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Identification of dopamine neuron progenitors in the embryonic midbrain and stem cell cultures - Studies on the role of neuronal subtype and differentiation state for cell replacemen in a rodent model of Parkinson's disease

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Identification of dopamine neuron progenitors in the embryonic midbrain and stem cell cultures

Studies on the role of neuronal subtype and differentiation state for cell replacement in a rodent model of Parkinson's disease

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Title and subtitle Identification of dopamine neuron progenitors in the embryonic midbrain and stem cell cultures - Studies on the role of neuronal subtype and differentiation state for cell replacement in a rodent model of Parkinson's disease

Abstract Parkinson's Disease (PD) is a neurodegenerative disorder where the dopamine producing neurons in the ventral mesencephalon (VM) progressively die and result in symptoms such as resting tremors, muscle rigidity, slowness and difficulties in initiating movements. Currently there is no cure for PD and the available drug treatments only offer symptomatic relief and are often associated with severe side effects. Thus, there is an obvious need for alternative therapies.

A promising alternative approach is cell replacement therapy, which aims to replace the lost dopamine-producing neurons by transplanting cells with equivalent properties. Clinical transplantations using cells obtained from foetal VM tissue have provided the proof-of-principle that cell replacement therapy can provide a long-lasting recovery. However, the use of foetal tissue involves moral and severe practical issues making it impossible to standardise the required quantity and quality of cells. In the context of developing an alternative cell source that can offer a consistent and expandable supply of mesDA neuron progenitors, this thesis has investigated the features of foetal VM cells that allow them to survive, innervate and function upon transplantation.

The foetal VM tissue obtained for transplantation contains mesDA neuron progenitors at different differentiation states. By identifying and isolating the mesDA neuron progenitors from different developmental time points and differentiation states, the work in this thesis has shown that the optimal differentiation state for transplantation changes from a proliferative to postmitotic progenitor during development. It is crucial that the mesDA progenitors are harvested within this window of opportunity for the cells to survive, integrate and function upon transplantation. Following transplantation, the foetal VM was shown to give rise to the two main mesDA neuron subtypes of the VM, namely A9 and A10. Furthermore, the data in this thesis demonstrates that a functional graft is explicitly dependent on the generation of A9 neurons, which were shown to be necessary for innervation and connection with the host tissue.

The knowledge obtained from the work with foetal VM was applied to embryonic stem (ES) cells as the alternative cell source. ES cells posses the feature of being renewable and have the potential to generate mesDA neurons. However, differentiating ES cell cultures also contain other cell types as well as immature cells, which frequently give rise to tumour-like structures within the grafts. The approach of identifying and isolating the ES cell derived mesDA progenitors prior to transplantation resulted in grafts enriched with mesDA neurons without the concomitant tumour-like growths.

The goal of obtaining a safe and alternative cell source for PD cell replacement therapy has been brought closer with the work presented in this thesis. The use of stem cells is unquestionably promising and may one day be a reality in the clinical treatment of PD patients.

Key words: Parkinson's disease, ventral mesencephalon, dopaminergic neurons, cell replacement therapy, transplantation, FACS, cell sorting, GFP, Corin, Ngn2, Pitx3, Embryonic stem cells

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Cover: Immunohistochemical labelling of a coronal section of ventral mesencephalon in mouse at embryonic day 12.5. SOX2 (green) labels the dopamine neuron progenitors in the ventricular zone, NURR1 (red) labels the dopamine neuron progenitors in the intermediate zone and mantle zone. TH (blue) labels the newborn dopamine neurons.

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To my family

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SUMMARY

Parkinson's Disease (PD) is a neurodegenerative disorder where the dopamine producing neurons in the ventral mesencephalon (VM) progressively die and result in symptoms such as resting tremors, muscle rigidity, slowness and difficulties in initiating movements. Currently there is no cure for PD and the available drug treatments only offer symptomatic relief and are often associated with severe side effects. Thus, there is an obvious need for alternative therapies.

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The foetal VM tissue obtained for transplantation contains mesDA neuron progenitors at different differentiation states. By identifying and isolating the mesDA neuron progenitors from different developmental time points and differentiation states, the work in this thesis has shown that the optimal differentiation state for transplantation changes from a proliferative to postmitotic progenitor during development. It is crucial that the mesDA progenitors are harvested within this window of opportunity for the cells to survive, integrate and function upon transplantation. Following transplantation, the foetal VM was shown to give rise to the two main mesDA neuron subtypes of the VM, namely A9 and A10. Furthermore, the data in this thesis demonstrates that a functional graft is explicitly dependent on the generation of A9 neurons, which were shown to be necessary for innervation and connection with the host tissue.

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The goal of obtaining a safe and alternative cell source for PD cell replacement therapy has been brought closer with the work presented in this thesis. The use of stem cells is unquestionably promising and may one day be a reality in the clinical treatment of PD patients.

SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Stamcellsterapi för Parkinsons sjukdom

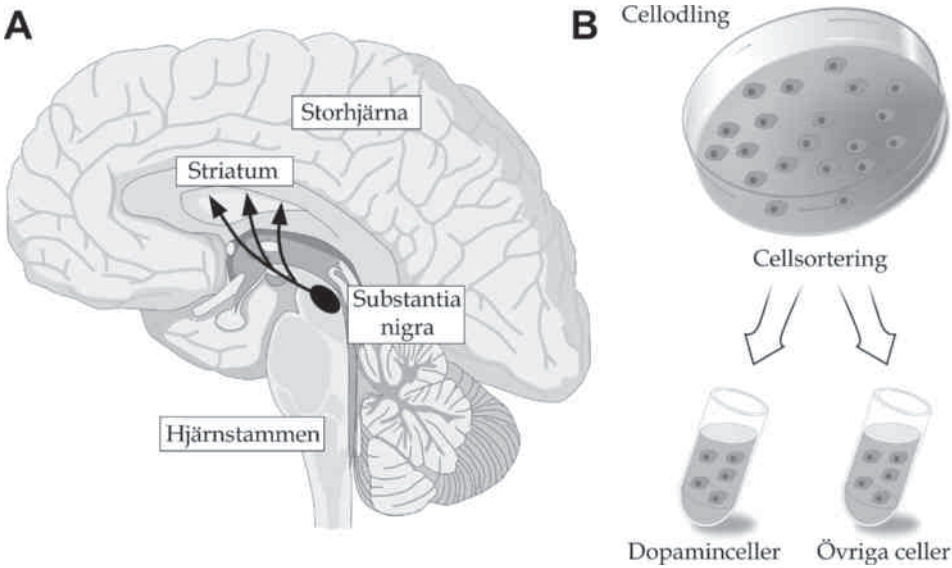
Hjärnan är uppbyggd av olika typer av celler som kommunicerar genom elektriska signaler eller genom att utsöndra signalsubstanser. När kommunikationen störs, till följd av sjukdom eller skada, kan det yttra sig genom att man drabbas av till exempel depression, får svårt att minnas eller att röra sig. Den här avhandlingen handlar om Parkinsons sjukdom, som innebär en brist på signalsubstansen dopamin.

Hjärnstammen innehåller flera grupper av dopaminceller som skickar utskott, s.k. axoner, till olika delar i storhjärnan (A). En grupp av hjärnstammens dopaminceller finns i substantia nigra och de skickar axoner till striatum där de utsöndrar sitt dopamin. Striatum är en del av storhjärnan och är viktig för att vi ska kunna röra oss normalt. Parkinsons sjukdom innebär att man gradvis förlorar dopaminceller i substantia nigra vilket därmed leder till dopaminbrist i striatum. Detta yttrar sig genom nedsatt rörelseförmåga där allt går långsamt och trögt, musklerna blir stela och man kan drabbas av skakningar i armar och ben. Symptomen är till en början milda men blir gradvis allvarligare allt eftersom fler dopaminceller dör. Den vanligaste behandlingen som erbjuds är medicinering med ämnen som kan höja nivåerna av dopamin i striatum, men den har ingen effekt på det oåterkalleliga sjukdomsförloppet. Efter ett antal år av medicinering drabbas patienterna av allvarliga biverkningar och det finns därför en stor efterfrågan på alternativa behandlingsmetoder.

Cellterapi innebär att man försöker ersätta de förlorade dopamincellerna genom att transplantera nya, friska celler till hjärnan. Redan på 1980-talet genomfördes de första transplantationerna av dopaminceller från fostervävnad till patienter med Parkinsons sjukdom och deras sjukdomstillstånd förbättrades avsevärt. Sedan dess har 300-400 patienter med Parkinsons sjukdom behandlats med cellterapi. Användandet av fostervävnad som källa för dopaminceller medför självklart etiska ställningstaganden och dessutom många praktiska hinder, såsom svårigheter att erhålla tillräckligt många celler vid en och samma tidpunkt och kvalitetstestning av cellerna innan transplantation. Det pågår därför forskning för att hitta alternativa källor av dopaminceller som undviker dessa hinder, men som fungerar lika effektivt för cellterapi som fostervävnad.

Nya rön har visat att stamceller utgör en mycket lovande källa till dopaminceller. En stamcell är en omogen cell som har två utmärkande egenskaper: de kan dela sig ett obegränsat antal gånger och har förmågan att mogna till flera olika celltyper. Dessa två egenskaper är fördelaktiga eftersom man kan generera ett stort antal celler när man behöver dem och de kan kvalitetstestas innan användning eftersom de odlas i laboratorium. Tyvärr har det visat sig vara svårt att styra stamcellernas mognad så att de enbart bildar dopaminceller, vilket resulterar i cellodlingar som består av flera oönskade celltyper. Vissa av dessa celler riskerar att fortsätta dela sig och bilda tumörer om de skulle transplanteras till hjärnan. En möjlig strategi för att undvika fortsatt celldelning och tumörbildning är att sortera ut dopamincellerna före transplantation med en metod som kallas cellsortering (B).

Avhandlingen syftar till att öka förståelsen för vad som är nödvändigt för att stamcellsterapi ska bli verklighet för behandling av Parkinsons sjukdom. Arbetet klargör i vilket mognadsstadium dopamincellerna bör befinna sig vid tidpunkten för cellsortering och transplantation och dessutom vilken specifik typ av dopamincell som åstadkommer en förbättring av sjukdomstillståndet efter transplantation.



Substantia nigra ligger i hjärnstammen och dess dopaminceller skickar axoner till till vardera hjärnhalvans striatum (A). Dopaminceller kan odlas fram från stamceller och sorteras ut från övriga celler (B).

GLOSSARY

Anterior	Anatomical term of nearer the head, opposite to posterior
Differentiation	A process when cells mature and becomes more specialised
Dorsal	Anatomical term relating to the upper side, or back of an animal, opposite to ventral
Ectopic	In an abnormal place of position
Heterogeneous	Diverse in properties or content
Homogeneous	No variation in properties or content
In vitro	Taking place outside an organism, for example a culture dish
In vivo	Taking place inside a living organism
Intermediate zone	Describing the location of the mesDA neuron progenitors in the developing VM that recently left the cell cycle and the ventricular zone
Lateral	Anatomical term for situated to either side of the middle
Mantle zone	Describing the location of the newborn mesDA neurons in the developing VM
Medial	Anatomical term for situated in the middle
Multipotent	Stem cell with the potential to give rise to multiple cell lineages (*latin* multus = much, many)
Neurogenesis	The birth of neurons
Neurogenic	A potential of generating neurons
Neuron	A specialised cell in the nervous system transmitting nerve impulses, also known as nerve cell
Pluripotent	Stem cell with the capacity to give rise to all cell lineages of the body (*latin* plures = several)
Posterior	Anatomical term of nearer the rear, opposite to anterior
Postmitotic	A cell that has exited the cell cycle and stopped dividing
Progenitor	A cell that is more committed than a stem cell, but can still be of a proliferating nature
Proliferating	A cell going through cell divisions and increase in numbers
Retrograde	Directed or moving backwards
Stem cell	A cell with the capacity to self-renew and to give rise to progeny with more restricted properties
Totipotent	A stem cell that has the capacity to generate a whole organism, including the extra embryonic cells (*latin* totus = whole)
Transgenic	Relating to an organism in which DNA from an unrelated organism has been artificially introduced
Unipotent	A stem cells with the capacity to give rise to only one cell lineage (*latin* unus = one)
Ventral	Anatomical term relating to the abdominal or underside of an organism, opposite to dorsal
Ventricular zone	Describing the location of the proliferating mesDA neuron progenitors in the developing VM, situated adjacent to the ventricular surface

ABBREVIATIONS

CNS	central nervous system
DA	dopaminergic
DNA	deoxyribonucleic acid
E	embryonic day
ES cells	embryonic stem cells
FACS	fluorescence activated cell sorting
GFP	Green fluorescent protein
HBSS	Hank's balanced salt solution
ICM	inner cell mass
IZ	intermediate zone
KPBS	potassium phosphate buffered saline
LIF	leukemia inhibitory factor
mesDA	mesencephalic dopaminergic
MFB	medial forebrain bundle
MZ	mantle zone
NS cells	neural stem cells (*in vitro* culturing)
NSC	neural stem cells
PBS	phosphate buffered saline
PD	Parkinson's Disease
PFA	paraformaldehyde
SNpc	substantia nigra pars compacta
VM	ventral mesencephalon
VTA	ventral tegmental area
VZ	ventricular zone

INTRODUCTION

Parkinson's Disease

Parkinson's Disease (PD) is a neurodegenerative disorder where the dopamine producing neurons located in the substantia nigra in the ventral mesencephalon (VM) are progressively lost. The first symptom often appears as a slight tremor in one extremity and as the disease advances the severity of the symptoms gradually increase, whereby tremors, rigidity, akinesia and bradykinesia spread throughout the body. Other symptoms such as depression, sleep disturbance, autonomic and cognitive dysfunction can also occur and occasionally manifest prior to the motor symptoms[1,2]. The aetiology of this relatively selective neurodegeneration is still largely unknown, however, age is a main risk factor. The average onset of PD is between the ages of 50-60 years with as much as 1% of people over 60 years of age being affected[3]. However, 10% of people diagnosed with PD are younger than 45 years, and onsets as young as 20 years have been reported[4,5], which indicates a more complex aetiology. In addition to age, environmental factors such as toxins from pesticides as well as familial gene abnormalities have been identified to cause susceptibility to develop PD (reviewed in Lees et al 2009[5]).

Neuropathology

The VM holds three nuclei of dopamine releasing neurons, namely, the retrorubral area (A8), the substantia nigra pars compacta (SNpc, A9) and the ventral tegmental area (VTA, A10)[6,7]. These nuclei innervate different areas of the brain, and accomplish functions distinct from one another. The A9 neurons of the SNpc mainly innervate the dorsolateral striatum, forming the nigrostriatal pathway where they supply dopamine that helps facilitate voluntary motor functions. The VTA neurons innervate the limbic areas of the ventro-medial striatum and cortical areas, forming the mesolimbic and mesocortical systems respectively, where dopamine is involved in regulating cognitive functions such as emotions. The A8 neurons projects along the same pathways as A9-A10 and mainly innervate limbic structures[8,9].

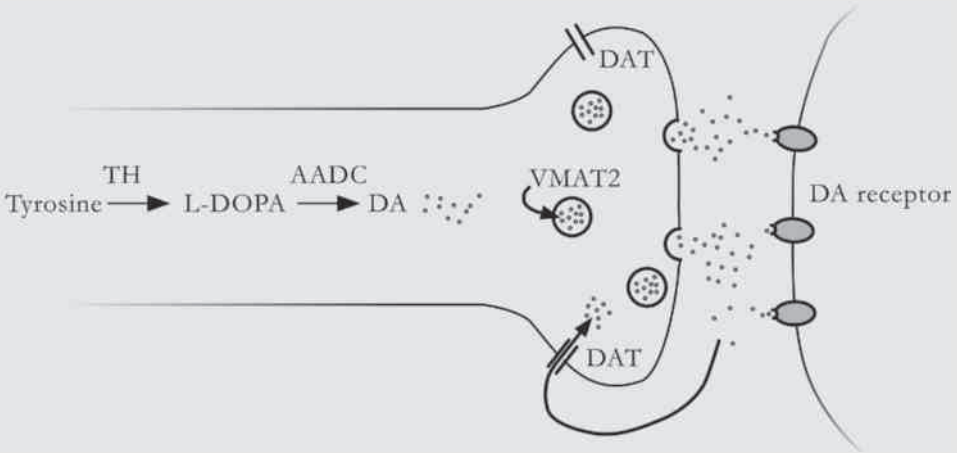
In PD, it is the mainly the A9 neurons that are lost. They degenerate in a retrograde manner, where the progressive cell loss is seen subsequent to their decreased striatal innervation. The cell loss and the inadequate levels of dopamine in the striatum are the underlying cause of the motor dysfunction and are characteristic hallmarks of PD.

The neurodegeneration seen in PD can be mimicked in animal models by using a neurotoxin specific for catecholamine neurons[10], resulting in motor deficits, which can be measured by several behavioural tests. The animal models used in this thesis are further described in the METHODS section.

Box I: Dopamine

Dopamine is synthesised from the amino acid tyrosine in two enzymatic steps. Firstly, tyrosine is converted to DOPA by Tyrosine hydroxylase (TH), DOPA is then converted to dopamine by Aromatic amino acid decarboxylase (AADC). Dopamine is then packaged into vesicles by the Vesicle monoamine transporter (VMAT) and upon an action potential dopamine is released into the synaptic cleft where it acts on the dopamine receptors of the receiving cell. The residual dopamine in the synaptic cleft is removed by re-uptake via the Dopamine transporter (DAT).

Dopamine was identified as a neurotransmitter in 1958[103] and the discovery was assigned to Arvid Carlsson, who was awarded the Nobel Prize in Physiology or Medicine in 2000. The importance of dopamine for controlling body movement was subsequently discovered and this led to the understanding that Parkinson's disease manifests as the lack of dopamine in the striatum. This knowledge enabled pharmacological treatment of the symptoms of Parkinson's disease with the dopamine precursor L-DOPA.



Schematic figure of a dopaminergic nerve terminal illustrating the synthesis, packaging, release and re-uptake of dopamine.

Treatment options

When motor symptoms manifest and a patient is diagnosed with PD, the neurodegeneration has often been ongoing for several years. In such patients the striatal dopamine levels have decreased by 60-80% and 50-60% of the DA neurons in the VM are already lost[11]. Currently there is no cure for PD and the available treatments in the clinic address the symptoms, such as L-DOPA treatment or deep brain stimulation, and have no effect on the progression of the disease. The principle areas of research to improve the treatment of

PD patients involve symptomatic, neuroprotective and restorative approaches. This thesis deals with cell replacement, which has the potential to restore the lost motor function by transplantation of functional mesDA neurons into the striatum of PD patients.

Cell replacement therapy in Parkinson's Disease

The first studies involving the grafting of embryonic VM tissue into the rodent brain were published in 1976[12,13] and only a few years later it was shown that grafted VM tissue pieces could improve the lost motor skills upon grafting in an animal model of PD[14,15]. This was the beginning of a new field of research aiming to restore the lost motor function seen in PD by cell replacement therapy.

The relatively selective neurodegenerative nature of PD makes it a suitable disease to treat with cell replacement therapy. The first clinical trial was performed in the 1980s using foetal VM tissue and gave proof-of-principle that cell replacement therapy for patients with PD can provide robust improvement of lost motor function. In some cases the striatal dopamine levels have been normalised and the L-DOPA treatment completely withdrawn[16]. Imaging of the dopamine uptake and autopsy of grafted patients have been analysed up to 16 years after transplantation, showing that the grafts can survive and innervate the host striatum over a long-term period[17-21]. To date, 300-400 patients with PD have received grafts with cells obtained from human embryonic VM tissue[22].

The grafted cells are placed in the striatum, which is the main innervation target of the mesDA neurons. The reason for not transplanting the cells in the VM, which is their natural anatomical location, is that the grafted cells show limited ability to extend axons along the mesostriatal pathway to reach their normal innervation targets. Furthermore, the VM of a PD patient is a pathologic area of the brain with ongoing cell death and high levels of oxidative stress, making it a fairly hostile environment for cells to be grafted. Due to the ectopic placing, the graft is distant from the afferent signals to the VM that normally regulate the dopamine secretion from the mesDA neurons in the SN. However, several studies have shown that the secretion of dopamine from the grafts is autoregulated, where the pre-synaptic dopamine receptor feedback makes sure that the dopamine level in the striatum is not overloaded[23,24].

In all clinical trials to date, grafted cells have been obtained from the VM of aborted embryos. The use of this cell source does not only involve ethical issues, but also logistical problems due to the need for several embryos per grafted hemisphere. This low availability of tissue, variations in age and quality of the obtained tissues, makes it impossible to standardise the procedure. These complications have promoted a search for alternative cell sources, where various stem cells have been proposed. In order to increase the knowledge of how to generate mesDA neurons from various stem cell sources, a lot of effort has been put into understanding the development of the VM and the mesDA neurons.

Development of the ventral mesencephalon

Due to the involvement of mesDA neurons in PD, and their potential role in cell replacement therapy, the development of the VM has been extensively studied. This has provided insight into the complex nature of mesDA neurogenesis and the multiple factors and genes involved. Such knowledge is of great importance in the pursuit of *in vitro* generated mesDA neurons from alternative cell sources.

Patterning of the ventral mesencephalon

The neuroectoderm and the subsequent neural tube are patterned both in an anterior-posterior and dorsal-ventral manner in order to form distinct structures of the central nervous system (CNS). The location and formation of the mesDA neurons was up until recently believed to be determined by the two organising centres known as midbrain-hindbrain organiser (MHO) and the floor plate (FP) (reviewed in Prakash et al 2004[25]). They are still identified as the key regulators of the DA domain, with the further knowledge that the FP cells are more than only organising cells and are in fact the mesDA progenitors[26,27].

The midbrain-hindbrain organiser. The border between the midbrain and hindbrain is visible as a tightened strip of the neural tube and is established by the non-overlapping expression of two homeodomain genes, *Otx2* and *Gbx2*. OTX2 expression is maintained by WNT1[28] and occupies the anterior parts of the neural tube, eventually forming the fore- and midbrain, and suppresses the formation of hindbrain by inhibiting *Nkx2.2*[29,30]. GBX2 is expressed in the posterior domains, which eventually forms the hindbrain and spinal cord, and suppresses *Otx2* and thereby the formation of midbrain[31]. These counteracting properties result in the formation of a distinct border between the midbrain and hindbrain where the MHO is established and secretes its key organising factor known as Fibroblast growth factor 8 (FGF8). Cells in and around the MHO express multiple transcription factors, such as PAX2/5, EN1/2 and LMX1B, which are also essential in the neural patterning and maintenance of the MHO[25].

The floor plate. Along the ventral midline of the developing neural tube, stretching from the posterior diencephalon to the spinal cord lies a narrow line of radial glia-like cells, called the floor plate (FP) cells. The FP is classically referred to as cells with organising capacities carried out by two features: the secretion of factors and functioning as a scaffold for the migrating neural progenitors. At all levels, the FP cells secrete Sonic hedgehog (SHH), which induces ventral patterning and neural specification. The FP cells also express proteins such as Corin, GLAST, FOXA2 and Netrin-1[26,27,32,33]. Despite many similarities, the cells forming the FP show morphological and molecular differences along the axes of the FP. Recent studies have revealed that FP cells at the level of mesencephalon (mesFP) possess neurogenic properties and are in fact the cells that eventually become the mesDA neurons[26,27,34]. This neurogenic property is unique to the mesFP and is not shared by other FP cells[27,35].

Taken together, the location and the formation of the VM and its DA neurons are determined by the combination of secreted factors from the FP and the MHO, SHH and

FGF8 respectively. They act together with WNT1 in a concentration dependent manner to determine the location where the mesDA neurons are formed.

Neurogenesis of the mesDA neurons

The birth of neurons is called neurogenesis, and refers to the process when a post-mitotic neuron is formed from its neural progenitor. During mesDA neurogenesis, the DA domain in the VM consists of three defined zones (see fig 1): the ventricular zone (VZ), which contains the proliferating mesDA neuron progenitors; the intermediate zone (IZ), which contains early post-mitotic mesDA neuron progenitors; the mantle zone (MZ), which holds the young mesDA neurons. In mouse, the mesDA neurogenesis is initiated at embryonic day (E) 10.5, when the vast majority of the DA domain consists of VZ and only a few progenitors have become post-mitotic. Over the next three days, the majority of mesDA neurons will be born[36] by exiting the cell cycle and migrating ventrally along radial glia through the IZ towards the MZ[37,38], as they simultaneously acquire many of the features of a mature mesDA neuron. As they reach the MZ, they migrate laterally along tangential axons[38], which give rise to the characteristic anatomy of the VTA and SNpc. The birth of the SNpc mesDA neurons has been shown to peak around E11.5, which is approximately a day earlier than the VTA mesDA neurons[6].

The mesDA neurogenesis is dependent on the correct expression of many transcriptions factors, both during the early patterning of the VM and during the specification of the mesDA neurons (summarised in table I). Many of the transcription factors important during mesDA specification are induced/repressed by, or act in collaboration with, secreted factors such as SHH[39], FGF8[40], and WNT1/3A/5A[41].

Establishment of the nigrostriatal pathway

Once the mesDA neurons have been generated and migrated to their final destination in the VM, they start to project axons that will be directed towards their targets by a combination of multiple axon guidance molecules and cellular interactions. The precise series of molecular and cellular events during the establishment of the mesDA projection circuits are largely unknown, but several families of guidance molecules of importance have been identified e.g. Ephrins, Netrins, Semaphorins, Slits and their individual receptors[49], as well as neurotrophic factors and morphogens (for an extensive review see Van den Heuvel et al 2008[50]).

As the individual mesDA axons exit the mesencephalon, they form large axon bundles, termed the medial forebrain bundles (MFB), from which the nigrostriatal pathways derivate. As the nigral axons reach the striatum, they innervate it in a lateral to medial manner. Initially, the mesDA neurons situated in the SNpc and the VTA do not show a strong preference for innervating the dorsal or ventral striatum, respectively, which is seen in adulthood. The preference is subsequently accomplished by axonal pruning during late embryonic and early postnatal development, when the VTA and SNpc neuron projections innervating the dorsal and ventral striatum, respectively, are selectively eliminated[51].

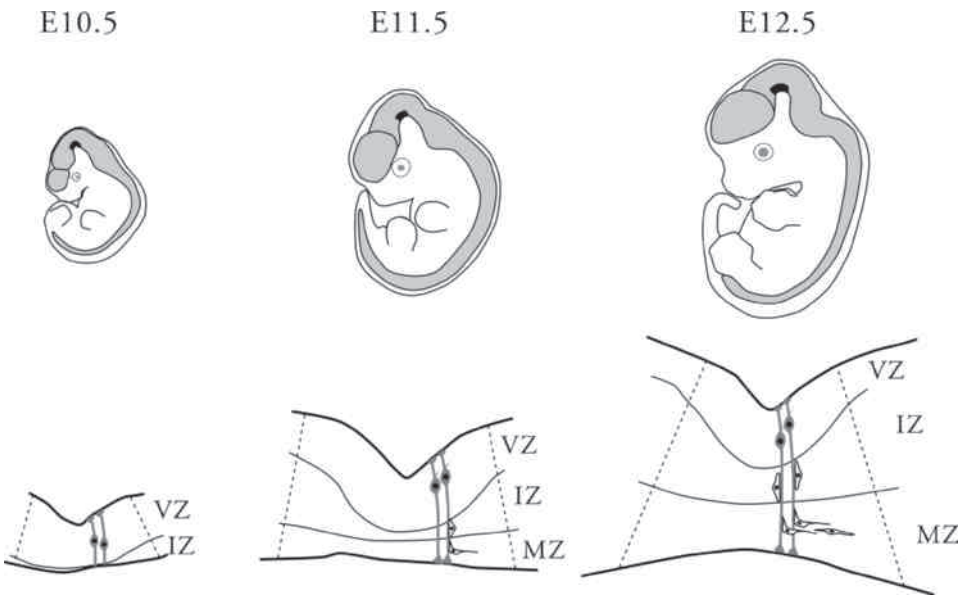


Figure 1. Neurogenesis of the mesDA neurons. The forebrain and neural tube ar indicated as light gray in mouse embryos 10.5 and 12.5 days old. The VM (black) is illustrated in a coronal cross-section. At E10.5 the DA domain (within dashed lines) mainly consists of proliferating mesDA neuron progenitors and only a few cells have exited the cell cycle and started their ventral migration. Two days later, at E12.5, the VM has increased in size and the DA domain contains proliferating and postmitotic mesDA neuron progenitors in VZ and IZ respectively, as well as newborn mesDA neurons situated in the MZ.

Table 1

Transcription factor	Expression during mesDA neurogenesis	Function in mesDA specification
EN1/2	IZ/MZ	Required for early VM patterning, generation of mesDA neurons and their maintenance in adulthood[108-110]
FOXA1/2	VZ/IZ/MZ	Induces the expression of *Shh*, *Lmx1a/b* and *Th*, inhibits serotonergic and GABAergic fates, maintains mesDA neurons in adulthood[111-113]
LMX1A	VZ/IZ/MZ	Induces *Msx1a* and *Ngn2*[44]
LMX1B	IZ/MZ	Maintains the expression of *Wnt1* and *Fgf8* during VM patterning, important for the co-expression of TH and PITX3 in mature mesDA neurons[114]
MSX1	VZ	Induces *Ngn2* and inhibits *Nkx6.1*[44]
NGN2	VZ	Specifies mesDA neuron fate by suppressing *Sox2* and inducing *Nurr1*[45-47]
NURR1	IZ/MZ	Induces genes important for DA synthesis and release: *Th*, *Aadc*, *Vmat*, *Dat*[115-118]
OTX2	VZ/IZ/MZ	Maintains mid-hindbrain border, induces *Ngn2* while suppressing *Nkx2.2*[29,30,119]
PITX3	MZ	Induces *Th*, important for the function of NURR1 and maintains adult mesDA neurons[90,120,121]

Note: This is a selection of transcription factors and their functions and should not be considered as a complete summary of all important factors during mesDA neurogenesis.

Stem cells

The term *stem cell* includes all cells that have the potential to self-renew and to give rise to progeny with more restricted properties[33,52,53]. There are many types of stem cells that display different levels of commitment, and the capacities of different stem cells are generally defined by their potency. Stem cell research has generated much excitement partly due to the potential use in cell replacement therapies for many different diseases including PD.

Potencies of stem cells

A cell that is capable of generating an entire organism, including extra-embryonic tissues, is said to be *totipotent*. The fertilized egg, called the zygote, holds this capacity as well as its initial daughter cells, called blastomeres. As the blastomeres continue to cleave and divide they eventually give rise to the blastocyst, which is a hollow structure enclosing the inner cell mass (ICM). The ICM has the potential to give rise to all cell lineages of the body, and are referred to as *pluripotent* stem cells. The ICM can be isolated for *in vitro* culturing and are then referred to as embryonic stem (ES) cells - this will be discussed further in the section EMBRYONIC STEM CELLS.

A *multipotent* stem cell can give rise to multiple lineages and a classical example is the hematopoietic stem cell, which can give rise to all cells in the blood system. Neural stem cells are also referred to as multipotent, since they can give rise to the major cell types in the CNS[54] i.e. neurons, astrocytes and oligodendrocytes. A more restricted stem cell that can only give rise to one lineage, such as the hepatocytes in the liver or the spermatogonial cells in the testis, is called *unipotent*.

Neural stem and progenitor cells

There are three main available sources of neural stem cells (NSC) namely the developing and adult neural tissue, as well as differentiating ES cell cultures. During embryonic development, NSCs are present in all areas of the CNS, but in adulthood they are confined to restricted areas. Neural stem cells obtained from sub-dissections of embryonic or adult neural tissue are traditionally maintained and expanded *in vitro* as neurospheres but with time the cultures undergo senescence and their multipotency decline as their ability to form neurons decreases. However, a novel method of long-term maintenance of NSC has been developed, where cells are maintained as attached monolayer cultures and are referred to as NS (neural stem) cells[55]. This approach obtains a more homogenous culture compared to the neurosphere system and have been reported to maintain their multipotency following long-term expansion[56,57].

The term *progenitor* is used to define cells that are more committed than a stem cell, but can still be of a proliferative nature[52]. In this thesis, cells with the potential of forming mature mesDA neurons will be referred to as mesDA neuron progenitors, which include both proliferating and early post-mitotic cells.

Embryonic stem cells

The studies of the developing VM have provided insight into the multiple genes and factors involved of mesDA neurogenesis. This knowledge is of great importance in the pursuit of recapitulating *in vivo* development by *in vitro* differentiation of renewable cell sources towards the fate of mesDA neurons.

Pluripotency of embryonic stem cells

During the development of the early embryo, the blastocyst contains the pluripotent ICM that gives rise to the three germ layers and thus all the tissues of the body. The ICM only maintains its pluripotency for a short time before it differentiates as the blastocyst implants into the uterus wall[58]. However, when the ICM is isolated for *in vitro* culturing[59,60], they can be maintained in a self-renewing pluripotent state in the presence of Leukaemia Inhibiting Factor (LIF) and are referred to as embryonic stem (ES) cells (fig 2). Based on the morphology and expression of multiple proteins known for pluripotency such as OCT4[61], NANOG[62,63] and SOX2[64], the ES cells show no sign of senescence or crisis over time, and can be expanded as a rather homogenous and undifferentiated culture as opposed to primary neural tissue.

Following *in vitro* expansion, the pluripotency of the ES cells can be demonstrated *in vivo* by re-introducing the ES cells into a blastocyst. Once integrated in the ICM, pluripotent ES cells will participate in the development of all three germ layers as well as the germline[65].

Differentiation of ES cells

ES cells rapidly loose their ability to self-renew and start to differentiate upon withdrawal of LIF. The early differentiation protocols involved the formation of cellular aggregates, called embryoid bodies (EB)[66,67] in which similarities to embryonic development were observed in the aspects of generated cell types[68]. The EBs are subsequently allowed to attach and continue maturation and will generate multiple mature cell types. In order to bias the cellular outcome towards a neural lineage, different soluble factors are added during differentiation. Retinoic acid has been used to increase the neural yield from EBs[69,70,71], and factors known to be necessary during the mesDA neurogenesis *in vivo*, such as SHH and FGF8[40], have been shown to increase the yield of DA neurons upon differentiation[72]. Recent protocols have been shown to be less heterogeneous that the EB protocols. However, the cultures still contains many different cell types in addition the mesDA neurons.

ES cells can be differentiated towards a neural fate as a monolayer culture, using DA neuron inducing factors SHH and FGF8[73]. This approach avoids the formation of EBs, instead the ES cells attach directly to the flat plastic surface of the culture vessel.

Another approach that favours the generation of neurons, particularly DA neurons, is based on co-culture of the ES cells with bone marrow derived stromal cells e.g. PA6 cells[74], and does not involve the formation of EBs or the use of added soluble factors. The PA6 cells provide a stromal cell derived inducing activity (SDIA), which is carried

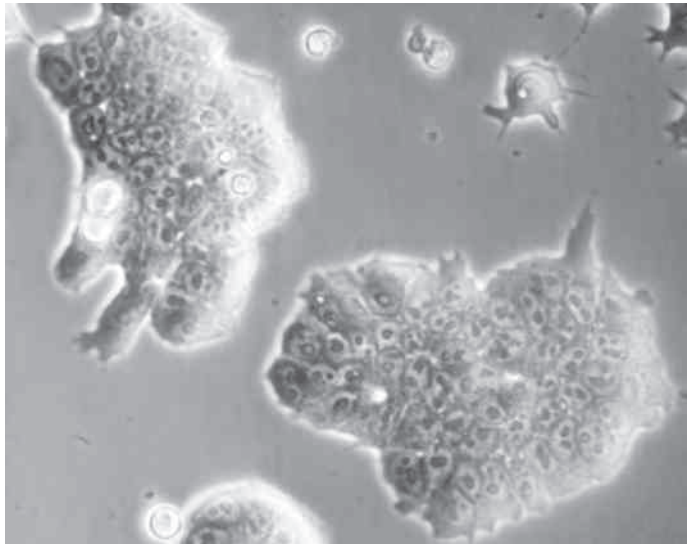


Figure 2. Expanding ES cells. Brightfield image of ES cells maintained in a pluripotent, self-renewing state by the presence of LIF.

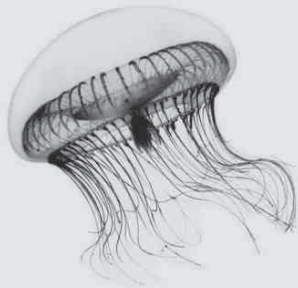
out by both cell surface activity and secreted factors. Although the SDIA is not yet fully characterised a recent study revealed the first known soluble factors directly involved in generating DA neurons[75]. It has also been suggested that the cell surface activity enhances the general neurogenesis while the secreted factors are primarily accountable for the DA induction[76].

Despite the successful use of cellular factors for neural induction when differentiating ES cells, it has proven difficult to obtain a uniform response. Upon differentiation conditions, ES cells leave their pluripotent state in an unsynchronised way and in addition to generating neural cells they also generate many other cell types, resulting in heterogeneous cell cultures. Consequently, differentiating ES cells give rise to un-synchronised cultures in both a spatial and temporal manner. Therefore, isolation of the generated cells of interest has been suggested as a suitable approach to avoid the wide variety of cellular outcome.

Identification of the mesDA neurons and their progenitors

ES cells are a promising alternative cell source for cell replacement therapy of PD due to their ability to self-renew and the potential to form neurons similar to mesDA neurons. However, the heterogeneous nature of differentiating ES cells allows immature, proliferating cells, as well as cells of the other germ layers, to remain within the cultures and these cells possess the risk of causing overgrowing grafts. Thus, the desired cells needs to be isolated from the bulk of various cells in the culture prior to grafting.

Box II: Green fluorescent protein



The Aequorea victoria *jellyfish measures between 8-20cm in diameter and GFP is expressed along its rim.*

The Green fluorescent protein (GFP) is a protein that exhibits bright green fluorescence (emission peak with a wavelength of 509nm) when exposed to blue light (major excitation peak at 395 nm). GFP was found to be expressed in the jellyfish Aaequorea victoria already in 1962[104], but it was not cloned until 1994[105]. Today recombinant GFP is found in laboratories all over the world and is one of the most frequently used reporter molecule for the expression of target proteins.

The purpose of the bioluminescence in the Aequorea victoria is unknown and any further studieshave been hampered by a collapse in the population in their natural habitat outside the Californian coastline. The story of GFP truly highlights the importance of basic research on even the most obscure organism.

The importance of GFP was recognized in 2008 when the Nobel Committee awarded Osamu Shimomura, Marty Chalfie and Roger Y. Tsien the Chemistry Nobel Prize "for the discovery and development of the green fluorescent protein." (for a more detailed review on the history and nature of GFP see Tsien 1998[106]).

Reporter ES cell lines and mice

Genetically modified mice, also called transgenic mice, offer an invaluable tool to investigate the role of different genes during development and are commonly used to study the gain or loss of function due to over-expression or deletion of a gene. When a transgenic cell line or mouse carry a gene that codes for a marker protein, they are generally referred to as reporter cell lines or mice.

Gene targeting offers the possibility to insert a gene coding for a reporter protein, such as Green fluorescent protein (GFP, described in Box II), under the endogenous transcriptional control of the gene of interest. The result being that GFP is only present in cells where the targeted gene is expressed. In order to ensure that the gene is incorporated at the desired locus and not randomly in the genome, the targeting vector is tailored to accomplish homologous recombination. The generated reporter ES cell line can subsequently be used to create a reporter mouse (see Box III).

Reporter cell lines and mice have become useful tools to identify and study the development of the mesDA neurons both *in vivo* and *in vitro*. Transgenic GFP expression provides the possibility of monitoring gene expression in living cells and also allows the analysis of cells by flow cytometry and physical sorting based on their GFP expression. Flow cytometry and cell sorting have played a crucial role in the work covered in this thesis are further described in the Methods section.

Box III: Transgenic mice

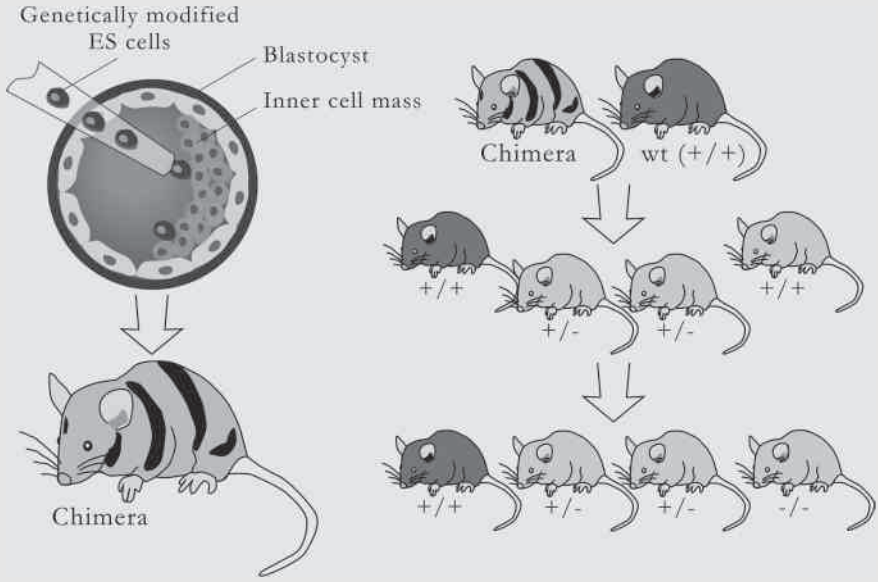
Homologous recombination allows one allele of a targeted gene to be altered without affecting any other locus in the genome. Any introduced linearized DNA will randomly integrate into the genome while homologous recombination is a rare event and the targeting vector, carrying the marker gene, have to be carefully designed. The targeting vector therefore includes the sequence to be inserted flanked by DNA sequences homologous to the integration site and contains elements allowing for subsequent positive and negative selection. The positive selection will include cells that have obtained the construct independently of integration site, and a negative selection will exclude cells in which the construct is randomly integrated leaving only the cells in which homologous recombination has occurred.

Once the ES cells with the homologous recombination are obtained, they will be clonally expanded and analysed with southern blotting, to insure the correct insertion. Once the cell line with the knocked-in construct is obtained, it can be used to generate a transgenic mouse.

The transgenic ES cells are injected into a blastocyst, which is then returned to the uterus. If the introduced ES cells integrate with the ICM they will be involved in generating the organism. The resulting organism, which is a blend of cells originating from the host ICM and the foreign ES cells, is called a chimera and can be identified at early adulthood by using genetic coat colour as markers i.e. introducing ES cells which are derived from a brown mouse into a blastocyst of white mouse will generate off-spring with a mixed coat colour.

If the germ cells in the chimera are derived from the transgenic ES cells, the genetic alteration will pass onto the offspring, following Mendelian ratios. The generated offspring will be carrying the alteration in one of its two alleles and are termed heterozygote (+/-). In order to generate a mouse homozygote (-/-) for the altered gene, two heterozygote mice can be bred with each other, giving rise to wild-type (1:4), heterozygote (1:2) and homozygote (1:4) offspring.

The discoveries that led to the possibility of gene targeting in mice were assigned to Mario R. Capecchi, Martin J. Evans and Oliver Smithies, which were awarded the Nobel Prize in medicine 2007 for their discoveries of “principles for introducing specific gene modifications in mice by the use of embryonic stem cells”.



AIMS OF THE THESIS

The overall aim of the work presented has been to increase our knowledge of the mesDA neuron progenitor in the context of cell replacement therapy for Parkinson's Disease patients, particularly the role of neuronal subtype in functional recovery. Emphasis has been placed on defining the window of opportunity for successful transplantation of embryonic tissue and using this knowledge towards the creation of an alternative cell source from stem cell cultures. The aims of the thesis have been achieved by a series of transplantations using cells of murine origin and a rodent model of Parkinson's Disease.

RESULTS & DISCUSSION

Foetal tissue has repeatedly been demonstrated to hold the capacity to restore the lost motor functions upon transplantation into patients suffering from Parkinson's Disease (PD). Due to the ethical and severe logistically problems with the use of this type of tissue, alternative sources of cells for grafting have been proposed. In order to make the approach of alterative cell sources successful, there are several questions that need to be answered. What is the specific cell type needed to evoke the functional recovery? At what state of differentiation should cells be transplanted in order to survive and function? Do renewable cell sources have the potential to generate the desired cells? The results in this thesis attempts to address these questions and illustrate the importance of several factors required to make renewable cell sources successful.

mesDA progenitors from the VM: Defining the window of opportunity for transplantation (Paper I)

The foetal tissue for grafting in clinical trials have been obtained from different stages of mesencephalic dopaminergic (mesDA) neurogenesis, when the mesDA neuron progenitors in the ventral mesencephalong (VM) range from proliferating cells in the ventricular zone (VZ), the early post-mitotic cells in the intermediate zone (IZ), and the young mesDA neurons starting to acquire a fully mature phenotype in the mantle zone (MZ). Thus, the generated cell suspensions from the sub-dissections of VM will contain a mix of VZ, IZ and MZ cells. When aiming to generate mesDA neuron progenitors from alternative cell sources intended for the use in cell replacement therapy, it is important to know at what state of differentiation they should be harvested for grafting, in order for them to survive, innervate and function in the host brain. This window of opportunity for grafting was investigated by conducting a series of transplantation rounds with isolated mesDA neuron progenitors from mouse VM at various states of differentiation.

Ventricular Zone potential at E10.5 is diminished at E12.5

At E10.5 the developing VM consists mainly of proliferating mesDA neuron progenitors in the VZ, with only a small number of post-mitotic cells, forming the first IZ cells. As the mesDA neurogenesis continues, the VM increases in size and at E12.5 it consist of the proliferative VZ cells, the early post-mitotic progenitors making up the IZ and the young mesDA neurons in the MZ.

In order to isolate and investigate the capacity of the VZ cells to survive and function upon grafting, the expression of Corin was used; a cell surface marker expressed on the FP cells along the neural tube[27]. The VZ progenitors in the VM were detected by Corin at both E10.5 and E12.5 in a medial-lateral gradient, where the strongest labelling is seen in the most medial cells and decreases laterally to the midline. Due to this gradient, cell sorting resulted in the two fractions: Corin^{high}, which contained the strongest labelled cells,

and the Corin$^{neg\text{-}low}$, which mainly contains the Corin negative cells, but also cells in which Corin is weakly detected.

Upon grafting of the E10.5 Corin^{high} and Corin$^{neg\text{-}low}$ fractions into the striatum of a rat model of PD, approximately the same number of mesDA neurons was generated. This can be explained by the expression-gradient of Corin that results in the VZ mesDA progenitors in both fractions, demonstrating that both the medial and the lateral parts of the dopaminergic domain have the capacity to survive grafting and mature into mesDA neurons. However, when grafting the Corin^{high} and Corin$^{neg\text{-}low}$ fractions from E12.5 VM tissue, the Corin^{high} cells poorly survived the grafting and generated only a few mesDA neurons, while the Corin$^{neg\text{-}low}$ cells generated enough mesDA neurons to evoke a functional response. Thus indicating that the mesDA progenitors in the VZ at E12.5 do no longer posses the ability to generate mesDA neurons upon grafting, and that this ability is instead found in the Corin$^{neg\text{-}low}$ cells, which contain the mesDA neuron progenitors from the IZ and MZ.

These results show that despite similar protein expression in the VZ cells at E10.5 and E12.5 i.e. SOX2, LMX1A, MSX1, Corin, NGN2[27,44,46,64], the capacity to survive and generate functional mesDA neurons upon grafting is different. This declined of capacity of the VZ progenitors at E12.5 to generate mesDA neurons could be due to the late stage of mesDA neurogenesis, when the majority of the mesDA progenitors have left the VZ. At this time point a combination of environmental and intrinsic factors could have induced the VZ cells to shift from neurogenesis to gliogenesis. However, the grafted Corin^{high} cells from E12.5 did not give rise to grafts rich in glia, which indicates that the cells in medial most part of the VZ have most likely become non-dividing scaffold cells.

At E12.5 the potential is greatest within the Intermediate Zone

With the knowledge that the mesDA progenitors have left the VZ at E12.5, the capacity of the IZ cells was investigated. In order to isolate the IZ cells, embryos from the *Ngn2*-GFP mouse[77] were used in which previous studies have shown that the GFP^{pos} cells from the E12.5 VM tissue represents cells from the DA domain[45,46]. Upon grafting, the mesDA neurons were all obtained from the GFP^{pos} cell fraction without any form of enrichment compared to unsorted VM cells (cells that were passed through the FACS but not sorted)[78]. NGN2 is expressed in the VZ cells at low levels and as the cells leave the cell cycle and migrate ventrally to the IZ, it has a transient peak of expression. The GFP protein stays in the cells longer than NGN2, resulting in high levels of GFP in IZ cells named GFP^{high}, while the GFP appears at lower levels or not at all in the VZ and MZ. By a stringent sorting of GFP^{high} cells and subsequent grafting we detected a 2-fold increase in the number of generated mesDA neurons compared to unsorted VM cells and reversed the impaired behaviour investigated by amphetamine rotations. These data demonstrate that when isolating the IZ at E12.5, and thereby excluding the VZ and MZ cells, the grafts are significantly enriched in mesDA neurons compared to unsorted cells from VM. Together with the data from the VZ transplantation, the transplantable mesDA progenitors are located in the VZ at E10.5 and the IZ at E12.5. This indirectly indicates that the MZ cells at E12.5 do not

largely contribute to the formation of mesDA neurons upon grafting. However, in order to investigate the potential of the MZ cells they were also isolated and grafted.

Mantle Zone potential is inadequate at E12.5

The capacity of the MZ cells to survive and function was addressed by using the heterozygote *Pitx3*-GFP mouse[79], which will be referred to as *Pitx3*$^{WT/GFP}$. In the mouse brain, the expression of PITX3 is restricted to the DA neurons in the VM, where it correlates with the expression of TH[48], and the *Pitx3*$^{WT/GFP}$ animals show no variations from a wild type mouse in number of mesDA neurons[80]. Thus, the isolated the GFP^{pos} cells from the E12.5 VM of *Pitx*3$^{WT/GFP}$ embryos, contains the newborn mesDA neurons situated in the MZ, while the GFP^{neg} cells contain cells from the VZ and the IZ as well as cells lateral to the DA domain included in the sub-dissection.

Upon grafting, the GFP^{pos} cells survived poorly and consequently had no impact on the motor deficit. However, the few cells present in the grafts displayed mesDA neuron characteristics expressing both GFP (*Pitx3*) and TH. However, the GFP^{neg} cells gave rise to the same number of TH expressing neurons as an unsorted graft i.e. the removal of the mature MZ cells does not affect the ability of the grafted cells to generate mesDA neurons, allowing the conclusion that the MZ cells do not hold the capacity to survive and function upon grafting. Taken together, the data shows that the transplantable cells at E12.5 are the early post-mitotic mesDA progenitors from the IZ.

The transplantable mesDA neuron progenitor travels through the VM

These results show that the differentiation state of the mesDA neuron progenitors at the time for harvesting is important for the outcome of cell transplantation. The window of opportunity for successful generation of mesDA neurons upon grafting is open from early mesDA neurogenesis in the proliferative VZ at E10.5, to the post-mitotic mesDA neurogenesis in the IZ at E12.5 (fig 3). It is most likely the same mesDA progenitors that have the capacity in the VZ that have made their way to the IZ two days later. However, the progenitors that have reached the MZ at this stage are too mature to survive the transplantation procedure.

However, it cannot be ruled out that the isolation process affects the mesDA progenitors to different degrees depending on their state of differentiation. It is likely that more mature MZ cells, which already at E12.5 have extended processes, suffer greater physical trauma due to the cell sorting procedure compared to an immature mesDA progenitor. However, the enrichment of the mesDA progenitors, and thereby the exclusion of neighbouring cells from the VM sub-dissection, does not seem to affect the capacity of the generated mesDA neurons to innervate the host striatum, as measured by the innervation volume per mesDA neuron, based on the expression of TH.

Can the window of opportunity be translated into renewable cell sources?

The results are encouraging for the use of renewable cell sources for cell replacement therapy in PD, since the opportunity for successful grafting includes both the proliferating

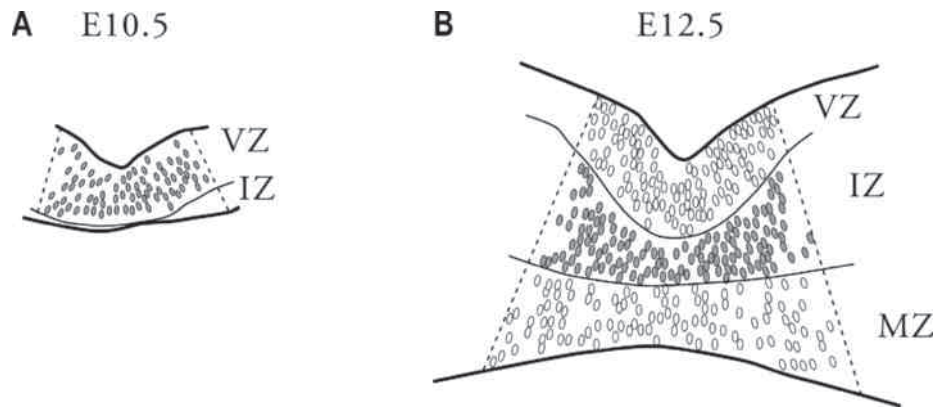


Figure 3. The journey of the transplantable mesDA neuron progenitors through the VM. The transplantable mesDA neuron progenitors (filled ovals), i.e. the mesDA neuron progenitors in the developing VM that survive, innervate and function upon grafting. At E10.5 the transplantable mesDA neuron progenitors are proliferative and situated in the VZ. At E12.5 however, the transplantable mesDA neuron progenitors are postmitotic and situated in the IZ and are likely to represent the progenitors located in the VZ at E10.5.

and the post-mitotic progenitors. One should bear in mind that the physical trauma of dissociating cells from a culture dish is not as harsh as the dissociation of the VM tissue. Indeed, a recent study showed that when sorting *Pitx3* expressing cells from differentiating ES cells (using the *Pitx3*-GFP ES cell line)[79], the cells could survive transplantation and reverse rotational PD behaviour[81], which indicates that the window of opportunity might be even wider when using renewable cell sources for cell replacement therapy in PD.

The road to recovery: mesDA neuronal subtype is critical (Paper II)

The VM contains two subtypes of DA neurons: the substantia nigra pars compacta (SNpc) contains the A9 neurons and the ventral tegmental area (VTA) contains the A10 neurons. The A9 neurons project into the dorsal-lateral parts of the striatum where they are involved in voluntary motor function. The A10 neurons predominately innervate the medial-ventral parts of the striatum where they are implicated in cognitive regulation[82]. In PD, it is mainly the A9 neurons that are degenerated, but the transplantation of embryonic VM tissue generates grafts containing a mix of the two main DA neuronal subtypes. Since it had not been directly addressed previously, and it is necessary to know what cell to strive for from alternative cell sources, we investigated the importance of these two subtypes for functional recovery upon grafting by using the *Pitx3*-GFP mouse[79].

VM tissue sourced from a transgenic mouse lacking A9 neurons

In order to study the impact of the two subtypes upon grafting, we made use of the *Pitx3*-GFP mouse. Since the expression of *Pitx3* in the CNS is confined to the mesDA

neurons, they are the only cells expressing GFP in the brain of the *Pitx3*-GFP mouse. GFP expression is seen throughout the cytoplasm of mesDA neurons, thus allowing GFP to be used as a reporter of the grafted mesDA neurons as well as their fibre outgrowths.

The heterozygote animals (*Pitx3*$^{WT/GFP}$) were used as a control since the number of mesDA neurons is unaffected and the VM contains a normal distribution of A9 and A10 neurons[80]. Animals that are homozygous for GFP (*Pitx3*$^{GFP/GFP}$) lack expression of *Pitx3* and consequently only generate half the amount of mesDA neurons. The lower number of mesDA neurons in the *Pitx3*$^{GFP/GFP}$ is due to the selective loss of A9 neurons at the time of mesDA neurogenesis, while the A10 neurons are left relatively intact[80]. This results in a loss of dopamine innervating fibres in the dorsal-lateral parts of striatum, where the A9 neurons normally project while the innervation targets of the A10 neurons are left only moderately affected.

Thus, by transplanting cells from embryonic VM tissue, obtained from either *Pitx3*$^{GFP/GFP}$ or *Pitx3*$^{WT/GFP}$, allowed us to compare the function of grafts containing only A10 neurons or both A9 and A10 neurons, respectively. In order to ensure that any differences in functional read-out would not be due to number of the mesDA neurons in the grafts, twice the amount of cells from the *Pitx3*$^{GFP/GFP}$ VM tissue was grafted compared to the *Pitx3*$^{WT/GFP}$. This resulted in grafts with similar numbers of GFP^{pos} cells (mesDA neurons) from both genotypes.

Neuronal subtype composition in grafts

In the VM, the A9 and A10 neuronal subtypes have a similar protein profile, expressing markers such as PITX3, NURR1, TH and FOXA2. A few weeks after birth however, the subtypes differ in the expression of the potassium channel subunit GIRK2[83,84] and the calcium binding protein Calbindin[85,86]. The A9 neurons express GIRK2 while the A10 neurons express Calbindin and thus by counting the TH$^+$/GIRK2$^+$ and TH$^+$/Calbindin$^+$ cells we could identify the A9 and A10 neurons in the grafts, respectively.

The composition of the neuronal subtypes in the grafts reflected their individual phenotype, where the *Pitx3*$^{WT/GFP}$ cells served as control and gave rise to grafts with a normal composition of A9/A10 neurons, while the *Pitx3*$^{GFP/GFP}$ cells generated grafts mainly containing A10 neurons. These data confirm the approach of generating grafts rich in A10 or both A9 and A10 neurons, by using the *Pitx3*$^{GFP/GFP}$ and *Pitx3*$^{WT/GFP}$ tissue, respectively.
This shows that the disruption of the physical location of the mesDA progenitors in the embryonic VM upon dissociation and grafting, does not affect their ability to mature into their individual neuronal subtype.

A9 neurons are necessary for functional recovery

The behavioural recovery was investigated by the amphetamine-induced rotation test as well as the cylinder test. Upon grafting, behavioural recovery was only obtained in animals receiving *Pitx3*$^{WT/GFP}$ cells. The amphetamine rotations were restored by eight weeks and the forelimb skills were normalised by 12 weeks, while no improvement was observed in animals grafted with cells from the *Pitx3*$^{GFP/GFP}$ VM tissue. Since the

Pitx3$^{GFP/GFP}$ grafts contain a similar number of mesDA neurons, the failure of motor recovery is not due to a lower number of mesDA neurons but instead indicate that in the absence of A9 neurons, the A10 neurons alone cannot restore motor function. Therefore, other factors known to be crucial for functional outcome following cell replacement therapy were investigated, namely the grafts ability to innervate and connect into the host tissue.

A10 neurons fail to innervate and connect with the host striatum

The ability of the grafted cells to innervate and connect with the host tissue is known to be crucial factors in order for a graft to generate a functional response. Therefore, we evaluated the density of innervating TH$^+$ fibres in the host striatum from the *Pitx3*$^{WT/GFP}$ and the *Pitx3*$^{GFP/GFP}$ grafts compared to the intact striatum. Only the *Pitx3*$^{WT/GFP}$ cells substantially innervated the host striatum, particularly the dorso-lateral part, which normally receives its dopaminergic innervation exclusively from the A9 neurons. The innervation capacity corresponded to the behavioural response, where recovery was only seen in the animals grafted with the *Pitx3*$^{WT/GFP}$ cells, which innervated the host striatum.

The connectivity of the grafts with the striatum was evaluated by the expression of c-Fos, the expression of which reflects dopamine transmission following intra-striatal grafts[87]. Also this measurement corresponded to the behavioural recovery, where the area lateral to the graft had an increased number of c-Fos expressing cells only in animals grafted with the *Pitx3*$^{WT/GFP}$ cells.

These data show that the presence of the A9 mesDA neurons is essential in order for the grafts to be able to innervate and connect with the host tissue. This is in line with previous studies correlating the number of GIRK2 expressing cells to the functional recovery following transplantation[88]. However, the data does not exclude a specific role of the A10 neurons for recovery following cell therapy, but on their own they cannot reverse the lost motor function.

The fact that the A10 neurons generated from the *Pitx3*$^{GFP/GFP}$ VM lack the expression of PITX3 could potentially decrease their ability to innervate the host upon grafting. However, it is unlikely to be the case since in the adult *Pitx3*$^{GFP/GFP}$ animals the innervation pattern of A10 is seemingly unaffected and the processes have reached their targets within the medial-lateral parts of the striatum. In addition, many of the target genes of PITX3 encode proteins that are involved in the synthesis and secretion of dopamine[80,89,90], but it has previously been shown that the A10 neurons present in the VM of *Pitx3* deficient mice have the ability to secrete dopamine and to fire action potentials[91,92], indicating that the A10 neurons in the *Pitx3*$^{GFP/GFP}$ animals have a functional dopamine secretion machinery. Thus, it is unlikely that the absence of behavioural response from the *Pitx3*$^{GFP/GFP}$ grafts are due to the lack of dopamine secretion and are instead a result of the poor connection into the striatal areas in which motor function is facilitated.

This knowledge is vital for the generation of mesDA neurons from alternative cell sources, since the challenge is not only to generate mesDA progenitors but they should specifically mature into a cell corresponding to the A9 neuronal subtype in order to function upon cell replacement therapy.

The expression of many axon guidance molecules necessary for the mesDA neurons to project and connect to their targets during development, is sustained into adulthood where they are believed to play a role in maintaining and stabilising the mesDA connections with the striatal neurons[107]. The presence of different axon guidance cues is likely to be essential for the grafted cells to be able to innervate the host tissue. However, since the A9 and A10 neurons normally innervate distinct brain regions they may respond differently to the same axonal guidance signal. This could explain why the environment might appear chemo-attractive to the grafted A9 neurons, while the A10 neurons are unresponsive or experience the signals as chemo-repulsive.

mesDA neuron subtypes can be separated at an embryonic state (Paper I)

During mesDA neurogenesis it is difficult to distinguish the A9 and A10 neuronal subtypes since the VTA and SNpc nuclei do not become anatomically prominent until around E17.5 in mouse and the expression of GIRK2[84] and Calbindin[85] can only be used to identify the two subtypes postnatally. However, in PAPER I where the Corin^{high} and Corin$^{neg\text{-}low}$ fractions from E10.5 VM tissue was isolated and grafted, the Corin$^{neg\text{-}low}$ cells only generated A9 neurons, indicating that the A9 progenitors are located towards the lateral part of the dopamine domain at this stage, while the grafts from the Corin^{high} cells generated both A9 and A10 neurons indicating that the subtypes are intermingled in the medial parts of the dopamine domain. This separation between the A9 and A10 neurons correlates with studies showing that the neurogenesis of the A9 neurons peak prior to that of A10, and that progenitors seem to leave the cell cycle in a lateral to medial manner[36], thereby positioning the A9 neurons mainly towards the lateral parts of the dopamine domain already during mesDA neurogenesis. Thus, even though the expression of known transcription factors in the mesDA neuron progenitors at E10.5 appears homogenous throughout the dopamine domain they are patterned already at this stage into generating distinct subtypes.

Combined with the results from PAPER II, showing that the A9 neurons are necessary to evoke a functional response upon cell replacement therapy, it is encouraging that enrichment of A9 neuronal progenitors can be achieved. The possibility of using Corin as a marker to separate A9 and A10 neurons are unlikely to be maintained for renewable cell sources, since the generation of the subtypes seems to be highly dependant on the developmental stage rather then a difference in protein expression.

Generating mesDA neurons from embryonic stem cells (Paper III)

Embryonic stem (ES) cells are suggested as a possible alternative source to the foetal tissue for replacement therapy of PD. They have the ability to self-renew and to generate DA neurons both *in vitro* and when grafted into animal models of PD. However, the pluripotency of ES cells have proven difficult to control with the differentiation protocols available today. Also, the maturation of ES cells upon differentiation occurs in an unsynchronised manner, leaving undifferentiated ES cells in the culture during differentiation.

When grafting such heterogeneous cultures, they do give rise to DA neurons, but they frequently maintain their proliferative capacity causing tumour-like structures as well as the formation of other cell types. In an attempt to remove the unwanted cell types prior to grafting, the mesDA progenitors were isolated by FACS.

The importance of cell surface markers

Several studies have performed cell sorting in attempts to isolate the required DA neuron progenitors by using reporter cell lines[81,93,94]. In order to bring the approach of cell sorting towards the clinic, the method of labelling DA neuron progenitors cannot involve the presence of the GFP proteins so far used in reporter cell lines. A more suitable approach would be to identify the desired cells by taking advantage of native cell surface markers. However, cell surface markers expressed only on the mesDA neuron progenitors are yet to be defined and the few known cell surface markers on mesDA progenitors are also expressed on many other cell types. The FP marker Corin as well as th neuron e radial glia marker GLAST is expressed in the mesFP cells, however, both are also expressed in other parts of the CNS and other parts of the body. With the knowledge from PAPER I, that Corin can be used to isolate the mesDA neuron progenitors from developing VM, we wanted to investigate its capacity to do the same from differentiating ES cells.

Corin is expressed in differentiating ES cells

During embryonic development, Corin is expressed throughout the FP and overlaps with other proteins expressed in the FP such as FOXA2[95,96]. In the mesFP it also overlaps with LMX1A, which is expressed in mesDA neuron progenitors as well as the mature mesDA neurons[44]. This expression pattern was detected also in ES cells upon eight days of SDIA differentiation, where Corin was co-expressed with both FOXA2 and LMX1A – indicating that mesFP cells were generated. However, these cultures also demonstrated heterogeneity by containing cells expressing FOXA2 and LMX1A not co-expressing Corin, which likely represents cells of a more mature state when Corin is absent, or cells of another choice of fate since LMX1A and FOX2 is not restricted to the FP cells and are important for the development of many other tissues. This heterogeneity in the differentiating ES cell cultures underline the importance of a thorough investigation of the mesDA character of the ES cell derived Corin expressing cells.

Enrichment of mesDA neuron progenitors

Proteins that are expressed and commonly used for identification of mesDA neurons are not restricted to the VM but are also expressed in other types of neurons or even in other parts of the body. This is not problematic when generating mesDA neurons from subdissections of the VM that is known to contain the mesDA neuron progenitors. However, when aiming to generate mesDA neurons from ES cells that have the capacity to generate many other cell fates than DA neurons, it becomes vital to characterise the expression mesDA markers. In order to resolve the mesDA character of the ES cell derived Corin^{high} cells, they where investigated in parallel with the Corin^{high} cells from E12.5 VM tissue.

The gene expression of the ES cell derived Corin^{high} cells were similar to the expression pattern of the E12.5 VM derived Corin^{high} cells, indicating that Corin enriches for cells with a mesDA character. The Corin^{high} fraction is enriched in cells expressing *Corin*, *Shh*, *Nestin, Lmx1a/b, En1, Msx1* and *Wnt5a*, while the expression of the hindbrain marker *Gbx2* is undetectable, indicating that they share the same molecular characteristics to the mesDA progenitors in the developing VM. In addition to enriching for mesDA neuron progenitors, the isolation of Corin expressing cells also excluded the remaining pluripotent ES cells still present in the cultures, since neither OCT4 nor NANOG was detected in the Corin^{high} fraction while they where strongly expressed in the Corin$^{neg\text{-}low}$ and the unsorted ES cells.

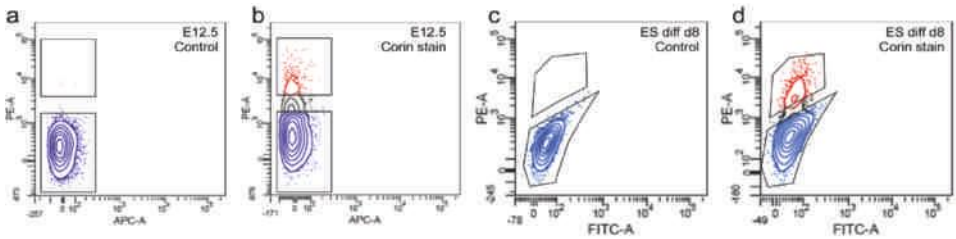


Figure 4. Sorting cells from E12.5 VM tissue and differentiating ES cells. Corin expressing cells are detected in a gradient manner in both VM tissue (b) and differentiating ES cells (d) and are sorted into Corin^{high} and Corin$^{neg\text{-}low}$ fractions. In order to define the fractions, dorsal mesencephalon was stained in parallel to VM as a negative control (a) and a portion of the differentiating ES cells were only incubated with the secondary antibody (c).

mesDA neurons are generated upon grafting

Upon grafting of the ES cell derived Corin^{high} and Corin$^{neg\text{-}low}$ fractions, they gave rise to grafts of considerable different size even though the same number of cells were grafted. The Corin^{high} fraction gave rise to small grafts, while the Corin$^{neg\text{-}low}$ fraction generated large grafts with a proliferative character reflecting the difference in heterogeneity in the two fractions. Both fractions gave rise to mesDA neurons, but a further indication of the enrichment of mesDA neuron progenitors was that the density of mesDA neurons were more than four times higher in the Corin^{high} grafts compared to the Corin$^{neg\text{-}low}$ grafts. Also, the Corin^{high} cells demonstrated an ability to generate both A9 and A10 neurons, indicating that using Corin to isolate mesDA progenitors from differentiating ES cells, we can indeed generate the specific cell types needed for functional cell replacement therapy.

Corin expression is conserved in human neural development

During mesDA neurogenesis in the developing human VM, Corin was found to have a similar expression pattern to mouse. Corin is expressed on cells with radial glia morphology along the ventral medial midline of the neural tube, from the VM and along the spinal cord. The expression pattern overlaps with the FOXA2 and LMX1A expressing

cells in the same way observed in mouse. Upon differentiation of human ES cells, Corin is expressed in cells within neural structures called neural rosettes and co-express the neural progenitor protein Nestin.

The knowledge that Corin is conserved in the human mesDA progenitors opens up the possibility to use it as a cell surface marker for the isolation of mesDA progenitors from human tissue and differentiating ES cells, which is important when continuing the quest to generate suitable cells from alternative cell sources for cell therapy for Parkinson's Disease.

CONCLUDING REMARKS & FUTURE PERSPECTIVES

Over the last decades we have come a long way in our understanding of what is needed to achieve a functional cell replacement therapy for Parkinson's disease (PD). Human foetal tissue is so far the only clinically tested and proven cell source to restore motor function in PD patients, but it cannot be made widely accessible and commonly practiced in the clinic due to the limited availability. Considering that around 1% of all people over the age of 65 suffer from PD and the average life expectancy is increasing, there is a clear need of an alternative source of renewable human mesDA neurons.

Embryonic stem (ES) cells can be expanded to unlimited numbers and supplied on demand, which makes them appealing for use in cell replacement therapy. The pluripotent nature of ES cells is a double-edged sword - the fact that ES cells have the capacity to generate almost any cell type is the very same feature that makes these cells difficult to control. Even though our understanding of ventral mesencephalon development and mesencephalic dopaminergic (mesDA) neurogenesis has drastically increased during recent years, the detailed knowledge of what is needed to generate homogenous mesDA neurons from ES cells remains elusive. Hence, the development of improved culturing techniques, and novel enrichment and cell sorting steps is crucial for the progression of research towards the goal of using ES cells in the clinic.

Considering the successful isolation of mesDA neuron progenitors and restoration of the rodent PD model described in this thesis, the cell surface maker Corin could serve as a good starting point in the quest to isolate the mesDA neuron progenitors from alternative cell sources. It is thus most encouraging that the expression of the cell surface marker Corin is conserved in humans. Additionally, the evidence that A9 mesDA neurons are necessary for functional recovery upon grafting should promote further research on alternative cell sources to preferentially generate A9 over A10 neurons. This will be challenging because apart from the fact that the A9 neurogenesis peaks earlier than A10, it is unknown if any molecular differences exist between the two subtypes at an early developmental stage. Further research is required to reveal key factors that direct mesDA neuron progenitors to become the A9 or A10 neuronal subtypes.

Major research efforts are needed to make stem cell-based therapy a clinically competitive treatment for PD patients. In addition to generating sufficient numbers of human ES cell derived mesDA neurons *in vitro*, the cells have to prove at least equally potent upon grafting compared to their foetal derived equivalent in order to be considered for clinical use. They should demonstrate long-term survival in the PD brain, functional innervation of the host tissue, and release dopamine in a regulative manner. Thus, a future where ES cells are used in a clinical setting is faced with challenges that we shall slowly but surely continue to unravel.

METHODS

This section is devoted to the description of different techniques used in this thesis, in a more explanatory manner than what is done in the included papers.

Housing and treatment of animals was in agreement with the guidelines set by the Ethical Committee for the Use of Laboratory Animals at Lund University.

Embryonic tissue

In order to obtain embryos of a specific age the mice where time mated, where the morning of vaginal plug was counted as embryonic day (E) 0.5. The mothers were sacrificed by cervical dislocation at E10.5, E11.5 or E12.5 and embryos removed into ice-cold L15 media, in which they were kept during the dissection of the VM (fig 5). The dissections were performed under a light microscope (Leica) equipped with a fluorescence lamp and a filter for GFP enabling genotyping of the embryos obtained from the *Ngn2*-GFP and *Pitx3*-GFP mice.

If the embryos where obtained for histological analysis, they where removed from the uterus and placed in PBS containing 4% paraformaldehyde (PFA, Merck) for 24 hours at 4°C. Embryos were cryo-protected in PBS containing 30% sucrose until saturated, and

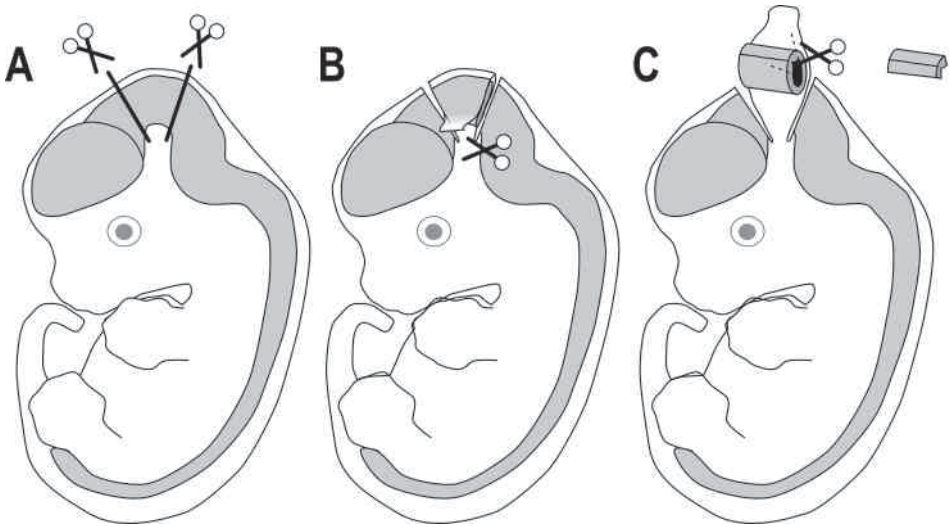


Figure 5. Sub-dissection of VM: The initial incisions are made to free the part of the neural tube containing the VM (A, B). The cut out cylinder can be 'rolled' out and the ventral midline is clearly visible as a thin line (C). The VM piece is then obtained by cutting close to both sides of the midline (C).

were then sectioned coronally at a thickness of 14-16 μm onto superfrost plus glass (Menzel) using a cryostat (Microm).

ES cell culturing

Cells were passaged every second day using trypsin and plated at a density of $4x10^4$ cells/cm^2 onto gelatine-coated plastics. In order to maintain the pluripotency of the ES cells, the expansion media (table 2) was supplemented with leukaemia inhibitory factor (LIF), which was produced in-house and used at a dilution needed to maintain ES cells in an undifferentiated state.

Differentiation on PA6 stromal cells was carried out as previously described with minor modifications[74]. On the day prior to differentiation, PA6 cells were irradiated at 50Gy and plated at a density to coat the culture flask in ES expansion medium. The following day, the medium was removed and the attached PA6 carefully rinsed with PBS before the ES cells were added at a density of 60 cells/cm^2 in differentiation medium (table 2). Upon 4 days of differentiation, half of the differentiation medium was renewed.

Cells were fixed for immunocytochemistry at day eight of differentiation by removing medium and applying cold PBS containing 4% PFA for 15 minutes, followed by three rinses in PBS.

Table 2

ES expansion medium	**Final concentration**	**100ml**
DMEM (Gibco)	1x	86ml
Penicillin/Streptomycin (Gibco)	1x	1ml
Glutamate (200mM)	2mM	1ml
NEAA (Gibco)	1x	1ml
Sodium puryvate (100mM, Gibco)	1mM	1ml
FCS	10%	10ml
β-Mercaptoethanol	0.1μM	0.2μl
LIF	-	As required
ES differentiation medium		
Same as the ES expansion medium, without LIF and FCS is replaced with KSR		
Both media can be stored at 4°C for 4 weeks.		

Preparation of cells for grafting

VM tissue

In order to obtain a single cell suspension, the dissected VM pieces where incubated in HBSS containing 0.1% trypsin and 0.5mg/ml DNAse (SigmaAldrich) at 37°C for 15min (except when staining for Corin, when 1:2 Accutase was used) followed by 4 rinses in HBSS containing 0.05% DNAse. The tissue was gently dissociated in the DNAse solution using a 200μl pipette until a single cell suspension was obtained (approximately 20 E12.5 VM pieces or 40 E10.5 VM pieces per 200μl DNAse solution). If the cells were grafted without sorting, they were counted at this step, spun down (60g, 5minutes) and resuspended in HBSS into the required cell concentration for transplantation.

If the cells where going to be sorted based on GFP expression, not including any prior antibody-labeling, the cell suspension was run through a 70μm filter (Falcon) to remove possible aggregates and diluted into 3x10^6 cells/ml in PBS containing 1% FCS.

ES cells

On day eight of PA6-differentiation cells were detached from the culture dish using Accutase (PAA Laboratories) diluted 1:2 in PBS containing 0.5mg/ml DNase. Cells were diluted with PBS and carefully pipetted until a single cell suspension was obtained, spun down (230g, 5minutes) and re-suspended acording to the protocol for staining against Corin.

Labelling Corin expressing cells

The ES cells were stained for Corin on day eight of PA6-differentiation and cells from mouse embryonic VM at E10.5 and E12.5. After obtaining a single cell suspension (see above), cells where filtered (70μm) and prepared into a concentration of 1x10^6 cells/100μl blocking solution (HBSS containing 10% FCS) and incubated with rabbit anti-Corin antibody (1:50, custom made from Abbomax) in for 30-40 minutes on ice. The suspension was diluted 1:10 in the blocking solution, spun down (230g, 5min), re-filtered and incubated for 30 minutes at 4°C with goat anti-rabbit Alexa488 (1:500, Molecular Labs) or donkey anti-rabbit PE (1:500, BD Pharmingen). The secondary antibody was rinsed off by diluting the suspension 1:10 in HBSS and spun down. Cells were re-suspended into 3x10^6 cells/ml in PBS containing 1-2% FCS (Sigma-Aldrich) and filtered before adding the 7-AAD.

Sorting cells by FACS

All FACS procedures were carried out using a FACS Diva flow cytometer (BD) with a cooling system keeping cells at 4°C. The samples where kept in polystyrene tubes (BD) and cells were gated based on forward and side scatter in order to exclude cell debris and doublets and dead cells were excluded on the basis of 7-AAD. The gates for sorting the different fractions are described Materials and methods in Paper I and III. Cells were sorted with PSI (pounds per square inch) 23 and a 70 μm nozzle, at a rate of approximately 1500 events/second. The collection tubes (polypropylene, BD) contained 1ml PBS containing 1-2% FCS, in order to avoid that cells attach to the tube.

Following sorting, the collection tubes were shortly vortexed, spun at 400g for 7min and cells re-suspended in 100μl PBS. The obtained cells were counted with tryphan blue exclusion, spun down (230g, 5min) and re-suspended into the required cell concentration for transplantation.

Principles of flow cytometry and cell sorting

The technique of flow cytometry provides a tool to analyse individual cells within a heterogeneous cell population. Thousands of cells per second can be analysed on multiple

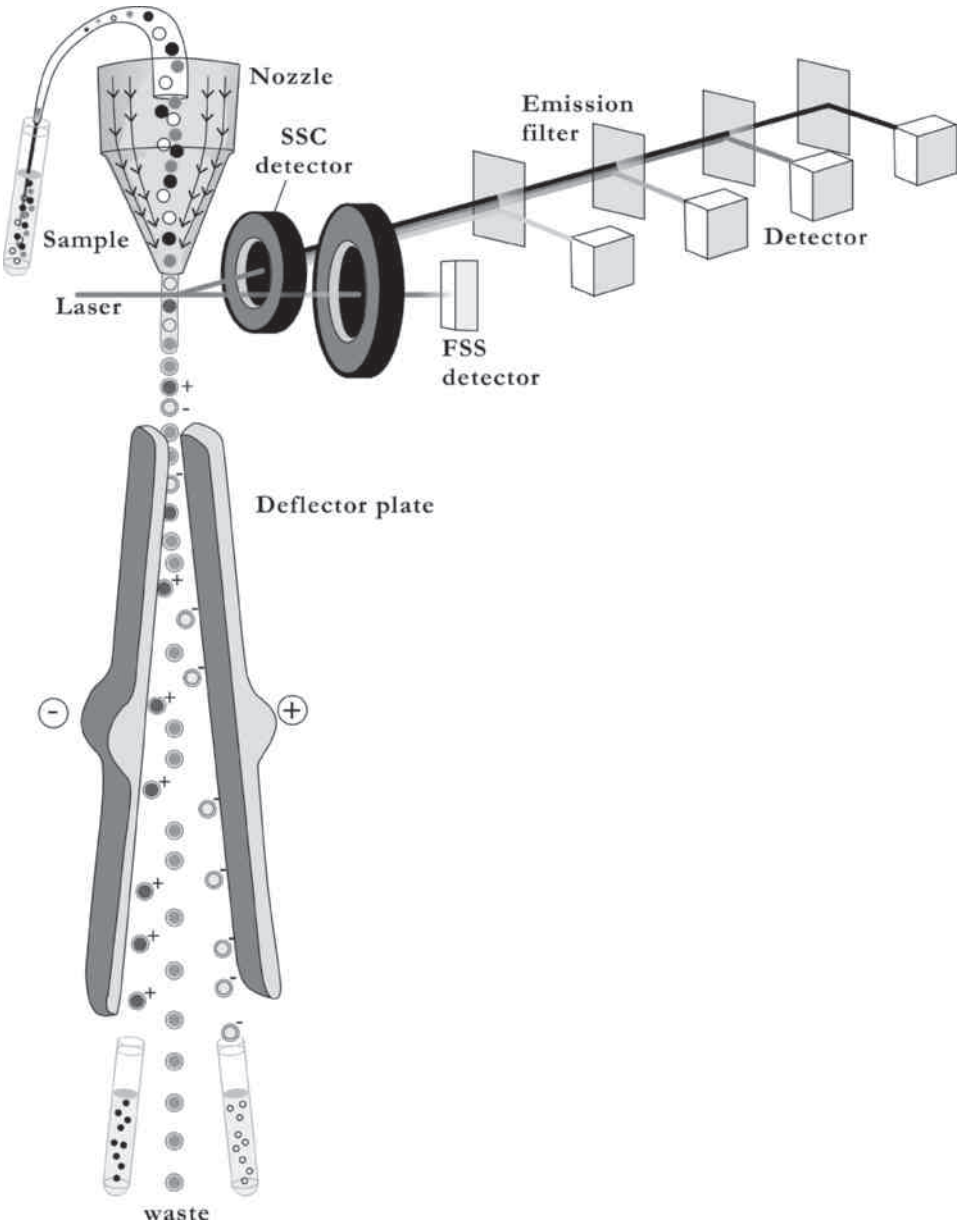


Figure 6. Schematic overview of the cell sorting technique. The procedure is described in the section PRINCIPLES OF FLOW CYTOMETRY AND CELL SORTING.

properties, such as size, shape, complexity and expression of fluorophores. Flow cytometry and cell sorting has mainly been used in the haematology field but is increasingly being applied to new fields of research (for further reading about flow cytometry and cell sorting[97,98]).

The cells in the sample are injected into a flowing stream of sheath fluid, which by hydrodynamic focusing causes the cells to be ordered in a line of cells, one after the other. After emerging from the nozzle, cells will pass one or more laser beams. This is the point in the flow cytometer where the properties of each cell are detected, called interrogation point. As each cell crosses the laser beam, they cause the light to be scattered in all directions and by detecting and measuring the amount of scattered light in the forward direction, called forward scatter (FSC), the size of the cells is obtained, while the light scattered to the sides, called side scatter (SSC), provides information about the cells inner complexity. In addition, by using various lasers and emission filters, multiple fluorophores can be detected. The detected FSC, SSC and fluorescence are converted to electronic signals that are translated into digital data and visualised as dot-plots using computer software. These dot-plots are used to analyse the cell composition of the samples by setting gates around the cells of interest.

In order to sort out the cells of interest, fluorescence activated cell sorting (FACS) can be applied on the flow cytometer. The fluorescence can be acquired by using reporter cell lines or mice, but also by immunocytochemical labelling.

Within the acquired dot plots, the cells that should be sorted are identified and marked with so called gates. For example, gates can be set to define cells to be positive or negative for a fluorophore. These gates are then used as instructions for the flow cytometer to sort the positive and negative cells into different vials.

As the stream of cells emerges into air, it will eventually break up into droplets. However, applying a vibration to the nozzle with a known frequency and amplitude will control the formation of droplets by making it a stable process and ensuring a high probability of one cell per droplet. The distance between the laser beam and where the stream breaks up into droplets is constant under given conditions and is referred to as drop delay. By knowing the drop delay, the flow cytometer keeps track of which droplet holds what cell. As a droplet leaves the stream, it can simultaneously be given a positive or negative charge. If the droplet contains a cell within the gates for sorting it will be given a charge, but if it is outside the gates it will remain uncharged. This results in droplets with a positive, negative or no charge depending on which cell it holds. As the droplets continue to fall, they will pass through a static electrical field between two charged deflector plates. The droplets will be attracted to its opposite charged plate and thereby deflected into its collection tube. If the droplet is uncharged, it will pass straight by the deflector plates and be collected as waste.

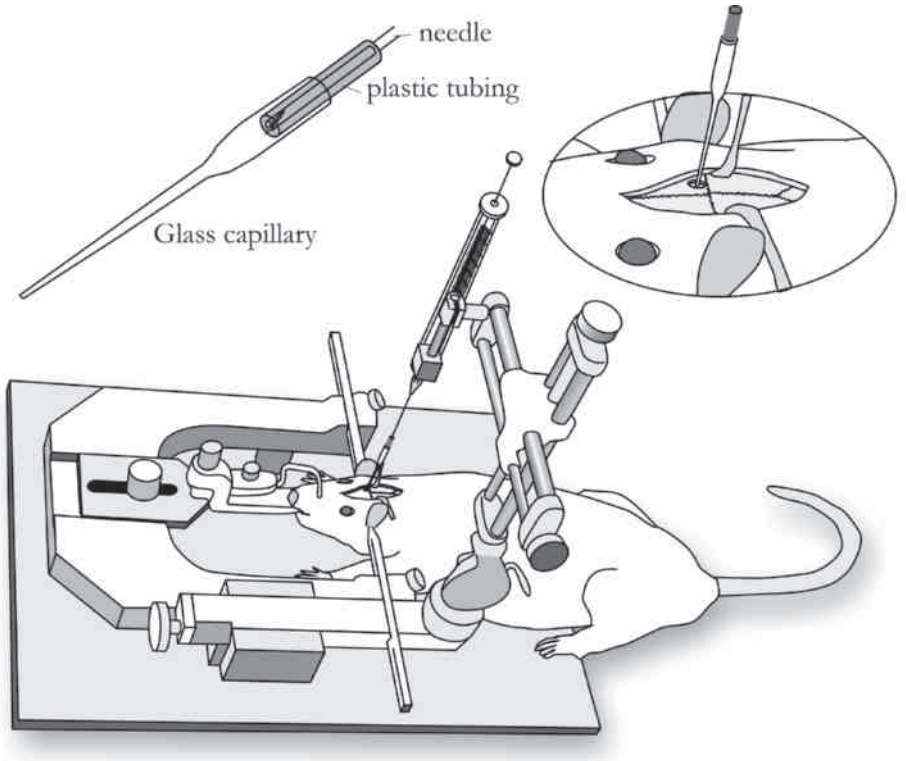


Figure 7. Illustration of the surgical procedure. Upon transplantation or partial lesion, a glass capillary was joined to the needle of the Hamilton syringe using plastic tubing. The scull was locked in flat position in a stereotactic frame and the anterior-posterior and medial-lateral coordinates measured from bregma, the crossing point of the saggital and coronal sutures of the scull.

Surgical procedures

All intracranial injections were performed using a Kopf stereotaxic frame (Kopf instruments, Tujunga, CA) and a Hamilton syringe (fig 7). The scull was locked in a flat position by keeping the tooth bar at -2.4 mm. The anterioposterior (AP) and mediolateral (ML) coordinates were measured from bregma and the scull was penetrated using a cavity drill (GertAB, Sweden). The dorsolateral (DV) coordinates were measured from the dural surface. When performing the partial lesion or transplantation, the Hamilton syringe was equipped with a glass capillary in order to obtain more precise injections, avoid tissue damage and reflux[99].

All surgeries were performed under general anesthetics using either 2% isofluorane gas (Isoba Vet Schering-Plough, Belgium) or an intra-peritoneal injection (6.3 ml/kg) of a 20:1 solution of Fentanyl (50μg/ml, Meda, Sweden) and Dormitor (1mg/ml, Orion Phar-

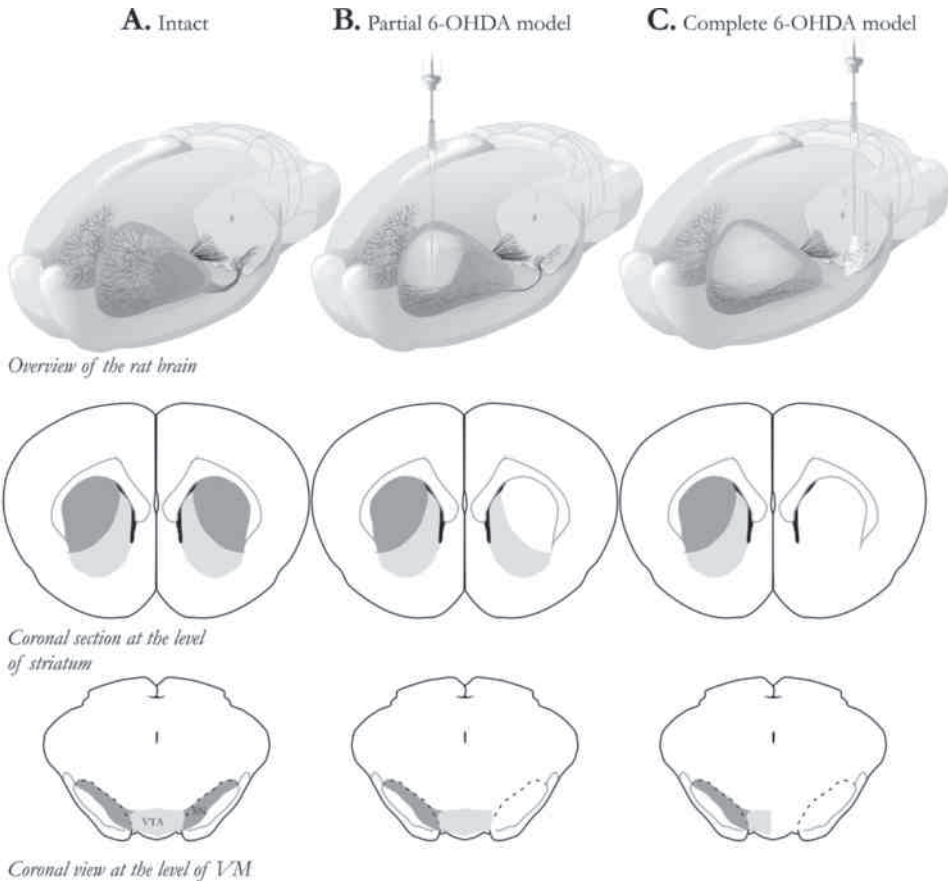


Figure 8. Illustration of the 6-OHDA animal models. Panel A shows the intact brain, which receives dopaminergic input from both SNpc (A9) and VTA (A10). The A9 and A10 mesDA neurons predominately innervate the dorso-lateral or ventral-medial parts of the striatum, respectively. The partial 6-OHDA lesion (Panel B) is achieved by injecting the toxin into the part of striatum that is innervated by the A9 mesDA neurons. The lesion mainly affects the A9 neurons (SNpc), while the VTA is left relatively intact. The complete 6-OHDA lesion (Panel C) abolish both the A9 and A10 neurons and is achieved by injecting the toxin into the medial-forebrain bundle.

ma, Finland). After surgery the animals were given analgesic by subcutaneously injecting (0.1 ml/kg) of Temgesic (0,3 mg/ml, Schering-Plough, Belgium) diluted 1:10 in saline.

Animal models of Parkinson's Disease

On of the most commonly used animal model of Parkinson's Disease is based on the intracerebral injection of the neurotoxin 6-hydroxy dopamine (6-OHDA)[10] (fig 8). The

6-OHDA enters cells via the dopamine transporter (DAT), which is preferably expressed on DA neurons, and leads to cell death by causing mitochondria malfunction and oxidative stress. In this thesis, we have made use of both a complete and partial 6-OHDA lesion model of PD.

6-OHDA is sensitive to light and oxidation and was therefore dissolved in 0.9% NaCl containing 0.02 mg/ml L-ascorbic acid (Sigma-Aldrich), kept in darkness on ice and was replaced every second hour. The concentration of 6-OHDA was 3.5μg free base per μl when generating both models. In order to avoid reflux, the syringe was left in place 2 minutes after each injection.

The complete 6-OHDA lesion model was obtained by injecting 4μl of the toxin into the medial-forebrain bundle (MFB), which is a fibre tract that holds both A9 and A10 neuron projections. Consequently, both the A9 and A10 neurons are affected and a near complete loss of both the striatal dopamine levels and the SNpc neurons are seen, as well as an 80% reduction of the VTA neurons[10].

The partial 6-OHDA lesion model was achieved by four injections of the toxin into the lateral striatum (2μl/injection), which is mainly innervated by the A9 neurons. The 6-OHDA is taken up by the axon terminals and is subsequently transported to the cell bodies. This results in a retrograde degeneration where the axons deteriorate within a week, while the majority of the cells die within 4 weeks of the injection. The partial lesion predominately affects the SNpc where 75-85% of the DA neurons are lost, while the VTA only looses 15-20% of its DA neurons[100].

Both of these PD models are unilateral, i.e. only affecting one side of the brain (and thus the opposite side of the body), allowing the intact side to serve as a control for the behavioural evaluation. The success of the lesion was evaluated by performance in the included behavioural tests and was used to select adequately lesioned animals for the studies.

Transplantation

Cells were prepared into the required concentration in PBS (Gibco) and kept on ice at all times. Cells were injected using a 5μl Hamilton syringe with an attached glass capillary at a rate of approximately 1μl/min and left in place for two additional minutes before withdrawal in order to avoid reflux of cells.

Grafted rats were immunosuppressed by daily intra-peritoneal injections of cyclosporine (Novartis) starting the day prior transplantation (15mg/kg for the first week, followed by 10mg/kg).

Perfusion

Rats were deeply anaesthetised with intra-peritoneal injection of sodium pentobarbital. The chest was opened and the animal transcardially perfused through the ascending aorta with 0.9% saline for 1 minute, followed by ice-cold PBS containing 4% PFA (pH 7.2 –

7.6) for 5 minutes (50ml/min). Brains were removed from the scull and post-fixed for 2h followed by cryo-protection by saturation in PBS containing 25% sucrose. Brains were sectioned on a freezing microtome (Leica) at a thickness of 35μm into 12 series.

Behavioural tests

By using unilateral models of PD, the behavioural tests allow the registration of the functional imbalance between the lesioned and intact side. Upon transplantation, the reduced imbalance is seen as a sign of recovery obtained by the graft.

Amphetamine-induced rotation test measures rotational asymmetry[101] and was used in PAPER I and II. Following intra-peritoneal injection of D-amphetamine sulphate (2.5 mg/kg) the animal is put in a rotometer bowl with a string attached around its waist, by which full body turns is recorded over 90 minutes (AccuScan instruments Inc.) and presented as net body turn per minute. The injection of amphetamine stimulates the release of dopamine and due the reduced dopamine levels in the lesioned striatum the animal will rotate ipsilateral to the lesioned hemisphere.

The cylinder test is an evaluation of the spontaneous motor function by measuring the use of the front paws[102] and was used in PAPER II. The animal is placed in a glass cylinder with a diameter of 20 cm and allowed to move freely. As it explores the environment, it uses either front paw to lean on the glass and was videotaped until it had at least 20 contacts. The recordings were used to score each animal, where the percentage of contacts with the impaired front paw (left) was calculated. Intact animals use their right and left paw at a similar frequency and thus get a score of 50%. Upon the partial 6-OHDA lesion, animals use their left paw between 10-25% of the contacts.

Immunochemistry

All procedures were performed in room temperature and the same protocol was used for fixed cells as well as for embryonic and adult tissue. Sections were incubated for 1 hour in blocking solution (KPBS with 0.25% triton-X and 5% normal serum of the species that the secondary antibody is generated in). The primary antibody was diluted in the in blocking solution and applied over night in room temperature. Following three rinses in KPBS the secondary antibodies, either biotinylated (Vector Labs) or fluorophore-conjugated secondary antibodies (Molecular Probes or Jackson Laboratories), where diluted in blocking solution and applied for 2 hours.

Biotinylated secondary antibodies were followed by 1 hour incubation with either streptavidin-horseradish peroxidase complex (ABC-elite kit, Vectastain) and subsequent timed exposure to di-amino-benzydine (DAB, 0.5mg/ml) with 0.01% H_2O_2, or fluorophore-conjugated streptavidin (Jackson Laboratories). When sections were stained with DAB, the endogenous peroxidase was quenched by 10 minutes pre-treatment with 3% H_2O_2 and 10% methanol in KPBS prior to the initial pre-incubation step.

Following the last labelling step the sections where rinsed three times in KPBS. The free-floating sections were mounted on gelatine-coated glass slides. Following staining with DAB, mounted sections were dehydrated in graduated solutions of ethanol, followed by xylene, before being cover-slipped with DPX (Merck) mounting media. The fluorescent-labelled sections or cells were cover-slipped with the anti-bleaching reagent PVA-DABCO.

Genotyping of animals

The animals used for breeding were genotyped by PCR using following primers.

Ngn2-GFP

Ngn2KI5: 5'-GGA CAT TCC CGG ACA CAC AC-3'
Ngn2KImut3: 5'-GCA TCA CCT TCA CCC TCT CC-3'
The generated product is 440 bp.

Pitx3-GFP

Pitx3 forward (mutant): 5'AGC CTC GAC TGT GCC TTC TA 3'
Pitx3 Reverse (common): 5' CCG GAG AGG CTG TGA ATT AC 3'
Pitx3 forward (wild-type): 5' TCC ATC GCC GCT TCT ATG GT 3'
The generated mutant product is 570 bp and the wild type product 390 bp.

qRT-PCR

The total RNA from the isolated cell fractions was isolated using the RNeasy Mini kit (Qiagen). Five nanograms of RNA was used for the reverse transcription performed with random primers (Invitrogen) and SuperscriptIII (Invitrogen) according to supplier's recommendations. SYBR green quantitative real-time PCR was performed with LightCycler 480 SYBR Green I Master (Roche) using standard procedures. Data was quantified using the DDCt-method and were normalised to Gapdh and β-actin expression. All data shown is normalised to β-actin. Gapdh-normalised data was close to identical with the β-actin data. Primers were designed using PrimerExpress software (Applied Biosystems). The efficiency of all primer pairs was confirmed by performing reactions with serially diluted samples. The specificity of all primer pairs was confirmed by analysing the dissociation curve.

Table 3

qRT-PCR primers, presented from 5' to 3'.

Corin
FW CCT TCT CCA GAG GAC CAG AG
RW TTG ACA CTG GCT GTG AGT GA

En1
FW CGT GGC TTA CTC CCC ATT TA
RW TCT CGC TGT CTC TCC CTC TC

Foxa2
FW CCG TTC TCC ATC AAC AA CCT
RW GGG GTA GTG CAT CAC CTG TT

Lmx1a
FW GAG ACC ACC TGC TTC TAC CG
RW GCC CGC ATA ACA AAC TCA TT

Lmx1b
FW CTT AAC CAG CCT CAG CGA CT
RW TCA GGA GGC GAA GTA GGA AC

Msx1
FW CCA AAA AGT GGC TGG AAG AG
RW CAA TTC TGC TGG GGA CCT TA

Nanog
FW GAT TTG TGG GCC TGA AGA AA
RW CAG GGC TGT CCT GAA TAA GC

Nestin
FW GAC CAC TTC CCT GAT GAT CCA
RW TCT AAA ATA GAG TGG TGA GGG TTG AG

Oct4
FW GTC CCA GGA CAT CAA AGC TC
RW AAT AGA ACC CCC AGG GTG AG

Shh
FW CCA ATT ACA ACC CCG ACA TC
RW AGT TTC ACT CCT GGC CAC TG

Wnt5a
FW GGA CCA CAT GCA GTA CAT CG
RW CCT GCC AAA AAC AGA GGT GT

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I grew up in a tiny community located in a region named Småland, which holds the most beautiful pine-tree forests in all of Sweden. The people that live within those forest are of a well-known character and go under the name Smålänningar, and are even described in the old Swedish encyclopaedia "Nordisk Familjebok" (1876-1926):

In Swedish
"Smålänningen är till sin natur vaken och intelligent, flitig och sträfsam, rask och hurtig, men likväl foglig till lynnet, händig och slug, hvilket allt medför åt honom den förmånen, att han äfven med små medel kan taga sig fram i livet."

In English
"Smålanders are alert and intelligent, hard-working and driven, energetic and cheerful, handy and wise, and in combination these qualities allow them to adjust and be able to make the most out of seemingly impossible situations."

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