 2005). However, recent studies have shown that this treatment also has adverse effects such as dyskinesias (Freed et al., 2001; Olanow et al., 2003; Snyder and Olanow, 2005) and that the Lewy pathology also appears after about 10 years in transplanted mDA cells (Kordower and Olanow, 2008; Li *et al.*, 2008). In addition, the use of fetal tissue carries with it the ethical dilemma of using a large number of aborted human fetuses (6-8) to treat one single patient. In order to circumvent this issue many studies are now focusing on developing CRT using different types of stem cells, such as neural stem cells (NSC), embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC). The advantage of these cells is that they can be propagated, standardized and optimized *in vitro* prior to transplantation.

The definition of a stem cell includes three basic properties; I) they are undifferentiated unspecialized cells; II) they have the ability to self-renew through cell division over long periods of time, and; III) have the capacity to differentiate into

specialized cell types (Thomson *et al.*, 1998). Based on these characteristics they are in many ways the most logical candidate cell type for CRT. However, we need to gain a better understanding of the networks of TFs and signaling molecules that govern DA differentiation in order to develop functional and robust differentiation protocols. Another hurdle to cross is how to improve survival and integration or excessive growth of the stem cell preparations once they are transplanted into the host.

1.5 CHEMOKINES AND CHEMOKINE RECEPTORS

1.5.1 Family of ligands

Chemokines (chemotactic cytokines) constitute a family of small (8-14 kDa) secreted peptides that bind and activate chemokine receptors. So far 53 human chemokines and 23 chemokine receptors have been identified (Zlotnik and Yoshie, 2000). The first chemokine to be discovered was CXCL8 (old nomenclature IL-8). This discovery was made in 1987, and CXCL8 became famous for its chemotactic activity on neutrophils (Walz *et al.*, 1987; Yoshimura *et al.*, 1987). Since then, chemokines has been best known for their role in immunresponse and immune trafficking. However, during recent years it has become more and more evident that these proteins also play a major role during the development of the CNS (Rossi and Zlotnik, 2000; Bajetto *et al.*, 2001b). The sequence homology among chemokine ligands is highly variable, ranging from less than 20% to over 90%, but all share very similar tertiary structures (Allen *et al.*, 2007). What all chemokines have in common is a chemokine domain at the N-terminus followed by an approximately 110 amino acid mucin-like stalk rich in Ser and Thr residues, a transmembrane domain, and a cytoplasmic tail. After translation most chemokine ligands are either secreted from the cell or produced in a membrane bound form, or a mix of both. Examples of the latter are CXCL1, CXCL16 and CX3CL1/Fractaline (Garton *et al.*, 2001; Gough *et al.*, 2004).

1.5.2 Classification of chemokines

Chemokines are classified into four groups and referred to, by the new nomenclature adopted in 2000, as the CXC, CC, C-, and CX3C subtypes. Sometimes these subgroups are also referred to as α, β, γ, and δ chemokines, respectively. The chemokine

classification is based on the localization and organization of the first conserved cystein residues in the N-terminus of the protein followed by the letter L (for ligand) and then a number (Murphy *et al.*, 2000; Bacon *et al.*, 2002). The CXC-chemokines (α) and the CC-chemokines (β) are the two largest groups and contain four conserved cysteines in the N-terminus. In the CXC-family the two first cysteines are separated by one amino acid (X) and in the CC-family the first two cysteines are adjacent. The CX3C (δ) include only one member, called CX3CL1 (previously known as fractalkine), in which the first two cysteines are separated by three amino acids (Bazan *et al.*, 1997).

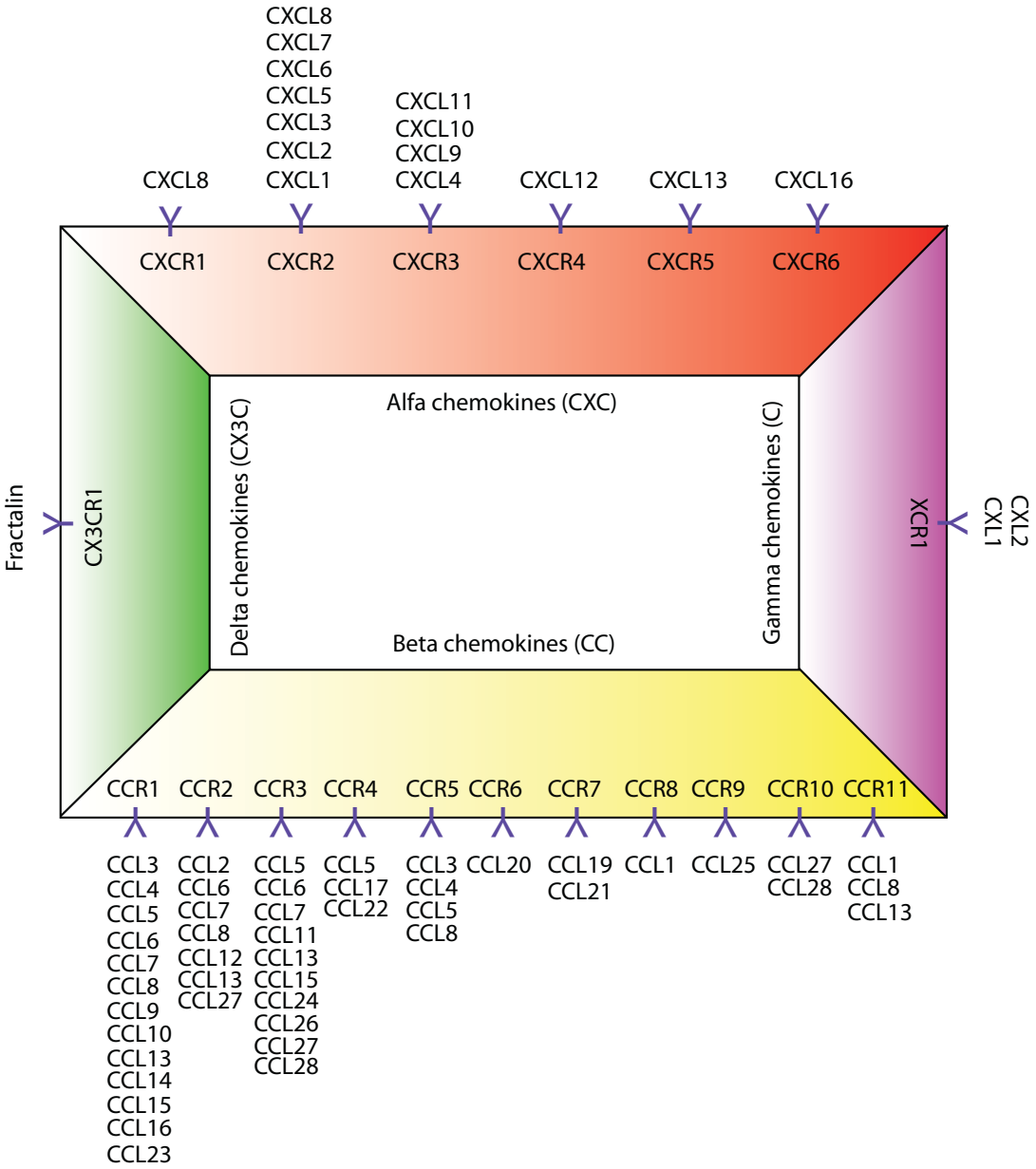


Figure 3. Chemokine receptor classification and ligand specificity. The four families CXC (α), CC (β), C (γ) and CX3C (δ) are depicted.

*The receptor CXCR7 (also referred to as RDC1) is not included in the scheme since it has yet to be approved as a proper chemokine receptor.

Finally, the C-chemokines (γ) is also a small subgroup with only two members; XCL1 (previously known as lymphotactin-a) and XCL2/ (previously known as lymphotactin-b). This subfamily contains only two of the four conserved cysteines found in the other subfamilies (Kelner *et al.*, 1994; Kennedy *et al.*, 1995; Yoshida *et al.*, 1995). The α-

chemokines can be further subdivided into two groups: ELR or non-ELR, depending on the presence or absence of an ELR (glutamic acid-leucine-arginine) motif at the N-terminal of the protein. The ELR motif precedes the CXC, and appears to be chemotactic for endothelial cells and to endow specificity for neutrophil angiogenesis and chemotaxis. A general rule is that chemokines that are lacking the ELR motif (i.e. CXCL4 and CXCL9-11) are instead non-angiogenic and attract monocytes and lymphocytes (Strieter *et al.*, 1995a; Strieter *et al.*, 1995b). However, one exception to the rule is CXCL12 (previously known as SDF-1), which has been shown to induce neovascularization *in vivo* (Bajetto *et al.*, 2001a).

1.5.3 Chemokine receptors

The chemokine receptors have been designated CXC, CC, C or CX3C, based on the chemokine class by which they are activated, followed by the letter R (for receptor), and a number corresponding to the chronological order in which they were identified. 19 chemokine receptors have been cloned so far, including six CXC receptors (CXCR1- CXCR6), 11 CC receptors (CCR1-CCR11), and two single receptors each for fractalkine and lymphotactin, called CX3CR1 and XCR1, respectively. Chemokine receptor classes typically possess about 50% sequence homology among themselves and less than 30% homology with the other classes. The relationships between chemokines and their receptors are complex, as individual chemokines can activate several different chemokine receptors, and conversely, individual chemokine receptors can often be activated by several different chemokines. Until recently, it was believed that there were still sets of unique ligand–receptor interactions existing. For example: CXCL12 with CXCR4; lymphotactin/XCL1 with XCR1; and fractalkine/CX3CL1 with CX3CR1. However, in 2007 it was shown by Balabanian et al. that CXCL12 also bind to another GPCR, namely CXCR7. So, it remains to be determined whether or not the two last pairs also share the promiscuous trait (Lefkowitz, 2004). Moreover, some chemokine receptors are widely expressed, whereas others are restricted to certain specific cells or by specific activation at certain differentiation states (Luster, 1998).
Chemokine receptors are also known as G protein-coupled receptors (GPCRs). GPCRs are members of a superfamily of seven-transmembrane receptors (7TMRs) that mediate intracellular signals through heterotrimeric GTP binding proteins (Pierce *et al.*, 2002).

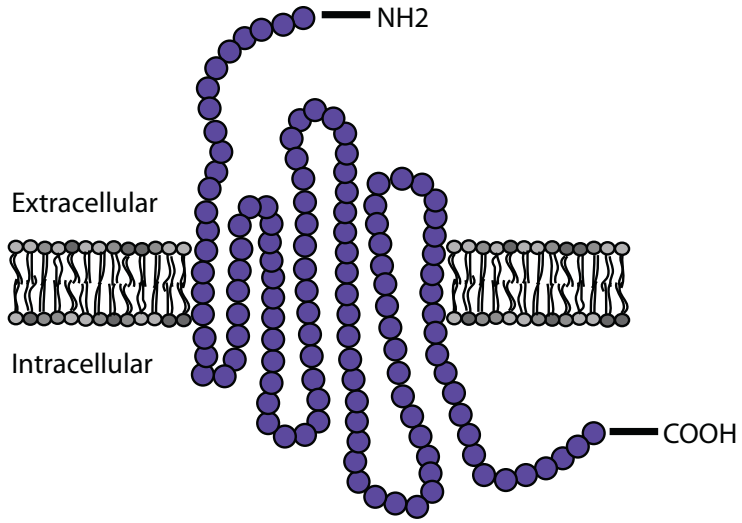


Figure 4. Illustration of a chemokine receptor with the seven helical membrane-spanning regions, an extracellular N-terminus, and an intracellular C-terminus.

As indicated by the name, these 7TMRs have seven helical membranespanning regions, with an extracellular N-terminus and an intracellular C-terminus. G proteins consists of three polypeptides, namely α, β, and γ. In the inactive state, the Gα subunit binds GDP. However, when the ligand binds to the binding pocket of the N-terminal, it allows the receptor to exchange the GDP for a GTP on the Gα-subunit. This in turn creates one complex of GTP bound to the Gα-subunit and one complex of Gβγ. The release of both the α- and βγ-subunits from the receptor initiates several signaling cascades that leads to a number of physiological responses (i.e. cell polarization, chemotaxis, cell migration, etc.). There are four major families of G proteins: Gs, Gi/o, Gq/11, and G12/13 (Wilkie *et al.*, 1992). However, the majority of responses mediated by chemokines can be inhibited by Pertussis toxin (PTX) (Kaslow, 1992), indicating that most of the G proteins involved are members of the Gi protein family. Initially, GPCRs were believed to signal as simple monomers. However, mounting evidence now indicates that many GPCRs, including several chemokine receptors, function as dimers or higher-order oligomers (Gurevich and Gurevich, 2008; Strange, 2008). Chemokine receptors can activate intracellular targets like adenylcyclase, phospholipase, or GTPases like Rho, Rac, and Cdc42 and pathways of major kinases like mitogen-activated protein kinase (MAPK) and phosphatidyl inositol-3 kinase (PI3-K). This diversity of signaling shows that chemokine receptors control a great spectrum of cellular functions (de Haas *et al.*, 2007).

1.5.4 Chemokine activity during CNS development

Chemokines and chemokine receptors are expressed in a constitutive or regulated manner by all the major cell types in the CNS (neurons, astrocytes, microglia and oligodendrocytes) (Belmadani et al., 2005; Khan *et al*., 2008; van Heteren *et al*., 2008; Tsai *et al*., 2002). In this regard, chemokines represent an innate system that mediates cellular communication and helps to establish and maintain CNS homeostasis (Bajetto *et al.*, 2002; Li *et al.*, 2008; Mélik-Parsadaniantz and Rostène, 2008). Chemokines regulates the development of forebrain, midbrain, hindbrain, neural crest and spinal cord (Araujo and Cotman, 1993; Horuk *et al.*, 1997; Tiveron *et al.*, 2006a). Chemokine activity has been reported to control key processes such as: neuronal migration, cell positioning, axon wiring, proliferation and differentiation (Edman *et al.*, 2008a; Edman *et al.*, 2008b). All these processes are essential for the establishment of neural networks in the different neuronal systems. For instance, the α-chemokine receptor CXCR2 has been shown to enhance the survival of hippocampal neurons, and together with its ligand, CXCL1, this signaling pair is involved in patterning the spinal cord by controlling the positioning of oligodendrocyte precursors (Araujo and Cotman, 1993; Horuk *et al.*, 1997; Tsai *et al.*, 2002; Stumm *et al.*, 2007). The expression of CXCL12, another α-chemokine ligand, has been thoroughly mapped in the cortex. It has been shown that projection neurons in the cortex express CXCL12 (Tiveron *et al.*, 2006b), and by binding to CXCR4, this duo regulates the tangential migration of CXCR4-expressing GABAergic interneurons as well as hem-derived Cajal-Retzius cells throughout the cortex (Tiveron *et al.*, 2006a). Among the β-chemokine family, expression of the receptors CCR1-5 and CCR9-11 have been detected in several brain regions (Bajetto *et al.*, 2001b; Edman *et al.*, 2008a). Most of the CC-ligands (β-chemokines) are weakly expressed in the normal brain, but their expression increases during damage and inflammation. Combined, these results illustrate that chemokines contributes to a number of diverse functions during embryogenesis.

1.5.5 Chemokine activity during midbrain development

As mentioned previously, there are a number of well-characterized factors that are involved in the complex construction of the midbrain. However, characterization of the complete molecular program that controls the generation of a progenitor cell residing at

the ventral midline of the midbrain to a mature DA neuron remains largely undefined. Thus, one might predict that new families of factors are likely to be involved in this process. The function of chemokines in DA neuron development is largely unknown. Very few studies have investigated the role of chemokine activity during midbrain development. Chong et al. (2001) showed by reverse transcription-polymerase chain reaction and *in situ* hybridization that the chemokine receptor, CXCR4b –a zebrafish homolog to human CXCR4- was expressed in the midbrain of zebrafish. One year earlier, CXCL15/WECHE, a ligand belonging to the α-chemokine family, was found to be expressed in the VM (Ohneda *et al.*, 2000). However, there is very little functional information available on the role of chemokines in the developing midbrain. Studies in our laboratory have shown that individual chemokines regulate different aspects of DA neuron development in the midbrain *in vitro*. For example, treatment of mouse and rat precursor cultures with CXCL1 and CXCL8 mainly increased cell proliferation and the number of postmitotic precursors population (Edman *et al.*, 2008b). On the other hand, treating the same cultures with CXCL6, CCL2 and CCL7 enlarged the number of differentiated TH+ cells. We have also demonstrated that CCL2 and CCL7 increased the neurite length of TH+ neurons (Edman *et al.*, 2008a). This established the fact that chemokines and chemokine receptors regulate three crucial aspects in early development of the DA neuron: the proliferation of DA progenitors, neurogenesis, and precursor differentiation into VM DA neurons. Furthermore, DA cell proliferation and maturation in the midbrain are known to, in sequence, follow radial and tangential migration (Hanaway *et al.*, 1971; Kawano *et al.*, 1995), send long axonal processes and establish complex neural networks that regulate functions as diverse as control of voluntary movements, emotions and cognition. Our laboratory has discovered is that that CXCL12/CXCR4 signaling regulates migration of A9-A10 DA neurons, neuritogenesis and the initial orientation of their processes (Paper III).

1.5.6 Chemokines in CNS disease

Chemokines are mainly known to mediate trafficking of inflammatory cells and to restrict spreading of inflammation (Hamann *et al.*, 2008). Conditions such as arthritis, atherogenesis, and inflammatory bowel syndrome are all caused by dysregulated chemokine signaling (Feng *et al*., 2000; Murdoch *et al.*, 2000; Baggeolini *et al*., 2001). In the CNS, chemokines are known to function as important mediators in the process of cell migration during certain pathological states. For example, it has been shown that neuronal CCL2 expression is induced upon ischemia, after axonal injury and in motorneurons in patients with amyotrophic lateral sclerosis (ALS) (Che, 2001; Baron, 2005; White, 2005; Henkel, 2006). In the case of brain injury (e.g. stroke or MS), the expression of both CCL2 and CXCL12, is up-regulated by cells surrounding the injury. Following the up-regulation, neural stem cells, neural- and oligodendrocyte progenitors expressing the CXCL12 and CCL2 receptors, CXCR4 and CCR2 respectively (Tran *et al.*, 2007), are attracted to the site of injury for repair purposes (Belmadani *et al.*; 2006). CCL21 is another chemokine that has proven to have an important role in brain damage. Biber et al. showed in 2001 and 2007 that this ligand is rapidly expressed in the ischemic core after brain ischemia. It has been also demonstrated that CCL21 is specifically expressed in neurons after spinal cord injury (Zhao *et al.*, 2007). These findings have suggested that damaged neurons secrete CCL21 in order to activate local microglia.

Chemokines have also been suggested to regulate the function of adult DA neurons. For example, it has been shown that CXCR4 is expressed both on DA neurons in the SN (Banisadr *et al.*, 2002a; Skrzydelski *et al.*, 2007a) and GABA axonal processes (Guyon et al., 2006). In the light of these data, Guyon and co-workers carried out whole cell patch clamp recordings in DA neurons from rat SNpc slices and discovered that CXCL12 (SDF-1α) increase DA release via CXCR4 (Guyon *et al.*, 2008). In addition to this, Patrik Kitabgi's lab has shown that DA release in the SNpc can be modulated upon CXCL12 (SDF-1α) stimulation, *in vivo*. Based on these findings Fuxe et al., speculates that chemokine receptors can interact with DA receptors of the SNpc (Fuxe *et al.*, 2008). This interaction would then lead to cross-talk between cytokines and DA via receptor-receptor interactions. It is possible that inadequate levels of DA and/or inflammation, such as that found in the SN of individuals with PD, may activate such a cross-talk and enhance lead a microglia-mediated neuroinflammatory response. The

expression patterns of chemokines in pathological conditions suggest that they may coordinate brain repair together with angiogenesis and leukocyte influx, giving them a central role in the overall response to injury (de Haas *et al.*, 2007). Moreover, adult midbrain dopamine (DA) neurons have been found to express CXCR4 and to modulate of DA neurotransmission and the activity of the nigrostriatal pathway in response to CXCL12 (Skrzydelski *et al.*, 2007b; Guyon *et al.*, 2008).

1.5.7 Chemokines as a possible source of treating neurodegenerative diseases

Since chemokines and their receptors play a newly recognised role as neuromodulators, they have now received much attention as possible targets in drug discovery. One possible application of chemokines could be in CRT for PD. Chemokines could be used in stem cell protocols to generate mDA neurons either *in vitro*, for transplantation, or *in vivo*, in the host striatum. Another application for chemokines could be in stem cell-based drug development, to improve cellular systems for drug testing. Our work has focused on the development of novel protocols to enhance the expansion and differentiation of stem/progenitor cells into DA neurons. Cells produced by this method may find a therapeutic application as tools for drug development in PD. A third application of chemokines could be as drug targets. The interest of both the pharmaceutical industry and the academic community in the potential of GPCRs is a well-established fact. By learning more about the signaling network of chemokine receptors one might be able find more potential GPCR-targets. A fourth application of chemokine inhibitors could be to prevent the recruitment of inflammatory cells after transplantation and to reduce the inflammatory response in PD.

We thus suggest that chemokines could be useful in many ways in the development of novel treatments for PD.

2 RESULTS AND DISCUSSION

One of the main objectives of our lab is to improve the number and quality of DA neurons in culture with the aim of developing DA cell transplantation strategies for PD. In order to do so, we must understand the signaling machinery that drives DA neurogenesis more precisely. Even though a number of factors already have been identified as crucial for proper DA neuron development, there are still missing pieces of the puzzle. A continued search for unidentified factors and their function is therefore needed.

When I first joined the lab, Dr. Helena Mira (a former posdoc. in the Arenas group) had initiated a project in which she set out to investigate the role of chemokines during DA development. At this point only a few papers had described the role of chemokines in CNS development and there was no functional information available on the role of chemokines during midbrain development. By performing a Chemokine-Gearray-membrane-assay containing 67 chemokines and chemokine receptor genes Dr. Mira found that a number of chemokine receptors, from both the α- and β-subfamily of chemokines, were expressed in the VM. In addition to this, another piece of data from the lab showed that two β-chemokines, CCL2 and CCL7 were regulated by the orphan receptor Nurr1 (Sousa *et al.*, 2006). These results were very intriguing as they all pointed out to an unsuspected role of chemokines in midbrain development and prompted us to further investigate what role chemokines play in the development of mDA neurons.

2.1 PAPER I: α-CHEMOKINES REGULATE PROLIFERATION, NEUROGENESIS AND DOPAMINERGIC DIFFERENTIATION OF VENTRAL MIDBRAIN PRECURSORS AND NEUROSPHERES

As mentioned previously, all the major cell types in the CNS express chemokines (Bajetto *et al.*, 2002), and at the commencement of this project a number of studies had presented evidence for chemokines being involved in guiding cellular communication in the CNS (Araujo and Cotman, 1993; Horuk *et al.*, 1997; Ma *et al.*, 1998; Bajetto *et al.*, 2001b; Bagri *et al.*, 2002; Tsai *et al.*, 2002). We decided to focus on one chemokine receptor in particular, namely CXCR2, and two of the ligands binding to it (i.e. CXCL6 and CXCL8). CXCR2 had been previously proven to enhance the survival of

hippocampal neurons as well as necessary for spinal cord patterning (Araujo and Cotman, 1993; Tsai *et al.*, 2002).

We first set out to analyse CXCR2 expression in the VM by PCR, and found that the receptor was indeed expressed during DA neurogenesis. This initial experiment indicated a possible function of α-chemokines in DA neuron development. Thereafter we treated rat VM E14.5 precursor cultures with either of the two ligands, CXCL6 or CXCL8, for 3 days in vitro (3DIV) and examined the number of cells expressing Nurr1 and/or TH, two markers that identify DA precursors (Nurr1 positive (+)/TH negative (-)), and DA neurons (Nurr1+/TH+). Dose response analysis revealed that both CXCL6 and CXCL8 increased the number of Nurr1+ cells, as well as the number of TH+ cells, in a dose-dependent manner. Additionally, CXCL6 (being endogenously expressed in rodents), but not CXCL8 (only expressed in *Homo sapiens*), increased the number of TH+ neurons out of the Nurr1 population, suggesting that CXCL6 has a predominant effect on the conversion of DA precursors into DA neurons. By quantifying bromodioxyuridine (BrdU) incorporation we assessed that CXCL8, but not CXCL6, increased the number of proliferating cells, indicating that CXCL8 promotes the neurogenic division of progenitors and that CXCL6 promotes the differentiation of Nurr+ precursors into DA neurons.

We next set out to examine whether CXCL6 and CXCL8 induced similar effects in mE11.5 VM neurosphere cultures. In presence of bFGF and EGF, CXCL8 induced cell proliferation in a similar manner as in precursor cultures. When removing bFGF and EGF, and therefore allowing the cells to differentiate on a poly-lysin-D covered plates, most VM neurospheres gave rise to cells belonging to the three different neural lineages: neurons (TuJ1+ cells), oligodendrocytes (MBP+ cells) and astrocytes (GFAP+ cells). As in proliferating neurospheres, CXCL8 increased the number of neurons, but did not affect the number of MBP+ cells or GFAP+ cells. Instead, CXCL6 induced a modest increase in MBP+ cells, but not in GFAP+ cells. Importantly, we found both CXCL6 and CXCL8 increased the number of TH+ cells per sphere by 50%, and the percentage of spheres that contain TH+ cells after 3DIV. These findings support the idea that CXCL6 and CXCL8 also increase the number of DA neurons in NSC preparations grown as neurospheres.

The pronounced effects created by the non-endogenous chemokine CXCL8 were very intriguing to us. Either the effects were non-specific or the CXCL8 expression mimicked another murine chemokine ligand. Firstly, we wanted to confirm that the effects induced by CXCL8 treatment were indeed specific. By adding a CXCL8

blocking antibody (MAB208, R&D systems) to our CXCL8 treated rat E14.5 primary cultures we detected a significant reduction in the number of TH+ neurons; a reduction that was not induced by cell death and was proven to be specific since it was not detected in CXCL6 treated cultures.

Given the well-known promiscuity between chemokine ligands and receptors we addressed the possibility that the antibody cross-reacted with an endogenous α-chemokine exhibiting similar structure/function to CXCL8. Our focus turned to CXCL1, a murine ortholog to the human CXCL8. CXCL1 shares the same CXCR2 receptor and has been proven to be involved in patterning of the spinal cord by controlling the positioning of oligodendrocyte precursors (Tsai *et al.*, 2002). By analysing rat E14.5 VM precursor cultures treated with CXCL1 for 3DIV we found that CXCL1 increased BrdU incorporation and the number of TH+ cells in a very similar manner to CXCL8. However, the number of Ki67+ cells, the labelling index or the number of TuJ1+ neurons did not increase upon CXCL1 treatment implying that CXCL1 does not affect overall proliferation or neurogenesis but rather has a specific effect on DA neurogenesis.

To further validate that CXCL1, CXCL6 and CXCL8 enhanced the number of functional DA neurons, we examined whether the TH+ cells born *in vitro* were capable of responding to a physiological stimuli, such as glutamatergic afferents. Since the glutamate receptor agonist AMPA is known to evoke a robust intracellular Ca^{2+} increase in neurons and it is well documented that the AMPA receptor is expressed in DA neurons (Liu *et al.*, 2005) we carried out intracellular Ca^{2+} flux experiments. We discovered that 90% of the DA neurons produced by CXCL1, CXCL6 or CXCL8 treatment could respond to physiological afferent stimuli and activate Ca^{2+} signaling in response to AMPA. This suggests that DA neurons generated *in vitro* are capable of responding to functional stimuli.

In sum, CXCL1, CXCL6 and CXCL8 regulate -by different mechanisms- three crucial aspects of DA neuron development: the proliferation of DA progenitors, neurogenesis and precursor differentiation into VM DA neurons. α-Chemokines could therefore be useful in the development of cell-based assays for drug development or CRT for neurodegenerative diseases affecting DA neurons, such as PD.

2.2 PAPER II: THE β-CHEMOKINES CCL2 AND CCL7 ARE TWO NOVEL DIFFERENTIATION FACTORS FOR MIDBRAIN DOPAMINERGIC PRECURSORS AND NEURONS

As previously mentioned, there were two experiments the particularly sparked our enthusiasm concerning the potential role of chemokines in the DA system: The chemokine focused array result, showing that the β-chemokine CCL7 is expressed in mouse VM at E10.5 and E12.5; and Dr. Sousa's results from the Affymetrix microarray showing that both CCL2 and CCL7 are regulated by the transcription factor Nurr1 (Sousa et al., 2007). In addition to our in-house data, Ji et al., demonstrated in 2004 that CCL2 and CCL7 are expressed by neurons and glia in the rat brain (Ji *et al.*, 2004b; Ji *et al.*, 2004a). Moreover, CCR2-immunoreactivity was found in TH+ DA neurons of the SNpc and VTA, suggesting a putative function in DA neuron development (Banisadr *et al.*, 2002b; Banisadr *et al.*, 2005). All together, these data prompted us to investigate the expression and function of CCL2 and CCL7, as well as their receptors, in the developing mouse VM.

Firstly, we confirmed by PCR that CCL2 and CCL7 are indeed expressed in the mouse VM during DA neurogenesis, Interestingly, when analysing the expression of these two ligands in Nurr1 knock-out mice, CCL7, but not CCL2, proved to be down-regulated in the null mutants. Since Nurr1 is a nuclear receptor required for DA neuron development (Zetterström *et al*., 1997; Saucedo-Cardenas *et al*., 1998; Castillo *et al*., 1998) our data indicated that CCL7 could mediate a number of events regulated by Nurr1 during VM development.

We then analysed the expression of the receptors binding to CCL2 and/or CCL7 (i.e. CCR1, CCR2, CCR3, CCR9, CCR11), and found that two out of the five receptors, CCR1 and CCR2, are expressed during the same developmental periods (mE10.5-P1).

We next treated rat VM E14.5 precursor cultures with either of the two ligands, CCL2 or CCL7, for 3DIV and examined the number of cells expressing Nurr1 and/or TH. Dose response analysis showed that CCL2 and CCL7 increased the number of TH+ cells in a dose-dependent manner, reaching maximal effects at 0.3 μg/ml CCL2 and 0.1 μg/ml CCL7. At these concentrations the number of Nurr1+ and TuJ1+ cells remained unchanged, however, both CCL2 and CCL7 increased the proportion of TH+/Nurr1+ cells in the cultures, denoting that CCL2 and CCL7 promote the differentiation of Nurr1+ precursor into TH+ neurons.

After a 6-hour-BrdU-pulse in rat E14.5 VM precursor cultures treated with either of the two β-chemokines, we analysed the number of cells that had incorporated BrdU as well as the number of cells that stained positive for active cleaved-caspase 3. No change in the number of BrdU+ cells nor the number active cleaved-caspase 3+ cells were detected, indicating that neither of the two chemokines exerted a proliferative or a survival-promoting effect.

We next set out to examine whether CCL2 and CCL7 may had an early effect in mE11.5 VM neurosphere cultures. However, in proliferative conditions (i.e. in the presence of bFGF and EGF) none of the two ligands enhanced differentiation of progenitors in the neurospheres. These results confirmed that CCL2 and CCL7 do not exert any mitogenic or neurogenic effect on proliferative progenitors. Combined, our results indicate that CCL2 and CCL7 mainly function during later stages of DA development by selectively enhancing the differentiation of Nurr1+ precursors into TH+ DA neurons.

In order to induce functional improvement from transplanting ESC into PD patients the transplant needs not only to survive, but also re-innervate the striatum in an accurate fashion (for review see Parish and Arenas 2007). We next examined whether CCL2 and CCL7 could promote neuritogenesis in DA neurons present, and found that both CCL2 and CCL7 increased the length of TH+ neurites (2-3 fold) in VM precursor cultures. Similar results were also obtained in mouse VM E11.5 neurosphere cultures, in which both chemokines increased neurite length by 2 fold.

In sum, our results indicate that CCL2 and CCL7 regulates important functions in the developing VM; the differentiation of DA precursors into DA neurons and DA neuritogenesis.

We propose that a future use application of beta-chemokines could be to improve and facilitate the differentiation of stem cells into DA neurons, which could then be used for DA drug discovery and for PD CRT.

2.3 PAPER III: CXCL12/CXCR4 SIGNALLING CONTROL THE MIGRATION AND THE INITIAL PROCESS ORIENTATION OF A9-A10 DOPAMINERGIC NEURONS

CXCL12 (Stromal cell-derived factor-1/SDF-1) is a member of the α-chemokine subfamily, and together with its cognate receptor, CXCR4, they represent the best-known chemokine ligand/receptor pair. CXCL12/CXCR4 signaling has been reported to regulate a number of processes essential for the establishment of neural networks in different neuronal systems, for example: neuronal migration, cell positioning and axon wiring (Paredes *et al.*, 2006; Stumm *et al.*, 2007; Tiveron and Cremer, 2008). Additionally, it has been reported that adult mDA neurons have been found to express CXCR4 and that this receptor modulates DA neurotransmission and the activity of the nigrostriatal pathway in response to CXCL12 (Skrzydelski *et al.*, 2007b; Guyon *et al.*, 2008). However, it was not known whether CXCL12/CXCR4 signaling regulates the development of A9-A10 TH+ DA neurons in the VM. Therefore, we sought to determine whether CXCL12 could also works as chemoattractant for DA neurons during VM development.

By performing *in situ* hybridization for CXCR4 and its ligand CXCL12 we were able to study their spatial and temporal expression during VM DA neuron development. We found that CXCR4 was strongly expressed in the mouse VM during the entire DA neurogenic period (i.e. E10.5-15.5). In contrast, CXCL12 was expressed in the meninges surrounding the VM during the same developmental stages. To characterize the cell compartment that expressed CXCR4, we performed additional *in situ* hybridizations for three genes encoding for well characterized DA transcription factors: Ngn2, expressed in DA progenitors and precursors (Kele *et al.*, 2006), Lmx1b, expressed at low levels in DA progenitors and at high levels in DA precursors and neurons (Smidt *et al.*, 2000), and Nurr1, expressed in DA precursors and neurons (Zetterström *et al.*; Castillo *et al.*, 1998; Saucedo-Cardenas *et al.*, 1998). CXCR4 expression was very similar to that of Lmx1b, and the area of high CXCR4 expression level coincided with that of Nurr1 at E11.5. These findings suggested that DA precursors and DA neurons both express high levels of CXCR4.

To further characterize the expression of CXCR4 we used a CXCR4-GFP transgenic mouse strain (Gong *et al.*, 2003). By performing double immunohistochemistry

experiments using different DA markers such Nurr1, TH, and Lmx1a, we show that CXCR4 expression is found in DA cells at all stages of differentiation.

These observations suggest that as the DA progenitors undergo neurogenesis and give rise to DA precursors, they concurrently up-regulate the expression of CXCR4. This up-regulation of CXCR4 could provide DA precursors with competence to respond to directional cues derived from the meninges, such as CXCL12.

Since DA neurons express CXCR4 and given that CXCL12/CXCR4 signaling has previously been shown to regulate migration and axon path finding of a number of different cell types (Balabanian *et al.*, 2005; Odemis *et al.*, 2005) we wanted to elucidate whether CXCL12 also functioned as a chemoattractant for DA neurons residing in the VM. To test this, we performed organotypic cultures of mouse E11.5 midbrain slices and placed agarose beads, coated with PBS or recombinant CXCL12, dorsal to the VM and close to the midline. Interestingly, CXCL12 was found to exert an attractant effect on the TH+ neurites both after 24h and 48h. Since CXCL12 is expressed at high levels in the meninges, we next examined whether the meninges themselves could exert a chemoattractive effect on TH+ cells. Midbrain slices were co-cultured for 72 hours with VM meningeal explants. Neurite outgrowth from the explants was examined every 24 hours using a 2-photon laser-scanning microscope (2PLSM). After 72 hours, almost 80% of the neurites extending from the ventral midline region of the VM reached their target. Moreover, TH immunohistochemistry revealed that many of these neurites originated from DA neurons.

To investigate whether the attractive influence of CXCL12 on TH+ VM cells was mediated via its receptor CXCR4, and not another receptor capable of binding CXCL12, co-cultures were treated with a highly specific CXCR4 antagonist, AMD3100 (Lazarini *et al.*, 2000; Gerlach *et al.*, 2001; Rubin *et al.*, 2003). In the presence of AMD3100 the neuritogenic and chemoattractant effect of the meninges decreased by 3-fold and decreased neurite length by more than 40%. Thus, our results suggest that CXCL12/CXCR4 signaling regulates both the initial ventral orientation of TH+ processes and process outgrowth from VM DA neurons *in vitro*.

Very few studies have investigated the cues necessary for the migration and positioning of DA precursors and neurons. We therefore set out to expand our understanding of this crucial developmental process and investigated the role of CXCL12/CXCR4 signaling in the migration of DA neurons *in vivo*. First, we focally electroporated the developing midbrain *in utero* (at mE12.5) with plasmids encoding for GFP or CXCL12. Thereafter we analysed the number and distribution of TH+ cells at mE14.5. In control embryos

(electroporated with pCAGIG-GFP), the number and position of TH+ cells in the electroporated and contralateral sides were similar. In contrast, in embryos electroporated with CXCL12, we found an increased number of TH+ cells in relatively more lateral and ventral positions than either the contralateral side or control embryos electroporated with GFP. These results demonstrated that CXCL12 also regulates the migration of DA neurons in the VM *in vivo*.

To further verify these findings, we analysed the distribution of TH+ cells and their processes in CXCR4 mutant embryos. At this stage of development, however, all TH+ cells had reached the MZ in wt embryos, whereas many cells were still located in the IZ of CXCR4 mutant embryos. In addition, we found that the number of DA neurons in the IZ exhibiting elaborated and misguided processes in dorsal position increased by more than 4-fold in mutant embryos compared to wt embryos. These results indicate that CXCR4 is required *in vivo* for the migration of DA neurons, a process that may require the correct orientation of their processes toward the MZ. Based on these findings, we propose that CXCL12/CXCR4 signaling is required for normal radial migration of postmitotic cells in the DA lineage.

Interestingly, when we analysed the distribution of TH+ cells and their processes in CXCR4 null mutants we did not find major changes in the total number of TH+ cells residing in the VM compared to wt at E11.5. Nevertheless, we found that the number of DA neurons in the IZ exhibiting elaborated and misguided processes in dorsal position increased by more than 4-fold in mutant embryos compared to wt embryos. These results imply that CXCR4 is required *in vivo* for the migration of DA neurons. Based on these findings, we propose that CXCL12/CXCR4 signaling is required for normal radial migration of postmitotic cells in the DA lineage.

In summary, our findings provide the first evidence that CXCL12 works as a chemoattractant in the VM floor plate and regulates the development of DA neurons in at least three critical steps: radial migration, tangential migration, and process outgrowth. We therefore suggest that CXCL12, by providing directional cues during DA neuron development and target innervation, may constitute an interesting tool for regenerative therapy, to improve the directed innervation/reinnervation of the striatum in PD.

3 CONCLUSIONS

Increasing evidence suggests that chemokines serve several important functions in the nervous system including regulation of neuroimmune responses, neurotransmission, neuronal survival and CNS development. However, the function of chemokines in DA neuron development was largely unknown. Since one of the main research areas in our laboratory has been to investigate the molecular mechanisms governing DA neurogenesis and differentiation, we set out to first investigate whether chemokines could play a role in the development of DA neurons, and second, apply that knowledge to improve the efficiency of DA neuron production by neural stem cells. Based on the work hereby presented we conclude:

Paper I: α-chemokines are expressed in the developing ventral midbrain

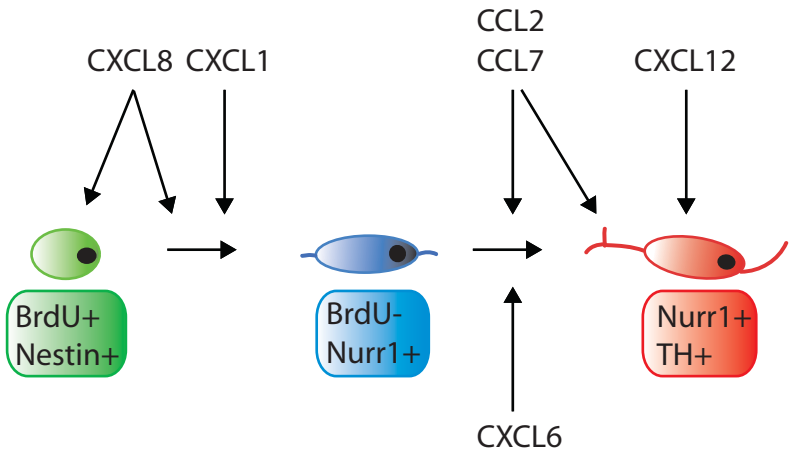
- CXCL1 and CXCL6 as well as their receptor CXCR2 are expressed in the VM during DA neurogenesis and differentiation.
- CXCL1 and CXCL8 induce proliferation
- CXCL6 induce differentiation

Paper II: β-chemokines are expressed in the developing ventral midbrain

- CCL2 and CCL7 as well as their receptors CCR1 and CCR2 are expressed in the ventral midbrain during DA neurogenesis and differentiation
- CCL7 is down-regulated in Nurr1 null mice
- CCL2 and CCL7 induce DA differentiation and neuritogenesis

Paper III: CXCL12/CXCR4 signaling controls DA neuron migration as well as process orientation.

- CXCR4 is expressed in the VM during DA neurogenesis, and its ligand CXCL12 is expressed in the meninges surrounding the VM during the same developmental stages.
- CXCL12 promotes neuritogenesis in TH+ cells
- CXCR4 null mice showed that TH+ neurons do not complete their radial migration, and that their cell processes are disoriented.



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5 REFERENCES

Albéri, L., Sgadò, P. & Simon, H.H. (2004) Engrailed genes are cell-autonomously required to prevent apoptosis in mesencephalic dopaminergic neurons. *Development* **131**, 3229-3236.

Alexandre, P. & Wassef, M. (2005) Does the isthmic organizer influence D/V patterning of the midbrain? *Brain Res. Brain Res. Rev.* **49,** 127-133.

Allen, S., Crown, S. & Handel, T. (2007) Chemokine: Receptor Structure, Interactions, and Antagonism. *Annual Review of Immunology* **25**, 787-820.

Andersson, E., Jensen, J., Parmar, M., Guillemot, F. & Björklund, A. (2006a) Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2. *Development* **133**, 507-516.

Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., Perlmann, T. & Ericson, J. (2006b) Identification of intrinsic determinants of midbrain dopamine neurons. *Cell* **124**, 393-405.

Ang, S. (2006) Transcriptional control of midbrain dopaminergic neuron development. *Development* **133**, 3499-3506.

Araujo, D.M. & Cotman, C.W. (1993) Trophic effects of interleukin-4, -7 and -8 on hippocampal neuronal cultures: potential involvement of glial-derived factors. *Brain Res.* **600**, 49-55.

Bacon, K., Baggiolini, M., Broxmeyer, H., Horuk, R., Lindley, I., Mantovani, A., Maysushima, K., Murphy, P., Nomiyama, H., Oppenheim, J., Rot, A., Schall, T., Tsang, M., Thorpe, R., Van Damme, J., Wadhwa, M., Yoshie, O., Zlotnik, A., Zoon, K. & Nomenclature, I.W.S.o.C. (2002) Chemokine/chemokine receptor nomenclature. *J. Interferon Cytokine Res.* **22**, 1067-1068.

Baggiolini, M. (2001) Chemokines in pathology and medicine. *J. Intern. Med.***250,** 91-104.

Bagri, A., Gurney, T., He, X., Zou, Y.R., Littman, D.R., Tessier-Lavigne, M. & Pleasure, S.J. (2002) The chemokine SDF1 regulates migration of dentate granule cells. *Development* **129**, 4249-4260.

Bajetto, A., Barbero, S., Bonavia, R., Piccioli, P., Pirani, P., Florio, T. & Schettini, G. (2001a) Stromal cell-derived factor-1alpha induces astrocyte proliferation through the activation of extracellular signal-regulated kinases 1/2 pathway. *J. Neurochem.* **77**, 1226-1236.

Bajetto, A., Bonavia, R., Barbero, S., Florio, T. & Schettini, G. (2001b) Chemokines and their receptors in the central nervous system. *Frontiers in neuroendocrinology* **22**, 147-184.

Bajetto, A., Bonavia, R., Barbero, S. & Schettini, G. (2002) Characterization of chemokines and their receptors in the central nervous system: physiopathological implications. *J. Neurochem.* **82**, 1311-1329.

Balabanian, K., Lagane, B., Infantino, S., Chow, K.Y., Harriague, J., Moepps, B., Arenzana-Seisdedos, F., Thelen, M. & Bachelerie, F. (2005) The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J. Biol. Chem.* **280**, 35760-35766.

Banisadr, G., Fontanges, P., Haour, F., Kitabgi, P., Rostene, W. & Parsadaniantz, S. (2002a) Neuroanatomical distribution of CXCR4 in adult rat brain and its localization in cholinergic and dopaminergic neurons. *Eur. J. Neurosci.* **16**, 1661–1671.

Banisadr, G., Gosselin, R., Mechighel, P., Kitabgi, P., Rostène, W. & Parsadaniantz, S. (2005) Highly regionalized neuronal expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) in rat brain: evidence for its colocalization with neurotransmitters and neuropeptides. *J. Comp. Neurol.* **489**, 275-292.

Banisadr, G., Quéraud-Lesaux, F., Boutterin, M., Pélaprat, D., Zalc, B., Rostène, W., Haour, F. & Parsadaniantz, S. (2002b) Distribution, cellular localization and functional role of CCR2 chemokine receptors in adult rat brain. *J. Neurochem.* **81**, 257-269.

Baron, P. (2005) Production of monocyte chemoattractant protein-1 in amyotrophic lateral sclerosis. *Muscle Nerve* **32**, 541-544.

Bayer, S.A., Wills, K.V., Triarhou, L.C., Ghetti, B. (1995) Time of neuron origin and gradients of neurogenesis in midbrain dopaminergic neurons in the mouse. *Exp. Brain Res.* **105**, 191-199.

Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R., Zlotnik, A. & Schall, T.J. (1997) A new class of membrane-bound chemokine with a CX3C motif. *Nature* **385**, 640-644.

Belmadani, A., Tran, P., Ren, D. & Miller, R. (2006) Chemokines regulate the migration of neural progenitors to sites of neuroinflammation. *J. Neurosci.* **26**, 3182-3191.

Biber, K., Sauter, A., Brouwer, N., Copray, S.C. & Boddeke, H.W. (2001) Ischemia-induced neuronal expression of the microglia attracting chemokine Secondary Lymphoid-Tissue chemokines (SLC). *Glia* **34**, 121-133.

Biber, K., Neumann, H., Inoue, K. & Boddeke, H.W. (2007) Neuronal "On" and "Off" signals control microglia. *Trends Neurosci.* **30**, 596–602

Björklund, A., Dunnett, S.B (2007) Dopamine neuron systems in the brain: an update. *Trends Neurosci.* **30**, 194-202.

Björklund, A. & Dunnett, S. (2007) Fifty years of dopamine research. *Trends Neurosci.* **30**, 185-187.

Blaess, S. Corrales, J.D., Joyner, A.L. (2006) Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. *Development* **133**, 1799-1809.

Bonilla, S., Hall, A., Pinto, L., Attardo, A., Götz, M., Huttner, W.B., Arenas, E. (2008) Identification of midbrain floor plate radial glia-like cells as dopaminergic progenitors. *Glia* **56**, 809-820.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T.M., Rubenstein, J.L., Ericson, J. (1999) Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**, 622-627.

Broccoli, V, Bonicinelli, E., Wurst, W. (1999) The caudal limit of Otx2 expression positions the isthmic organizer. *Nature* **401**, 164-168.

Candy, J., Perry, R., Perry, E., Irving, D., Blessed, G., Fairbairn, A. & Tomlinson, B. (1983) Pathological changes in the nucleus of Meynert in Alzheimer's and Parkinson's diseases. *J. Neurol. Sci.* **59**, 277-289.

Carlsson, A., Lindqvist, M., Magnusson, T. & Waldeck, B. (1958) On the presence of 3-hydroxytyramine in brain. *Science* **127**, 471.

Castillo, S.O., Baffi, J.S., Palkovits, M., Goldstein, D.S., Kopin, I.J., Witta, J., Magnuson, M.A. & Nikodem, V.M. (1998) Dopamine biosynthesis is selectively abolished in substantia nigra/ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene. *Mol. Cell Neurosci.* **11**, 36-46.

Che, X. (2001) Monocyte chemoattractant protein-1 expressed in neurons and astrocytes during focal ischemia in mice. *Brain Res.* **9**. 171-177.

Chong, S.W., Emelyanov, A., Gong, Z. & Korzh, V. (2001) Expression pattern of two zebrafish genes, cxcr4a and cxcr4b. *Mech. Dev.* **109**, 347-354.

Chung, S., Hedlund, E., Hwang, M. Kim, D.W., Shin, B.S., Hwang, D.Y., Jung Kang, U., Isacson, O., & Kim, K.S. (2005) The homeodomain transcription factor Pitx3 facilitates differentiation of mouse embryonic stem cells into AHD2-expressing dopaminergic neurons. *Mol. Cell Neurosci.* **28**, 241-252.

Cotzias, G., Van Woert, M. & Schiffer, L. (1967) Aromatic amino acids and modification of parkinsonism. *N. Eng. J. Med.* **276**, 374-379.

Crossley, P., Martinez, S. & Martin, G. (1996) Midbrain development induced by FGF8 in the chick embryo. *Nature **380***. 66-68.

Danielian, P. & McMahon, A. (1996) Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development. *Nature* **383**. 332-334.

Davidson, D., Graham, E., Sime, C. & Hill, R. (1988) A gene with sequence similarity to Drosophila engrailed is expressed during the development of the neural tube and vertebrae in the mouse. *Development* **104**, 305–316.

Dawson, T. & Dawson, V. (2003) Molecular pathways of neurodegeneration in Parkinson's disease. *Science* **302**. 819-822.

de Haas, A., van Weering, H., de Jong, E., Boddeke, H. & Biber, K. (2007) Neuronal Chemokines: Versatile Messengers In Central Nervous System Cell Interaction. *Mol. Neurobiol.* **36**, 137–151.

Di Porzio, U., Zuddas, A., Cosenza-Murphy, D. & Barker, J. (1990) Early appearance of tyrosine hydroxylase immunoreactive cells in the mesencephalon of mouse embryos. *Int. J. Dev. Neurosci.* **8**, 523-532.

Dunnett, S.B., Björklund, A., Lindvall, O. (2001) Cell therapy in Parkinson's disease – stop or go? *Nat. Rev. Neurosci.* **2**, 365–369.

Edman, L., Mira, H. & Arenas, E. (2008a) The beta-chemokines CCL2 and CCL7 are two novel differentiation factors for midbrain dopaminergic precursors and neurons.
Exp Cell Res. **314**, 2123-2130.

Edman, L., Mira, H., Erices, A., Malmersjö, S., Andersson, E., Uhlén, P. & Arenas, E. (2008b) Alpha-chemokines regulate proliferation, neurogenesis, and dopaminergic differentiation of ventral midbrain precursors and neurospheres. *Stem Cells* **26**, 1891-1900.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. & Briscoe, J. (1997) Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Farkas, L.M., Dünker, N., Roussa, E., Unsicker, K., Krieglstein, K. (2003) Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in vitro and in vivo. *J. of Neurosci.* **23**, 5178-5186.

Feng, L. (2000) Role of chemokines in inflammation and immunoregulation. *Immunol. Res.* **21**, 203-210.

Foster, G.A., Schultzberg, M., Kökfelt, T., Goldstein, M., Hemmings, H.C. Jr., Ouimet, C.C., Walaas, S.I., & Greengard, P. (1988) Ontogeny of the dopamine and cyclic adenosine-3':5'-monophosphate-regulated phosphoprotein (DARPP-32) in the pre- and postnatal mouse central nervous system. *Int. J. Dev. Neurosci.* **6**, 367-386.

Friling, S., Andersson, E., Thompson, L., Jönsson, M., Hebsgaard, J., Nanou, E., Alekseenko, Z., Marklund, U., Kjellander, S., Volakakis, N., Hovatta, O., El Manirad, A., Björklund, A., Perlmann, T. & Ericson, J. (2009) Efficient production of mesencephalic dopamine neurons by Lmx1a expression in embryonic stem cells *Proc. Natl. Acad. Sci. USA.* **106,** 7613-7618.

Fuxe, K., Tarakanov, A., Goncharova, L. & Agnati, L. (2008) A new road to neuroinflammation in Parkinson's disease? *Brain Res. Rev.* **58**, 453-458.

Gardner, C.A., Darnell, D.K., Poole, S.J., Ordahl, C.P. & Barald, K.F. (1988) Expression of an engrailed-like gene during development of the early embryonic chick nervous system. *J. of Neurosci.* **21**, 426–437.

Garton, K.J., Gough, P.J., Blobel, C.P., Murphy, G., Greaves, D.R., Dempsey, P.J. & Raines, E.W. (2001) Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). *J. Biol. Chem.* **276**, 37993-38001.

Gerlach, L.O., Skerlj, R.T., Bridger, G.J. & Schwartz, T.W. (2001) Molecular interactions of cyclam and bicyclam non-peptide antagonists with the CXCR4 chemokine receptor. *J. Biol. Chem.* **276**, 14153-14160.

Gilbert, S., F (2003) *Developmental Biology.* Sinauer Associates.

Gildenberg, P. (2005) Evolution of neuromodulation. *Stereotact. Funct. Neurosurg.* **83**, 71-79.

Gong, S., Zheng, C., Doughty, M.L., Losos, K., Didkovsky, N., Schambra, U.B., Nowak, N.J., Joyner, A., Leblanc, G., Hatten, M.E., & Heintz, N. (2003) A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* **425**, 917-925.

Gough, P.J., Garton, K.J., Wille, P.T., Rychlewski, M., Dempsey, P.J. & Raines, E.W. (2004) A disintegrin and metalloproteinase 10-mediated cleavage and shedding regulates the cell surface expression of CXC chemokine ligand 16. *J. Immunol.* **172**, 3678-3685.

Guo C, Qiu, H.Y., Huang, Y., Chen, H., Yang, R.Q., Chen, S.D., Johnson, R.L., Chen, Z.F. & Ding, Y.Q. (2007) Lmx1b is essential for Fgf8 and Wnt1 expression in the isthmic organizer during tectum and cerebellum development in mice. *Development* **134**, 317-325.

Gurevich, V.V. & Gurevich, E.V. (2008) GPCR monomers and oligomers: it takes all kinds. *Trends Neurosci.* **31**, 74-81.

Guyon, A., Skrzydelsi, D., Rovère, C., Rostène, W., Parsadaniantz, S.M. & Nahon, J.L. (2006) Stromal cell-derived factor-1alfa modulation of the excitability of rat substantia nigra dopaminergic neurons: presynaptic mechanisms. *J. Neurochem*. **6,** 1540-1550.

Guyon, A., Skrzydelski, D., Rovère, C., Apartis, E., Rostène, W., Kitabgi, P., Mélik Parsadaniantz, S. & Nahon, J.L. (2008) Stromal-cell-derived factor 1alpha /CXCL12 modulates high-threshold calcium currents in rat substantia nigra. *Eur. J. Neurosci.* **28**, 862-870.

Hamann, I., Zipp, F. & Infante-Duarte, C. (2008) Therapeutic targeting of chemokine signaling in Multiple Sclerosis. *J Neurol Sci* **274**, 31-38.

Hammond, R., Blaess, S. & Abeliovich, A. (2009) Sonic Hedgehog Is a Chemoattractant for Midbrain Dopaminergic Axons. *PLoS ONE* **4**, e7007.

Hanaway, J., McConnell, J.A. & Netsky, M.G. (1971) Histogenesis of the substantia nigra, ventral tegmental area of Tsai and interpeduncular nucleus: an autoradiographic study of the mesencephalon in the rat. *J. Comp. Neurol.* **142**, 59-73.

Hedlund, E., Pruszaka, J., Lardaroa, T., Ludwiga, W., Viñuelaa, A., Kima, K. & Isacson, O. (2008) Embryonic Stem Cell-Derived Pitx3-Enhanced Green Fluorescent
Protein Midbrain Dopamine Neurons Survive Enrichment by Fluorescence-Activated Cell Sorting and Function in an Animal Model of Parkinson's Disease *Stem Cells* **26**, 1526–1536.

Henkel, J. (2006) The chemokine MCP-1 and the dendritic and myeloid cells it attracts are increased in the mSOD1 mouse model of ALS. *Mol. Cell Neurosci.* **31**, 427–437.

Horuk, R., Martin, A.W., Wang, Z., Schweitzer, L., Gerassimides, A., Guo, H., Lu, Z., Hesselgesser, J., Perez, H.D., Kim, J., Parker, J., Hadley, T.J. & Peiper, S.C. (1997) Expression of chemokine receptors by subsets of neurons in the central nervous system. *J. Immunol.* **158**, 2882-2890.

Hynes, M., Porter, J.A., Chiang, C., Chang, D., Tessier-Lavigne, M., Beachy, P.A., & Rosenthal, A. (1995a) Induction of midbrain dopaminergic neurons by Sonic hedgehog. *Neuron* **15**, 35-44.

Hynes, M., Poulsen, K., Tessier-Lavigne, M. & Rosenthal, A. (1995b) Control of neuronal diversity by the floor plate: contact-mediated induction of midbrain dopaminergic neurons. *Cell* **80**, 95-101.

Hynes, M. & Rosenthal, A. (1999) Specification of dopaminergic and serotonergic neurons in the vertebrate CNS. *Curr. Opin. Neurobiol.* **9**, 26-36.

Hynes, M., Stone, D., Dowd, M., Pitts-Meek, S., Goddard, A., Gurney, A. & Rosenthal, A. (1997) Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. *Neuron* **19**, 15-26.

Ikemoto, S. & Panksepp, J. (1999) The role of nucleus accumbens dopamine in motivated behaviour: a unifying interpretation with special reference to reward-seeking. *Brain Res. Rev.* **31**, 6-41.

Ji, J., He, B., Dheen, S. & Tay, S. (2004a) Expression of chemokine receptors CXCR4, CCR2, CCR5 and CX3CR1 in neural progenitor cells isolated from the subventricular zone of the adult rat brain. *Neurosci. Lett.* **355**, 236-240.

Ji, J., He, B., Dheen, S. & Tay, S. (2004b) Interactions of chemokines and chemokine receptor mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. *Stem Cells* **22**, 415-427.

Joksimovic, M, Yun, B.A., Kittappa, R., Anderegg, A.M., Chang, W.W., Taketo, M.M., McKay, R.D. & Awatramani, R.B. (2009) Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis. *Nat. Neurosci.* **Feb;12(2):125-31**.

Kandel, E.R., Schwartz, J.H. & Jessell, T.M. (2000) *Principles of neural science.* McGraw-Hill, Health Professions Division.

Kaslow, H.R. & Burns, D.L. (1992) Pertussis toxin and target eukaryotic cells: binding, entry, and activation. *FASEB J.* **6**, 2684-2690.

Kordower, J.H., Chu, Y., Hauser, R.A. & Olanow, C.W. (2008) Lewy body–like pathology in long-term embryonic nigral transplants in Parkinson's disease. *Nat. Med.* **14**, 483-485.

Kawano, H., Ohyama, K., Kawamura, K. & Nagatsu, I. (1995) Migration of dopaminergic neurons in the embryonic mesencephalon of mice. *Brain Res. Dev. Brain Res.* **86**, 101-113.

Kele, J., Simplicio, N., Ferri, A.L., Mira, H., Guillemot, F., Arenas, E. & Ang, S.L. (2006) Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. *Development* **133**, 495-505.

Kelner, G.S., Kennedy, J., Bacon, K.B., Kleyensteuber, S., Largaespada, D.A., Jenkins, N.A., Copeland, N.G., Bazan, J.F., Moore, K.W. & Schall, T.J. (1994) Lymphotactin: a cytokine that represents a new class of chemokine. *Science* **266**, 1395-1399.

Kennedy, J., Kelner, G.S., Kleyensteuber, S., Schall, T.J., Weiss, M.C., Yssel, H., Schneider, P.V., Cocks, B.G., Bacon, K.B. & Zlotnik, A. (1995) Molecular cloning and functional characterization of human lymphotactin. *J. Immunol.* **155**, 203-209.

Kim, J.H., Auerbach, J.M., Rodríguez-Gómez, J.A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S.H., Nguyen, J., Sánchez-Pernaute, R., Bankiewicz, K. & McKay, R. (2002) Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* **418**, 50-56.

Khan, M. Z., Brandimarti, R., Shimizu, S., Nicolai, J., Crowe, E. & Meucci, O. (2008) The chemokine CXCL12 promotes survival of postmitotic neurons by regulating Rb protein. *Cell Death Differ.* **15**, *1662-1672.*

Kringelbach, M.L., Jenkinson, N., Owen, S.L. & Aziz, T.Z. (2007) Translational principles of deep brain stimulation. *Nat. Rev. Neurosci.* **8**, 623–635.

Lazarini, F., Casanova, P., Tham, T.N., De Clercq, E., Arenzana-Seisdedos, F., Baleux, F. & Dubois-Dalcq, M. (2000) Differential signalling of the chemokine receptor CXCR4 by stromal cell-derived factor 1 and the HIV glycoprotein in rat neurons and astrocytes. *Eur. J. Neurosci.* **12**, 117-125.

Lee, S., Danielian, P., Fritzsch, B. & McMahon, A. (1997) Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. *Development* **124**, 959-969.

Lefkowitz, R.J. (2004) Historical review: a brief history and personal retrospective of seven-transmembrane receptors. *Trends Pharmacol. Sci.* **25**, 413-422.

Li, G., Adesnik, H., Li, J., Long, J., Nicoll, R.A., Rubenstein, J.L. & Pleasure, S.J. (2008) Regional distribution of cortical interneurons and development of inhibitory tone are regulated by Cxcl12/Cxcr4 signaling. *J. Neurosci.* **28**, 1085-1098.

Li, J.Y., Englung, E., Holton, J.L., Soulet, D., Hagell, P., Lees, A.J., Lashley, T., Quinn, N.P., Rehncrona, S., Björklund, A., Widner, H., Revesz, T., Lindavall, O. & Brundin, P. (2008). *Nat. Med.* **14**, 501-503.

Lindahl, R., Evces, S. (1984) Comparative subcellular distribution of aldehyde dehydrogenase in rat, mouse and rabbit liver. *Biochem. Pharmacol.* **33**, 3383-3389.

Lindvall, O., Rehncrona, S., Gustavii, B., Brundin, P., Astedt, B., Widner, H., Lindholm, T., Björklund, A., Leenders, K.L., Rothwell, J.C., Marsden, C.D., Johnels, B., Steg, G., Freedman, R., Hopper, B.J., Seiger, Å., Strömberg, I., Bygdeman, M. & Olson, L. (1988) Fetal dopamine-rich mesencephalic grafts in Parkinson's disease. *The Lancet* **31**, 1483-1484.

Lindvall, O. & Hagell, P. (2000) Clinical observations after neural transplantation in Parkinson's disease. *Prog. brain res.* **127**, 299-320.

Liu, A. & Joyner, A. (2001) EN and GBX2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region. *Development* **128**, 181-191.

Liu, Q., Pu, L. & Poo, M. (2005) Repeated cocaine exposure in vivo facilitates LTP induction in midbrain dopamine neurons. *Nature* **13**, 1023-1031.

Liu, X., Zhang, Z., Zhang, R., Gregg, S., Wang, L., Yier, T. & Chopp, M. (2007) Chemokine ligand 2 (CCL2) induces migration and differentiation of subventricular zone cells after stroke. *J. Neurosci. Res.* **85**, 2120-2125.

Luster, A.D. (1998) Chemokines: chemotactic cytokines that mediate inflammation. *New. Engl. J. Med.* **338**, 436-445.

Ma, Q., Jones, D., Borghesani, P.R., Segal, R.A., Nagasawa, T., Kishimoto, T., Bronson, R.T. & Springer, T.A. (1998) Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc. Natl. Acad. Sci. USA.* **95**, 9448-9453.

Maden, M. (2007) Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nat. Rev. Neurosci.* **8**, 755-765.

Marchand, R. & Poirier, L. (1983) Isthmic origin of neurons of the rat substantia nigra. *Neurosci.* **9**, 373-381.

Marin, F., Herrero, M.T., Vyas, S. & Puelles, L. (2005) Ontogeny of tyrosine hydroxylase mRNA expression in mid- and forebrain: neuromeric pattern and novel positive regions. *Dev. Dyn.* **234**, 709 -717.

Martinez, S., Crossley, P.H., Cobos, I., Rubenstein, J.L. & Martin, G.R. (1999) FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression. *Development* **6**, 1189-1200.

McMahon, A.P., Joyner, A.L., Bradley, A. & McMahon, J.A. (1992) The midbrain-hindbrain phenotype of Wnt-1-/Wnt-1- mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. *Cell* **4**, 581-595.

McMahon, A.P. & Bradely, A. (1990) The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **6**, 1073-1085.

Mélik-Parsadaniantz, S. & Rostène, W. (2008) Chemokines and neuromodulation. *J. Neuroimmunol.* **198**, 62-68.

Millet, S., Campbell, K., Epstein, D.J., Losos, K., Harris, E. & Joyner, A.L. (1999) A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. *Nature* **401**, 161-164.

Murdoch, C. & Finn, A. (2000) Chemokine receptors and their role in inflammation and infectious diseaseases. *Blood 95*, 3032-3043.

Murphy, P.M., Baggiolini, M., Charo, I.F., Hébert, C.A., Horuk, R., Matsushima, K., Miller, L.H., Oppenheim, J.J. & Power, C.A. (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol. rev.* **52**, 145-176.

Nunes, I., Tovmasian, L.T., Silva, R.M., Burke, R.E. & Goff, S.P. (2003) Pitx3 is required for development of substantia nigra dopaminergic neurons. *Proc. Natl. Acad. Sci. USA.* **100**, 4245-4250.

Odemis, V., Lamp, E., Pezeshki, G., Moepps, B., Schilling, K., Gierschik, P., Littman, D.R. & Engele, J. (2005) Mice deficient in the chemokine receptor CXCR4 exhibit impaired limb innervation and myogenesis. *Mol. Cell. Neurosci.* **30**, 494-505.

Ohneda, O., Ohneda, K., Nomiyama, H., Zheng, Z., Gold, S.A., Arai, F., Miyamoto, T., Taillon, B.E., McIndoe, R.A., Shimkets, R.A., Lewin, D.A., Suda, T. & Lasky, L.A. (2000) WECHE: a novel hematopoietic regulatory factor. *Immunity* **12**, 141-150.

Ono Y, N.T., Sakamoto Y, Mizuhara E, Minaki Y, Kumai M, Hamaguchi A, Nishimura M, Inoue Y, Hayashi H, Takahashi J, Imai T. (2007) Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development* **134**, 3213-3225.

Orentas, D.M., Hayes, J.E., Dyer, K.L. & Miller, R.H. (1999) Sonic hedgehog signaling is required during the appearance of spinal cord oligodendrocyte precursors.
Development **126**, 2419-2429.

Paredes, M.F., Li, G., Berger, O., Baraban, S.C. & Pleasure, S.J. (2006) Stromal-derived factor-1 (CXCL12) regulates laminar position of Cajal-Retzius cells in normal and dysplastic brains. *J. Neurosci.* **26**, 9404-9412.

Parish, C.L. & Arenas, E. (2007) Stem-cell-based startegies for the treatment of Parkinson's disease. *Neurodegener. Dis.* **4**, 339-347.

Piccini, P., Brooks, D.J., Björklund, A., Gunn, R.N., Grasby, P.M., Rimoldi, O., Brundin, P., Hagell, P., Rehncrona, S., Widner, H. & Lindvall, O. (1999) Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. *Nat. Neurosci.* **2**, 1137–1140.

Pierce, K.L., Premont, R.T. & Lefkowitz, R.J. (2002) Seven-transmembrane receptors. *Nat. Rev. Mol. Cell Biol.* **3**, 639-650.

Placzek, M. & Briscoe, J. (2005) The floor plate: multiple cells, multiple signals. *Nat. Rev. Neurosci.* **6**, 230-240.

Prakash, N. & Wurst, W. (2004) Specification of midbrain territory.*Cell Tissue Res.* **318**, 5-14.

Prakash, N. & Wurst, W. (2006) Development of dopaminergic neurons in the mammalian brain. *Cell Mol. Life Sci.* **63**, 187-206.

Rossi, D. & Zlotnik, A. (2000) The biology of chemokines and their receptors. *Annu. Rev. Immunol.* **18**, 217-242.

Rubin, J.B., Kung, A.L., Klein, R.S., Chan, J.A., Sun, Y., Schmidt, K., Kieran, M.W., Luster, A.D. & Segal, R.A. (2003) A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors. *Proc. Natl. Acad. Sci. USA.* **100**, 13513-13518.

Sacchetti, P., Brownschidle, L.A., Granneman, J.G. & Bannon, M.J. (1999) Characterization of the 5'-flanking region of the human dopamine transporter gene. *Brain Res. Mol. Brain Res.* **74**.

Sakurada, K., Ohshima-Sakurada, M., Palmer, T.D. & Gage, F.H. (1999) Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* **126**, 4017-4026.

Saucedo-Cardenas, O., Quintana-Hau, J.D., Le, W.D., Smidt, M.P., Cox, J.J., De Mayo, F., Burbach, J.P. & Conneely, O.M. (1998) Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc. Natl. Acad. Sci. USA.* **95**, 4013-4018.

Schwarz M, Alvarez-Bolado, G., Urbánek, P., Busslinger, M. & Gruss, P. (1997) Conserved biological function between Pax-2 and Pax-5 in midbrain and cerebellum development: evidence from targeted mutations. *Proc. Natl. Acad. Sci. U S A.* **94**, 14518-14523.

Simon, H., Saueressig, H., Wurst, W., Goulding, M. & O'Leary, D. (2001) Fate of midbrain dopaminergic neurons controlled by the engrailed genes. *J. Neurosci.* **21**, 3126–3134.

Simon, H.H., Bhatt, L., Gherbassi, D., Sgado, P. & Alberí, L. (2003) Midbrain dopaminergic neurons/determination of their developmental fate by transcription factors. *Ann. N Y Acad. Sci.* **991**, 36-47.

Skrzydelski, D., Guyon, A., Daugé, V., Rovère, C., Apartis, E., Kitabgi, P., Nahon, J.L., Rostène, W. & Parsadaniantz, S.M. (2007) The chemokine stromal cell-derived factor-1/CXCL12 activates the nigrostriatal dopamine system. *J. Neurochem.* **102**, 1175-1183.

Smidt, M.P., Smits, S.M., Bouwmeester, H., Hamers, F.P., van der Linden, A.J., Hellemons, A.J., Graw, J. & Burbach, J.P. (2004) Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3. *Development* **131**, 1145-1155.

Smidt, M.P., van Schaick, H.S., Lanctôt, C., Tremblay, J.J., Cox, J.J., van der Kleij, A.A., Wolterink, G., Drouin, J. & Burbach, J.P. (1997) A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. *Proc. Natl. Acad. Sci. U S A.* **94**, 13305-13310.

Smidt, M.P., Asbreuk, C.H., Cox, J.J., Chen, H., Johnson, R.L. & Burbach, J.P. (2000) A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nat. Neurosci.* **3**, 337-341.

Sousa, K.M., Mira, H., Hall, A.C., Jansson-Sjöstrand, L., Kusakabe, M. & Arenas, E. (2007) Microarray analyses support a role for Nurr1 in resistance to oxidative stress and neuronal differentiation in neural stem cells. *Stem Cells* **25**, 511-519.

Steece-Collier, K., Maries, E. & Kordower, J.H. (2002) *Proc. Natl. Acad. Sci. U S A.* **99**, 13972-13974.

Strange, P.G. (2008) Signaling mechanisms of GPCR ligands. *Current opinion in drug discovery & development* **11**, 196-202.

Strieter, R.M., Polverini, P.J., Arenberg, D.A. & Kunkel, S.L. (1995a) The role of CXC chemokines as regulators of angiogenesis. *Shock* **4**, 155-160.

Strieter, R.M., Polverini, P.J., Kunkel, S.L., Arenberg, D.A., Burdick, M.D., Kasper, J., Dzuiba, J., Van Damme, J., Walz, A. & Marriott, D. (1995b) The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J. Biol. Chem.* **270**, 27348-27357.

Stumm, R., Kolodziej, A., Schulz, S., Kohtz, J.D. & Höllt, V. (2007) Patterns of SDF-1alpha and SDF-1gamma mRNAs, migration pathways, and phenotypes of CXCR4-expressing neurons in the developing rat telencephalon. *J. Comp. Neurol.* **502**, 382-399.

Thomas, K.R. & Capecchi, M.R. (1990) Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature*, 847-850.

Thomson, J., Itskovitz-Eldor, J., Shapiro, S., Waknitz, M., Swiergiel, J., Marshall, V. & Jones, J. (1998) Embryonic Stem Cell Lines Derived from Human Blastocysts. *Science* **282**, 1145-1147.

Tiveron, M. & Cremer, H. (2008) CXCL12/CXCR4 signalling in neuronal cell migration. *Curr. Opin. Neurobiol.***18**, 237-244.

Tiveron, M., Rossel, M., Moepps, B., Zhang, Y., Seidenfaden, R., Favor, J., König, N. & Cremer, H. (2006a) Molecular interaction between projection neuron precursors and invading interneurons via stromal-derived factor 1 (CXCL12)/CXCR4 signaling in the cortical subventricular zone/intermediate zone. *J. Neurosci.* **26**, 13273-13278.

Tiveron, M.C., Rossel, M., Moepps, B., Zhang, Y.L., Seidenfaden, R., Favor, J., König, N. & Cremer, H. (2006b) Molecular interaction between projection neuron precursors and invading interneurons via stromal-derived factor 1 (CXCL12)/CXCR4 signaling in the cortical subventricular zone/intermediate zone. *J. Neurosci.* **26**, 13273-13278.

Tran, P.B., Banisadr, G., Ren, D., Chenn, A. & Miller, R.J. (2007) Chemokine receptor expression by neural progenitor cells in neurogenic regions of mouse brain. *J. Comp. Neurol.* **6**, 1007-1033.

Tsai, H.H., Frost, E., To, V., Robinson, S., Ffrench-Constant, C., Geertman, R., Ransohoff, R.M. & Miller, R.H. (2002) The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. *Cell* **110**, 373-383.

Turle-Lorenzo, N., Maurin, B., Puma, C., Chezaubernard, C., Morain, P., Baunez, C., Nieoullon, A. & Amalric, M. (2006) The dopamine agonist piribedil with L-DOPA improves attentional dysfunction: relevance for Parkinson's disease. *J. Pharmacol. Exp. Ther.* **319**, 914-923.

van den Munckhof, P., Gilbert, F., Chamberland, M., Levesque, D. & Drouin, J. (2006) Striatal neuroadaptation and rescue of locomotor deficit by L-dopa in aphakia mice, a model of Parkinson's disease. *J. Neurochem.* **96**, 160-170.

van Heteren, J.T., Rozenberg, F., Aronica, E., Troost, D., Lebon, P. & Kuijpers, T.W. (2008) Astrocytes produce interferon-alpha and CXCL10, but not IL-6 or CXCL8, in Aicardi-Goutières syndrome. *Glia* **5**, 568-578

Wagner, J., Akerud, P., Castro, D.S., Holm, P.C., Canals, J.M., Snyder, E.Y., Perlmann, T. & Arenas, E. (1999) Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes. *Nat. Biotech.* **17**, 653-659.

Wallén, A.A., Castro, D.S., Zetterström, R.H., Karlén, M., Olson, L., Ericson, J. & Perlmann, T. (2001) Orphan nuclear receptor Nurr1 is essential for Ret expression in midbrain dopamine neurons and in the brain stem. *Mol. Cell Neurosci.* **18**, 649-663.

Walz, A., Peveri, P., Aschauer, H. & Baggiolini, M. (1987) Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. *Biochem. Biophys. Res. Commun.* **149**, 755-761.

Wang, M.Z., Jin, P., Bumcrot, D.A., Marigo, V., McMahon, A.P., Wang, E.A., Woolf, T. & Pang, K. (1995) Induction of dopaminergic neuron phenotype in the midbrain by Sonic hedgehog protein. *Nat. Med.* **1**, 1184-1188.

White, F. (2005) Excitatory monocyte chemoattractant protein-1 signaling is up-regulated in sensory neurons after chronic compression of the dorsal root ganglion. *Proc. Natl. Acad. Sci. U S A.* **102**, 14092–14097.

Wilkie, T., Gilbert, D., Olsen, A., Chen, X., Amatruda, T., Korenberg, J., Trask, B., de Jong, P., Reed, R., Simon, M., Jenkins, N. & Copeland, N. (1992) Evolution of the mammalian G protein subunit multigene family. *Nat. Gen.* **1**, 85-91.

Winkler C, Kirik, D. & Björklund, A. (2005) Cell transplantation in Parkinson's disease: how can we make it work? . *Trends Neurosci.* **28**, 86-92.

Wise, R.A. (2004) Dopamine, learning and motivation. *Nat. Rev. Neurosci.* **5**, 483-494.

Wurst, W., Auerbach, A.B. & Joyner, A.L. (1994) Multiple developmental defects in Engrailed-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development* **120**, 2065-2075.

Wurst, W. & Bally-Cuif, L. (2001) Neural plate patterning: upstream and downstream of the isthmic organizer. *Nat. Rev. Neurosci.* **2**, 99-108.

Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T.M. (1991) Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell* **64**, 635-647.

Ye, W., Shimamura, K., Rubenstein, J.L., Hynes, M.A. & Rosenthal, A. (1998) FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-766.

Yoshida, T., Imai, T., Kakizaki, M., Nishimura, M. & Yoshie, O. (1995) Molecular cloning of a novel C or gamma type chemokine, SCM-1. *FEBS Lett.* **360**, 155-159.

Yoshimura, T., Matsushima, K., Oppenheim, J.J. & Leonard, E.J. (1987) Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL 1). *J. Immunol.* **139**, 788-793.

Zervas, M., Millet, S., Ahn, S. & Joyner, A. (2004) Cell Behaviors and Genetic Lineages of the Mesencephalon and Rhombomere 1. *Neuron* **43**, 345-357.

Zetterström, R.H., Williams, R., Perlmann, T. & Olson, L. (1996) Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. *Brain Res. Mol. Brain Res.* **41**, 111-112.

Zetterström, R.H., Solomin, L., Jansson, L., Hoffer, B.J., Olson, L. & Perlmann, T. (1997) Dopamine neuron agenesis in Nurr1-deficient mice. *Science* **276**, 248-250.

Zhao, P., Waxman, S.G. & Hains, B.C. (2007) Modulation of thalamic nociceptive processing after spinal cord injury through remote activation of thalamic microglia by cysteine cysteine chemokine ligand 21. *J. Neurosci.* **27**, 8893-8902.

Zlotnik, A. & Yoshie, O. (2000) Chemokines: a new classification system and their role in immunity. *Immunity* **12**, 121-127.