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PO Box 117
221 00 Lund
+46 46-222 00 00

Academic Dissertation

CELL REPLACEMENT THERAPY FOR PARKINSON'S DISEASE:

The importance of neuronal subtype, cell
source and connectivity for functional recovery

SHANE GREALISH

With the approval of the Faculty of Medicine of Lund University, this thesis will
be defended at 13:00 on January 27th, 2012 in Segerfalksalen,
Wallenberg Neuroscience Center, Lund, Sweden.

Faculty Opponent:

PROFESSOR JEFFREY D. MACKLIS,

Department of Stem Cell and Regenerative Biology,
Harvard University, Boston, USA.



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Abstract <p>Parkinson's disease (PD) is a neurodegenerative disorder characterised by motor deficits such as slowness in movement, difficulty in initiating movement and tremor at rest. The cause of these motor symptoms is the selective loss of mesencephalic dopaminergic (mesDA) neurons, located in the substantia nigra (SN). These neurons project axons to the striatum where they release dopamine, a neurotransmitter that controls voluntary movement. Current drug treatments restore the lost dopamine, while initially efficacious, the beneficial effects wear off resulting in severe side effects. Thus, there is a clear requirement for alternative therapeutic options.</p> <p>One such idea is cell replacement therapy (CRT). CRT aims to replace neurons that have degenerated in PD, with donor cells that have the potential to functionally re-integrate into the host circuitry. This involves transplantation of developing midbrain cells from aborted fetuses, (the part that form mesDA neurons), into the striatum of a PD patient. Clinical trials have demonstrated that CRT can provide long-lasting, significant clinical benefit. Although some patients do not respond as favourably. We still do not know what specific factors contribute to the success in transplantation i.e. what cells are responsible for motor recovery? Can the transplants reform damaged neuronal circuitry? Use of human fetal tissue raises several ethical issues, but are there alternative cell sources that can substitute effectively? The aim of this thesis was to understand how particular factors such as neuronal content, placement and cell source, affect functional outcome after transplantation into the rodent brain.</p> <p>In paper №1, I detail the neurodegenerative and behavioural outcomes in a mouse lesion model of PD, which can be used as a platform for the development of novel therapeutic strategies. I also describe the development of a novel behavioural task that is predictive of mesDA neuron cell loss in mice. Previously, it was thought that transplanted neurons could not extend axons over long distances rendering transplantation into the SN a non-viable approach. In paper №2, I describe how mesDA neurons transplanted in the adult SN of a PD mouse model, extended axons across millimetres into the striatum, functionally reforming the nigrostriatal pathway. In paper №3, I also identify the specific mesDA population (A9) that is critical for functional recovery, with transplants that lack A9 neurons failing to improve motor recovery. A potentially pre-clinical aspect of this thesis is detailed in paper №4 where I describe a robust protocol for the generation of functional mesDA neurons from human embryonic stem cells that are functional in a rat model of PD. No evidence of tumour formation was observed in the transplanted animals, a major concern when utilising a pluripotent cell source.</p> <p>Through understanding functional recovery in terms of neuronal subtype and connectivity, the work presented in this thesis aims to bring the prospect of CRT closer to the clinic. I also describe the generation of a very promising alternative cell source that could rival fetal tissue. Together this work contributes to making CRT a reality for the treatment of PD.</p>		
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LUND UNIVERSITY, LUND, SWEDEN.

CELL REPLACEMENT THERAPY FOR PARKINSON'S DISEASE:

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2012



NEUROBIOLOGY



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Cover: The cover illustrates two of the main dopaminergic neuron pathways in the adult midbrain, the nigrostriatal and mesolimbic pathways.

The **front cover** depicts an A9 dopamine neuron in the substantia nigra pars compacta extending an axon rostral along the medial forebrain bundle towards its target, the dorsolateral striatum. A dendrite is releasing dopamine on the GABAergic neurons of the substantia nigra pars reticulata.

The **back cover** details an A10 dopamine neuron in the ventral tegmental area with its axon coursing along the medial forebrain bundle to the nucleus accumbens.

Cover art beautifully made by Bengt Mattsson.

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ORIGINAL PAPERS AND MANUSCRIPTS

PAPER №1

Grealish, S., Mattsson, B., Draxler, P. and Björklund, A. (2010)

Characterisation of behavioural and neurodegenerative changes induced by intranigral 6-hydroxydopamine lesions in a mouse model of Parkinson's disease.

European Journal of Neuroscience. 31 (12): 2266–78.

PAPER №2

Thompson, L.H., Grealish, S., Kirik, D., and Björklund, A. (2009)

Reconstruction of the nigrostriatal dopamine pathway in the adult mouse brain.

European Journal of Neuroscience. 30 (4): 625–638.

PAPER №3

Grealish, S., Jönsson, M.E., Li, M., Kirik, D., Björklund, A. and Thompson, L.H. (2010)

The A9 dopaminergic neuron component in grafts of ventral mesencephalon is an important determinant for recovery of motor function in a rat model of Parkinson's disease.

Brain. 133 (Pt2): 482–95.

PAPER №4

Kirkeby, A.*, Grealish, S.*, Wolf, D., Wood, J., Nelander, J., Lundblad, M., Lindvall, O. and Parmar, M. (2011)

Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions.

Submitted for publication.

* These authors contributed equally.

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterised by motor deficits such as slowness in movement, difficulty in initiating movement and tremor at rest. The cause of these motor symptoms is the selective loss of mesencephalic dopaminergic (mesDA) neurons, located in the substantia nigra (SN). These neurons project axons to the striatum where they release dopamine, a neurotransmitter that controls voluntary movement. Current drug treatments restore the lost dopamine, while initially efficacious, the beneficial effects wear off resulting in severe side effects. Thus, there is a clear requirement for alternative therapeutic options.

One such idea is cell replacement therapy (CRT). CRT aims to replace neurons that have degenerated in PD, with donor cells that have the potential to functionally re-integrate into the host circuitry. This involves transplantation of developing midbrain cells from aborted fetuses, (the part that form mesDA neurons), into the striatum of a PD patient. Clinical trials have demonstrated that CRT can provide long-lasting, significant clinical benefit. Although some patients do not respond as favourably. We still do not know what specific factors contribute to the success in transplantation i.e. what cells are responsible for motor recovery? Can the transplants reform damaged neuronal circuitry? Use of human fetal tissue raises several ethical issues, but are there alternative cell sources that can substitute effectively? The aim of this thesis was to understand how particular factors such as neuronal content, placement and cell source, affect functional outcome after transplantation into the rodent brain.

In paper №1, I detail the neurodegenerative and behavioural outcomes in a mouse lesion model of PD, which can be used as a platform for the development of novel therapeutic strategies. I also describe the development of a novel behavioural task that is predictive of mesDA neuron cell loss in mice. Previously, it was thought that transplanted neurons could not extend axons over long distances rendering transplantation into the SN a non-viable approach. In paper №2, I describe how mesDA neurons transplanted in the adult SN of a PD mouse model, extended axons across millimetres into the striatum, functionally reforming the nigrostriatal pathway. In paper №3, I also identify the specific mesDA population (A9) that is critical for functional recovery, with transplants that lack A9 neurons failing to improve motor recovery. A potentially pre-clinical aspect of this thesis is detailed in paper №4 where I describe a robust protocol for the generation of functional mesDA neurons from human embryonic stem cells that are functional in a rat model of PD. No evidence of tumour formation was observed in the transplanted animals, a major concern when utilising a pluripotent cell source.

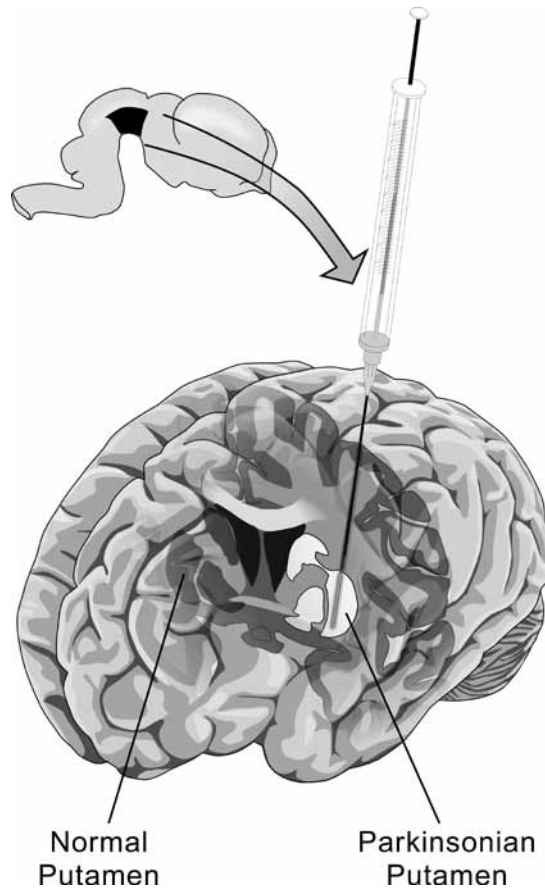
Through understanding functional recovery in terms of neuronal subtype and connectivity, the work presented in this thesis aims to bring the prospect of CRT closer to the clinic, I also describe the generation of a very promising alternative cell source that could rival fetal tissue. Together this work contributes to making CRT a reality for the treatment of PD.

LAY SUMMARY

Patients that suffer from Parkinson's disease (PD) have lost the faculty to consciously control their movement. Lifelong medication is needed, which relieves symptoms, but often with different efficiencies. Although these drugs, namely L-DOPA, provide amazing clinical benefit: as the disease progresses and the drug continues to cause more side effects, the mobility of the patient decreases. Apart from medication, there are little clinical options available to patients, therefore there is a clear need for new therapies. We know that dopamine neurons of the midbrain (mesDA neurons) are responsible for controlling movement, and that these cells are lost during the progression of PD. Therefore, might it be feasible to try and replace these lost cells?

The most successful approach is called cell replacement therapy. This involves taking the region of the developing brain that will form mesDA neurons (from a fetus donated from elective abortions) and grafting these developing neurons into a patients brain. Initial clinical trials have proved that patients can survive with functioning grafts for at least 14 years and in some cases stop taking L-DOPA altogether. However this is the best-case scenario and not all patients respond to the same extent. Additionally, it is important for the widespread application of cell replacement therapy to consider alternative cell sources for large-scale application, given the ethical implications arising from the source of the tissue. The work presented in this thesis aims to understand what factors affect functionality of transplanted mesDA neurons in a preclinical setting; namely where the neurons are placed, what neurons are responsible for recovery and what cells are used.

In order to ensure a functional effect, cells are typically transplanted into their destination or target structure (the striatum). In studies in mice we have discovered that if mesDA cells are transplanted into their site of origin (the midbrain), they can make functional connections to the striatum which is several millimetres away. This phenomenon is something that was previously believed to be unachievable. The next question was to understand what type of mesDA neuron is responsible for mediating motor recovery after transplantation. Here we found that if the mesDA neurons associated with motor control (A9) are missing from transplants, a PD animal model does not recover motor function. The final question addressed in this thesis is: can use cell sources other than human fetal tissue (i.e. human stem cells), to generate a true mesDA neuron. We found that we can generate mesDA neurons from human embryonic stem cells that are remarkably similar to their fetal counterparts. The stem cell derived mesDA neurons can survive transplantation, not generate tumours, and provide functional benefit.



Cell replacement therapy for Parkinson's disease. The mesDA neurons are located in the substantia nigra in the midbrain and send axons to innervate the putamen. These neurons die, resulting in the motor problems of Parkinson's disease. Taking newly formed mesDA neurons from fetuses and transplanting them into the depleted striatum of Parkinsonian patients provides therapeutic benefit.

The work in this thesis has contributed to advancing our understanding of how transplants of mesDA neurons elicit their beneficial effects, and what factors we should consider when using alternative cell sources such as stem cells. It is hoped that this work brings us closer towards a cell replacement therapy for Parkinson's disease.

SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Patienter som drabbats av Parkinsons sjukdom (PD) saknar förmågan att medvetet kontrollera sina rörelser. Livslång medicinering krävs, vilket lindrar symptomen och leder till att rörelsemönstret förbättras, men med varierad effektivitet. Trots att de läkemedel (t ex L-DOPA) som finns tillgängliga idag har fantastisk effekt, minskar deras effektivitet i takt med att sjukdomen fortgår och ger istället en högre grad bieffekter. Förutom medicinering finns det idag få andra möjligheter till behandling av sjukdomen och efterfrågan på nya behandlingsformer är stor. Vi vet att det är de dopaminerga nervcellerna i mellanhjärnan (mesDA nervceller), vilka ansvarar för kontrollen av rörelser, som dör av då PD fortskrider. Kan det därför vara ett bra alternativ till behandling att försöka ersätta dessa celler?

Ett lovande och framgångsrikt tillvägagångssätt har visat sig vara att ta ut den del av hjärnan som ger upphov till mesDA nervceller från donerade aborterade foster, och sedan injicera dessa friska nervceller till patienter med PD. Resultat från dessa kliniska studier har visat att cellerna kan överleva i minst 14 år och att vissa patienter helt har kunnat sluta sin medicinering med L-DOPA. Dessvärre har inte alla patienter reagerat lika positivt på behandlingen och för vissa patienter har det inte blivit någon förbättring alls. Det finns också ett etiskt dilemma i och med användandet av celler från aborterade foster och om cellterapi ska kunna bli ett alternativ till behandling i stor skala måste man undersöka alternativa sätt att tillhandage dessa typer celler. Arbetet som presenteras i denna avhandling har till uppgift att ge förståelse för vilka faktorer som påverkar funktionaliteten av transplanterade mesDA nervceller i ett preklinisk sammanhang, d.v.s. var nervcellerna är placerade, vilka nervceller som är viktiga för återhämtning och vilka celler som bör användas.

Det typiska är att cellerna transplanteras in i striatum, vilket är det område där cellerna och dopaminet har funktionella effekter. Vi upptäckte i studie på möss att om mesDA celler transplanteras in i mellanhjärnan, där de i vanliga fall är placerade i en frisk hjärna kan cellerna innervera striatum flera millimeter bort, något som man tidigare trodde var omöjligt. Vi undersökte också vilka typer av mesDA nervceller som ger förbättring och återhämtning efter transplantation. Det visade sig att om mesDA nervceller som associeras med motorisk kontroll (A9) saknades förbättrades inte det motoriska mönstret i de djur som transplanterats. En sista fråga som behandlas i denna avhandling är huruvida man kan använda andra källor för att generera rätt typ av mesDA nervceller än fetal vävnad, så som humana stamceller. I vår studie kom vi fram till att det är möjligt att generera mesDA nervceller från humana stamceller med egenskaper förvånansvärt lika de i ett foster. De mesDA nervceller som kommer från

stamcellerna kan överleva en transplantation, bildar inga tumörer, och bidrar till förbättrad funktion.

Arbetet i denna avhandling har bidragit till en ökad förståelse för hur transplantation av mesDA nervceller ger sin positiva effekt och vilka faktorer som bör tas hänsyn till om användandet av andra källor än fetal vävnad, så som stamceller, är önskvärt. Förhoppningen med detta arbete är att ta cellterapi som behandlingsform för Parkinsons sjukdom ett steg närmare kliniken.

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
CNS	central nervous system
DA	dopamine
DAT	dopamine transporter
E	embryonic day
GFP	green fluorescent protein
hESC	human embryonic stem cell
mesDA	mesencephalic dopamine
MFB	medial forebrain bundle
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
PD	Parkinson's disease
SHH	Sonic Hedgehog
SNC	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
TH	tyrosine hydroxylase
VM	ventral mesencephalon
VTA	ventral tegmental area

INTRODUCTION

INTRODUCTION

The mesencephalic dopaminergic neuron system

Dopamine (DA) within the central nervous system (CNS) is best known for its role in mediating pleasurable and rewarding experiences, but also in its control of voluntary movement. Within the midbrain, or mesencephalon, lies the largest population of DA-producing neurons within the CNS. Located in the most ventral aspect of the midbrain, the mesencephalic dopamine (mesDA) neurons comprise three main cell groups, designated A8, A9 and A10, as defined by Dahlström & Fuxe (Dahlstrom and Fuxe, 1964). mesDA neurons innervate various structures in the forebrain, and control a variety of physiological functions, most notably voluntary motor control (Fig. 1A). As understanding the morphological, anatomical and functional aspects of mesDA neurons is key to this thesis: I will provide an overview of the essential aspects of the neurobiology of the mesDA subtypes in relation to the work presented here. I will restrict my description to the rodent mesDA system, as earlier studies were performed in rat and newer studies using transgenic mice are of particular interest for the context of this work.

ANATOMICAL DESCRIPTION

The most posterior cell group is the A8 group, which contains the retrorubral area (RRA). The A9 group lies lateral to the midline and consists of the substantia nigra (SN), and has three distinct subdivisions; the pars compacta (SNc), pars reticulata (SNr) and the pars lateralis (SNI) (Halliday and Tork, 1986; Fig 1B). The most medial cell group A10, consists of the ventral tegmental area (VTA). The VTA is comprised of the parabrachial pigmented nucleus, the intrafascicular nucleus and the paranigral nucleus (Halliday and Tork, 1986; Fig. 1B).

These cell groups are all composed primarily of dopaminergic (DAergic) neurons. When describing them this way, all of the neurons uniformly express markers for DA synthesis and handling such as: tyrosine hydroxylase (TH) and amino acid decarboxylase (AADC) for DA synthesis, dopamine transporter (DAT) and vesicular monoamine transporter (VMAT2) for DA re-uptake and vesicularisation. However, more subtle differences can be observed using a more rigorous, morphological-based analysis of the cell bodies. According to German and colleagues, the rat midbrain contains around 45,000 TH⁺ neurons, 5% of these are located in the A8 group, while the remaining are almost equally split between A9 and A10 (German and Manaye, 1993). Therefore I will primarily discuss the A9 and A10 cell groups.

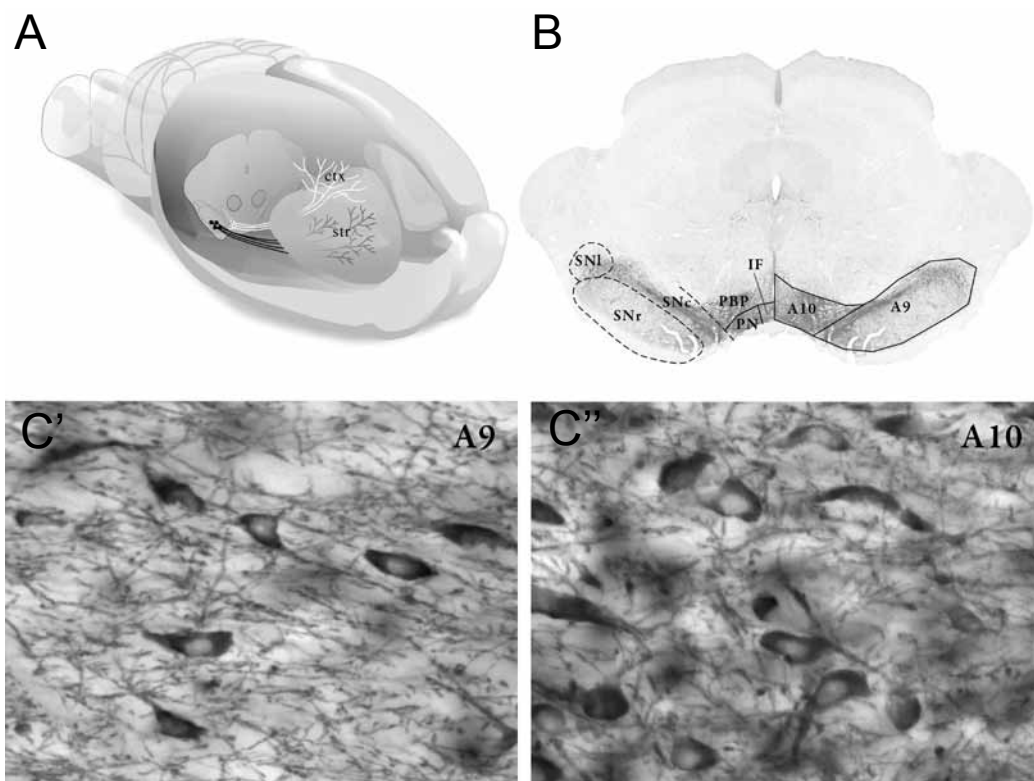


Figure 1. Overview of neuroanatomy of the rodent mesencephalic dopaminergic system. A depiction of the mesDA neuron somas located in the midbrain that project to forebrain structures (A). A coronal section of the midbrain immunohistochemically stained for TH reveals the nuclei within the midbrain that contain the mesDA neurons (B). Closer inspection of the cytoarchitecture of the DAergic neurons of the A9 (C') and A10 (C'') cell groups reveals distinct morphological differences (C). Ctx=cortex, IF=intrafascicular nucleus, PBP=parabrachial pigmented nucleus, PN=paranigral nucleus, RRA=retro-rubral area, SNc=substantia nigra pars compacta, SNl=substantia nigra pars lateralis, SNr=substantia nigra pars reticulata, Str=striatum.

The A9 cell group contains around 10,500 TH⁺ neurons, with 8% of those residing in the SN pars reticulata (SNr) (German and Manaye, 1993) and the majority located in the SN pars compacta (SNc). They are large, angular, fusiform neurons with an average cell diameter (as measured along the long axis) of 21.9µm (German and Manaye, 1993; Halliday and Tork, 1986; Fig. 1C'). These neurons innervate the dorsal striatum, and play a classical role in motor control (Bentivoglio et al., 2005).

The A10 cell group contains a similar number of TH⁺ neurons to A9, approximately 10,200 (German and Manaye, 1993). Comparable to A9 neurons, A10 cells are typically small, round, fusiform cells with an average cell diameter of 16.9µm (German and Manaye, 1993; Halliday and Tork, 1986; Fig. 1C”). A10 neurons innervate a number of ventral forebrain structures such as the nucleus accumbens (NAc), olfactory tubercle and medial prefrontal cortex (mPFC). Functionally, they have been described in the regulation of reward, addiction and cognitive function (Bentivoglio et al., 2005).

The description of the cell groups thus far has primarily been anatomical and cytoarchitectural, although these descriptions are not definitive. The original description by Dahlström and Fuxe in 1964 highlights:

“there is no distinct border–line medially between A9 and A10 and laterally between A9 and A8. Nor could any distinct border–line be seen between the ventro–lateral part of A10 and the ventromedial part of group A8”.

The detection method used in this seminal study was based on the fluorescence of catecholamines and indolamines when a condensation reaction occurs with formaldehyde, known as the Falck–Hillarp method (Falck et al., 1962). With the advent of newer technologies a further refinement in the nomenclature of the mesDA system has occurred.

FUNCTIONAL ANATOMICAL DESCRIPTION

This newer nomenclature is not solely based on where the cell somas reside, but was founded on a more functional basis, involving connectivity to target structures (as determined by retro– and antero–grade tracing) and the differential expression of proteins in subsets of anatomical nuclei. This aspect of mesDA neurobiology was pioneered by the work of Gerfen and colleagues (Gerfen et al., 1985; Gerfen et al., 1987). Although the striatum (the main target field of mesDA neurons) may seem homogenous in its appearance, Gerfen and others noted the presence of two main compartments that could be differentiated based on protein expression. The organisation and cytoarchitecture of the striatum is complex. It is composed of islands that are dense in opiate receptors (Herkenham and Pert, 1981), the patch compartment, intermingled within the matrix compartment, as detected by the presence of Calbindin and acetylcholinesterase (Gerfen et al., 1985; Graybiel, 1984; Graybiel and Ragsdale, 1978; Fig. 2A). Protein expression combined with tracing studies revealed that the neurons from the A8, A9 and A10 nuclei innervated patch and matrix compartments differentially resulting in a new nomenclature.

The DAergic neurons in the dorsal layer of SNc, the entire VTA and A8, preferentially innervate the striatal matrix. This functional anatomical unit is known as the dorsal tier. The ventral portion of the SNc, including the DAergic neurons of SNr, selectively innervates the patch compartment, and is named the ventral tier (Fig. 2A). The striatal compartments also send reciprocal innervation to their input structures. The striatal patch innervates the ventral tier, whereas the matrix innervates the globus pallidus and the SNr (Gerfen et al., 1987).

An elegant anterograde tracing study from Matsuda and colleagues was performed in SN DAergic neurons (Matsuda et al., 2009). In this study they over-expressed a palmitoylated form of GFP (thereby increasing its affinity for membranes) that allowed for clear demarcation of the somatodendritic and axonal membranes of the entire neuron. As compared to previous methods, this novel approach showed high-sensitivity of detection, especially of the axonal terminals. Previous estimations of SN DAergic axonal length was around 36mm (Matsuda et al., 2009; Prensa and Parent, 2001). Using this approach Matsuda and colleagues estimated that the dorsal tier SN DAergic neurons have an axonal length ranging from 140–780mm, with around 95% of their axonal fibres innervated matrix. The ventral tier neurons have an average axonal length of 500–610mm, and showed a preferential innervation of patch.

Included in this ventral and dorsal tier definition, there is also a more functional component. The most medial aspect of the dorsal tier (the VTA) innervates the matrix of the limbic striatum, including the NAc. The central dorsal tier (the medial aspect of SNc and the lateral aspect of the VTA) innervates the matrix of associative striatum, while the lateral dorsal tier (the lateral SNc) innervates the matrix of motor striatum (Bjorklund and Dunnett, 2007; Joel and Weiner, 2000; Fig. 2B). Within the ventral tier, the medial aspect innervates the patches of the associative striatum, while the lateral region innervates patches of motor striatum (Joel and Weiner, 2000). From these descriptions one can also relate the most lateral mesDA population with the motor striatum, hence controlling movement; the central (both medial SNc and lateral VTA) with the associative striatum, involved in attention; and finally the medial mesDA neurons innervating the limbic striatum, controlling reward and addiction (Fig. 2B). The medial mesDA neurons also innervate other areas, such as the prefrontal cortex, septum and amygdala. These three functional anatomical pathways are also known as the mesostriatal (Fig. 2Ci), mesolimbic (Fig. 2Cii) and the mesocortical (Fig. 2Ciii) pathways respectively.

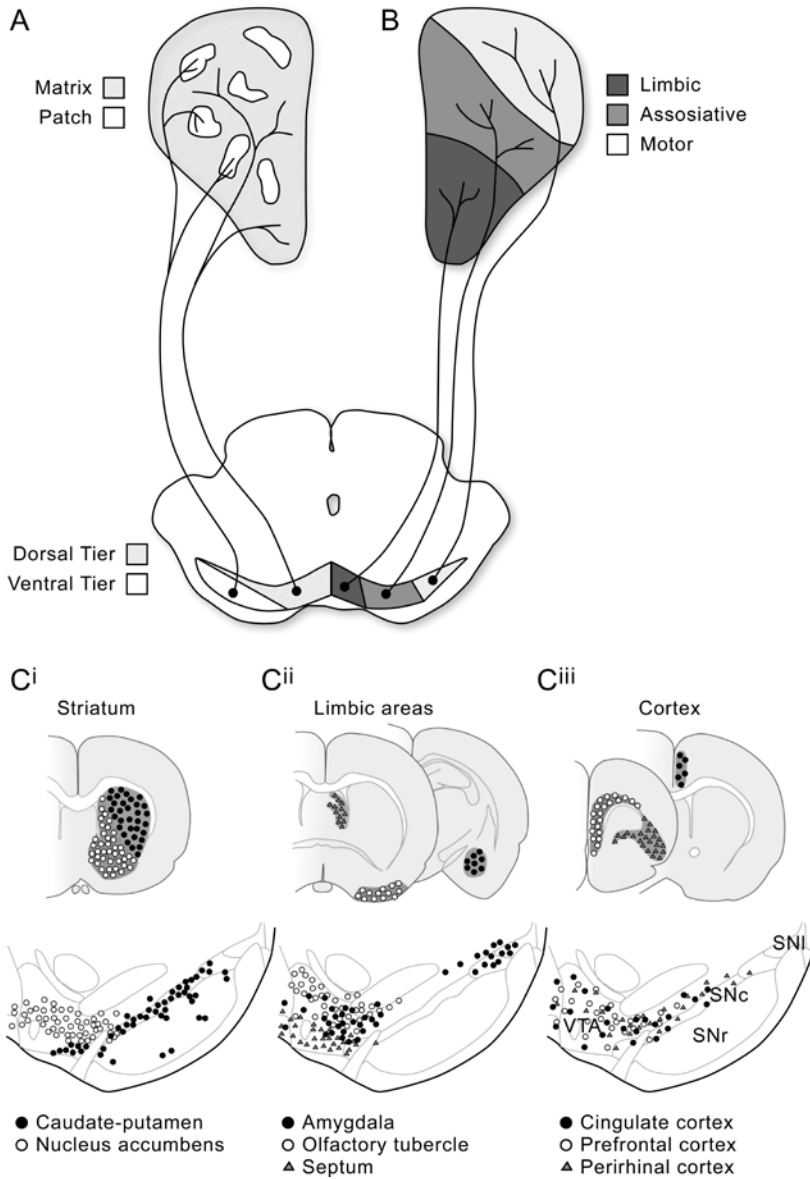


Figure 2. Overview of the functional neuroanatomy of the rodent mesencephalic dopaminergic neuron system. The striatal patch and matrix components are specifically innervated by the ventral and dorsal tier DAergic neurons respectively (A). The mesDA neurons innervating the limbic, associative and motor striatum are organised in a mediolateral fashion, respectively (B). The forebrain targets of the mesostriatal (i), mesolimbic (ii) and mesocortical (iii) pathways of the mesDA system (C).

Mesencephalic dopaminergic neuronal subtypes

So far our understanding of the mesDA neurons is based on their anatomical location, target of innervation and functional association, but can they be more easily identified? As highlighted by Dahlström and Fuxe, the anatomical dissociation of the nuclei cannot be clearly delineated in certain borders. Since there are clear differences in morphology and diameter, as discussed above, are there further phenotypic differences?

Gerfen and colleagues described that dorsal tier DAergic neurons selectively express the calcium-binding protein, Calbindin (Gerfen et al., 1985). This mirrors their innervation target, the striatal matrix that also expresses Calbindin. The Calbindin negative DAergic neurons innervate the patch compartment and comprise the ventral tier. In the mouse, the same expression pattern of Calbindin can be observed in the midbrain, with almost all of the VTA containing TH⁺/Calbindin⁺ neurons, this expression becomes absent when moving lateral into the SNc (Liang et al., 1996). Thus the medial SNc and VTA express Calbindin, suggesting a close functional relationship, despite anatomically segregated nuclei. Calbindin has been widely accepted as a marker for dorsal tier, A10 neurons of the mesolimbocortical pathway.

Gerfen also reported that the dorsal tier expresses relatively low levels of *Dat* mRNA when compared to the high-expressing ventral tier (Gerfen et al., 1985). This can be further expanded to differential functional isoforms of DAT. The DAT isoform present in the cell soma of mesDA neurons is a 50kDa (the non-glycosylated isoform) while the DAT predominantly found in axons and terminals is the 80kDa glycosylated isoform (Afonso-Oramas et al., 2009). DAT is found in similar levels in the dorsal and ventral SN, however the glycosylated (glyco-DAT) isoform is more prevalent in the ventral tier SNc. This was also true for the innervation target, the dorsal striatum, expressing higher levels of glyco-DAT when compared to ventral striatum. This was true across a number of species, including rats, monkeys and humans. Given this differential expression of glyco-DAT. Afonso-Omaras and colleagues also attributed a functional difference in dorsal and ventral striatal terminals. Preparations of synaptosomes revealed that the glyco-DAT expressing dorsal striatal terminals had a ³H-DA uptake efficiency (*V*_{max}) that was 23% higher than ventral striatum, despite a similar affinity. Thus ventral tier DAergic neurons selectively express glyco-DAT in their soma and terminals, and also have a higher DA-reuptake capacity, than dorsal tier DAergic neurons.

Glyco-DAT expressing neurons also express the G-protein-coupled inward rectifying potassium channel, GIRK2 (Di Salvio et al., 2010). GIRK2 was previously described as being more highly expressed in the SNc and dorsolateral VTA

and thus has been used to delineate the A9, ventral tier, nigrostriatal neurons (Chung et al., 2005; Inanobe et al., 1999; Mendez et al., 2005; Schein et al., 1998; Thompson et al., 2005).

The transcription factor OTX2 has been shown to be preferentially expressed in the VTA, with more than 65% of TH⁺ neurons co-expressing OTX2 (Chung et al., 2010; Di Salvio et al., 2009). All OTX2⁺/TH⁺ neurons were also shown to express Calbindin, the marker of dorsal tier, while OTX2 and glyco-DAT showed no overlap (Di Salvio et al., 2010). The majority of OTX2 expression was confined to ventral VTA, with a graded expression diminishing towards dorso-lateral VTA. Hence OTX2 can be used to delineate A10, dorsal tier, mesolimbocortical neurons.

A small subpopulation of OTX2⁺/GIRK2⁺ DAergic neurons in the dorsolateral VTA has also been described, suggesting an overlap between the nigrostriatal and mesolimbocortical pathways (Di Salvio et al., 2010). An elegant study performed by Di-Salvio and colleagues describes a potential mechanism regulating mesDA neuron subtype. Using conditional expression of OTX2-flox/GFP alleles in a *Dat-Cre* background, they were able to selectively delete OTX2 in DAergic neurons and trace the deletion using GFP. Conversely, they also generated an OTX2 over-expressing transgenic mouse. With a deletion of OTX2 expression in all DAergic neurons, no significant change in mesDA neuron number was observed; however GIRK2⁺/GFP⁺ and glyco-DAT⁺/GFP⁺ neurons were observed in the central VTA, suggesting a medial expansion of the A9 phenotype. This was independent of other transcription factors that are essential for mesDA survival and maintenance: PITX3, NURR1 and FOXA2, which will be discussed later. Over-expressing OTX2 in DAergic cells that do not normally express it in adulthood (A9 cell group) did not result in any changes in cell number or phenotype of the SNc. The only observation was an obliteration of GIRK2⁺/OTX2⁺ neurons in dorsolateral VTA. This suggests that OTX2 plays a role in suppressing a ventral tier, A9 phenotype only within the VTA.

Although these different studies describe disparate protein expression and anatomical location of the neurons, there has been little focus on how neuronal subtype directly relates to neuronal activity within the mesDA system. A comprehensive study was performed by Lammel and colleagues, investigating individual mesDA neurons on an electrophysiological, gene- and protein-expression and a histological level, following retrograde labelling from defined target structures (Lammel et al., 2008). Mesolimbocortical mesDA neurons, that innervate mPFC, basolateral amygdala or NAc core, cluster in medial VTA and all express Calbindin, and have an average cell diameter of 17.5µm. They express low *Dat/Th* and *Dat/Vmat2* mRNA ratios. Consistent with numerous previous, individual reports summarised above.

Mesolimbic and mesostriatal neurons that innervate the NAc lateral shell and dorsolateral striatum, respectively, are located both in the most medial aspect of the SNc, with lateral SNc neurons being selectively mesostriatal. They have on average a cell diameter of 21.5 μ m and 24 μ m respectively, much larger than their mesolimbocortical counterparts. 60% of the mesolimbic neurons expressed Calbindin, while none of the mesostriatal neurons did. There was also a higher ratio of *Dat/Th* and *Dat/Vmat2* than mesolimbocortical DAergic neurons, which was primarily due to the high levels of *Dat* mRNA. These SN neurons also fired action potentials at a frequency of 3–6Hz, although the mesolimbocortical neurons exhibit faster and more sustained action potential discharges than SN neurons, with the range of 10–15Hz. This study compiled previously described phenotypic differences and linked them with target–innervation with functional differences, providing a comprehensive overview of the system.

Origin of dopaminergic neurons

mesDA neurons are implicated in a number of neurological and psychiatric disorders. Therefore, understanding how they are formed, what regulates their specification and maintenance has the possibility to shed light on novel therapeutic strategies. As a consequence, the development of mesDA neurons is a topic that is of intense interest to the cell replacement therapy and neuroprotection fields. Here, I will provide an overview of some critical factors in mesDA neuron specification, differentiation and maintenance. I will also discuss what is currently known about mesDA subtype specification and comment on axonal extension and pathway formation.

DEVELOPMENT

The developing neural tube is comprised of three main anterior–posterior regions: forebrain, midbrain and hindbrain. The midbrain is the anatomical origin of mesDA neurons, and in this region there are two main signalling centres, or organisers, involved in the specification of mesDA neurons: they are the floor–plate and the isthmic organiser. The organising centres are primarily responsible for the secretion of proteins that extrinsically affect the induction, patterning and proliferation of the neural precursors within the mesDA region: SHH, WNT1, WNT5a, FGF8, TGF- α and - β (for overview see (Prakash and Wurst, 2006). Although, it is now understood that the floor–plate is not only an organiser, but is also the source of mesDA progenitors itself (Bonilla et al., 2008; Joksimovic et al., 2009; Kittappa et al., 2007; Ono et al., 2007). It is generally

Table 1. List of selected transcription factors and their roles in mesDA neuron specification and maintenance.

Transcription factor	Day of expression	Expression in adult	Role in mesDA development	Reference
En1/2	E9.5	Yes	Essential for specification as En2 null background results in a loss of progenitors. Essential for maintaining an isthmic organiser by influencing <i>Wnt1</i> , <i>Fgf8</i> and <i>Pax5</i> expression. Loss of a single allele of En1 results in progressive degeneration.	Simon et al., 2001 Liu et al., 2001 Sonnier et al., 2007
FoxA2	E8.5	Yes	Induces <i>Shh</i> , <i>Lmx1a/b</i> , <i>Ngn2</i> . Inhibits alternative fates by suppressing <i>Nkx2.2</i> . Regulates <i>Nurr1</i> and also controls survival in adulthood.	Ang et al., 1993 Kittappa et al., 2007 Ferri et al., 2007 Lin et al., 2009
Lmx1a	E9.0	Yes/ Decrease with age	Induces <i>Msx1</i> and <i>Ngn2</i> . Regulates <i>Wnt1</i> expression and cell cycle exit, thus loss of function results in less proliferation. Loss of both alleles results in a loss of PITX3 and NURR1 expression. Loss of <i>Lmx1a</i> and <i>Lmx1b</i> results in a loss of CORIN expression.	Andersson et al., 2006b Yan et al., 2011 Deng et al., 2011 Chung et al., 2009 Ono et al., 2007
Lmx1b	E8.0	Yes	Cooperates redundantly with <i>Lmx1a</i> . Not essential for LMX1A, FOXA2, TH or PITX3 expression.	Smidt et al., 2000 Yan et al., 2011 Deng et al., 2011
Msx1	E9.5	-	Induces <i>Ngn2</i> .	Andersson et al., 2006b
Ngn2	E10.5	No	Determines the number of postmitotic neurons, by suppressing <i>Sox2</i> and inducing <i>Nurr1</i> .	Andersson et al., 2006a Kele et al., 2006 Thompson et al., 2006
Nurr1	E10.5	Yes	Essential for the maintenance of neurons and also regulates the expression of <i>VMAT2</i> , <i>RET</i> , <i>DAT</i> and <i>TH</i> .	Wallen et al., 1999 Smits et al., 2003 Kadkhodaei et al., 2009 Saucedo-Cardenas et al., 1998
Otx2	E8.0	Yes, restricted to VTA	Defines <i>Wnt1</i> and <i>Fgf8</i> expression at isthmus. Induces <i>Ngn2</i> and <i>Mash1</i> , suppresses non-DA fates (<i>Nkx6.1</i> and <i>Nkx2.2</i>). Loss of expression results in loss of <i>Nurr1</i> , <i>Pitx3</i> and <i>TH</i> .	Brodski et al., 2003 Puelles et al., 2004 Prakash et al., 2006 Vernay et al., 2005
Pitx3	E11.5	Yes	Essential for the maintenance of A9, SNc neurons	Nunes et al., 2003 Hwang et al., 2003 Van der Munchhof et al., 2003

accepted that mesDA neurons are born around embryonic day 10–14 (E10–E14) in the mouse (Bayer et al., 1995). Some of the major intrinsic factors involved in mesDA specification and maintenance, and their critical functions are listed in Table 1.

One transcription factor of particular interest in this thesis is PITX3. In the CNS, PITX3 is selectively expressed in the TH⁺ neurons of the mesencephalon, during development and adulthood (Smidt et al., 1997). Making it a very attractive marker for identifying mesDA neurons. Loss of expression of *Pitx3*

does not affect the specification of mesDA progenitors, but when they become post-mitotic the SNc neurons fail to survive (Maxwell et al., 2005; Smidt et al., 2004; van den Munckhof et al., 2003). PITX3 also plays a role in potentiating the function of NURR1 in its role as “master regulator” of mesDA fate (Jacobs et al., 2009). Thus PITX3 is required for the maintenance of A9 neurons, but its precise role is still unknown.

SUBTYPE DEVELOPMENT

The intrinsic and extrinsic factors listed above impact on all mesDA neural precursors in the ventral mesencephalon (VM), thus there is currently no identifiable determinant of mesDA subtype. Similarly, there is no understanding into how mesDA neuron heterogeneity is acquired, despite a common origin, or how the precursors located in the medial aspect of the VM migrate to form the distinct and complex anatomical organisation of the SN and VTA.

A string of recent literature utilising genetic lineage fate mapping has outlined a possible spatiotemporal origin of mesDA subtypes, where it appears that birth-date and length of exposure to extrinsic signals determines subtype (Blaess et al., 2011; Brown et al., 2011; Hayes et al., 2011). All three studies have clearly demonstrated that mesDA progenitors are specified as early as E7.5, considerably earlier than previously believed (Bayer et al., 1995). The study by Brown and colleagues traced the *Wnt1*-lineage (as it is essential for mesDA neuron specification and is expressed at E8.0), and showed that it can contribute to the A8, A9 and A10 cell groups from E7.5 to E13.5. At all stages the *Wnt1*-lineage gave rise to TH⁺/Calbindin⁺ and TH⁺/GIRK2⁺ neurons, with no obvious bias (Brown et al., 2011). Thus progenitors expressing *Wnt1* contribute to all compartments and neuronal subtypes of the mesDA system.

Two studies investigated the contribution of the floor-plate and role of SHH signalling, its expression (*Shh-Cre* model) and responsiveness (*Gli1-Cre* model) in fate determination in the developing VM (Blaess et al., 2011; Hayes et al., 2011). Blaess and colleagues showed the peak contribution of the *Shh*-lineage and *Gli1*-lineage to the mesDA neuron compartment was progenitors labelled at E9.5 and E8.5, respectively. The peak of the *Shh*-lineage coincides with the highest number of *Shh*-expressing cells within the *Lmx1a*-expressing domain (Blaess et al., 2011). The *Gli1*-lineage begins to contribute to mesDA neurons one day earlier than the *Shh*-lineage, this earlier contribution being induced by SHH secreted from the notochord first, and then subsequent secretion from the floor-plate itself, *Shh*- and *Gli1*-lineages (Hynes et al., 1995).

Hayes and colleagues observed that cells of the *Shh*-lineage and *Gli1*-lineage contributed to the SNc first, with a peak at E9.5 and E8.5, respec-

tively. It was also reported that contribution to the VTA was later, with *Shh*-lineage exclusively contributing to VTA at E11.5 and the *Gli1*-lineage at E10.5. They also observed that progenitors destined to the VTA maintained an LMX1A⁺ progenitor-like state for longer, and become post-mitotic later than the SNc-bound mesDA neurons. Blaess and colleagues provided quantitative data showing that the first-born mesDA neurons form the SN, while the later phase comprise the VTA. This was also evident when analysing markers of mesDA subtypes. Labelling at E9.5 showed a larger contribution to GIRK2⁺/TH⁺ rather than to Calbindin⁺/TH⁺ neurons, where as this changed when labelling at E11.5, with a larger contribution to Calbindin⁺/TH⁺ neurons (Blaess et al., 2011). Again this demonstrates that A9, GIRK2⁺ neurons are born first and form the SN, while the A10, Calbindin⁺ neurons are born later, maintain a progenitor-like state for longer and migrate less laterally to form the VTA.

The day difference in total contribution outlined above is also evident in different nuclei, hence defining a temporal difference in mesDA neuron distribution to distinct anatomical structures. The authors also observed a tendency for the earlier progenitors (peak at E11.5) to give rise to more anterior mesDA neurons and the later (peak at E12.5) contributing to posterior. These analyses are consistent, with the early observations of Bayer using ³H-thymidine, where it was stated that the early-generated neurons are predominantly located the SN and the more anterior midbrain, whereas the later-generated neurons are mainly in the VTA and posterior midbrain (Bayer et al., 1995).

It should be noted that *Shh*- and *Gli1*-lineages also give rise to non-DAergic neurons within the midbrain: the *Shh*-lineage contributes to the red nucleus, and the *Gli1*-lineage also contributes to the oculomotor nucleus (Blaess et al., 2011). Both lineages also give rise to astrocytes (Blaess et al., 2011). Although from the studies discussed above indicate there is a clear spatiotemporal influence on the positional diversity of mesDA neurons, it is not completely mutually exclusive. Thus there must be further factors at play in the determination of the anatomical locale, and subtype specification of mesDA neurons.

FORMATION OF PATHWAYS

Following mesDA neurogenesis, newly born neurons begin to extend neurites and specify axons that project in a rostral direction. In the case of nigrostriatal pathway formation, guidance cues and intrinsic signalling mechanisms influence specified axons to fasciculate, giving rise to the medial forebrain bundle (MFB). Across development, these axons course ventrally towards their respective forebrain targets before innervating their target fields. This process begins

at E11.5 in mouse and DAergic axons begin to innervate the striatum between E15.5–E16 (Hu et al., 2004; Kolk et al., 2009). Although the ontogeny of mesDA projection systems has been characterised through elegant anatomical tracing studies, the molecular basis of nigrostriatal pathway formation is rather poorly understood in contrast to our knowledge of pre-mitotic mesDA neuronal development. Recently, a number of classical axon-guidance molecules and receptors have been implicated in the orientation and outgrowth of mesDA axons including, semaphorins, slits, netrins and ephrins (Van den Heuvel and Pasterkamp, 2008). Additionally, morphogens and growth factors have also been implicated in mesDA pathway formation (WNT5A and SHH). For an extensive review on neurite outgrowth and pathway formation, I refer the reader to a review by (Van den Heuvel and Pasterkamp, 2008).

SUBTYPE CONCLUSION

One thing that is highlighted in the summary above is that despite intense research into the diverse anatomical, functional and phenotypic aspects of mesDA heterogeneity, little is known about how these distinct subclasses establish their individual identities during development, or indeed how this process may be regulated.

The neurons of the dorsal and ventral tiers lie in neighbouring anatomical locale and using TH-immunohistochemistry alone, are almost undistinguishable. Proteins that mark subsets of mesDA neurons further confound the complexity, as no marker alone is absolutely exclusive to nigrostriatal or mesolimbocortical pathways. This further highlights the comments of Dahlström and Fuxe in the original definitions of midbrain DA cell groups with respect to the difficulty of clearly delineating A8, A9 and A10. Using sophisticated projection analysis, it was also found that neither dorsal nor ventral tier SN neurons exclusively innervate striatal patch or matrix, and on average 5% of a single neuron's axonal field innervates the opposite target (Matsuda et al., 2009). Thus single mesDA neurons can synapse with both patch and matrix, albeit to different extents. There is some evidence from retrograde tracing studies during development that the mesostriatal and mesolimbic pathways are not predetermined. At E15, 63% of all mesDA neurons innervated both dorsolateral and ventromedial striatum. This co-labelling declined overtime, and by birth (postnatal day, P0) no double-labelled neurons were observed (Hu et al., 2004). This data on pathway formation suggests that mesDA neurons extend axons in a non-specific manner at first, and that axonal pruning defines mesostriatal or mesolimbic identity. Hence implicating that mesDA neuronal subtype is something that is acquired,

not predetermined. Other observations such as the uniform expression of OTX2 in mesDA neurons during development, and its further restriction to dorsal tier mesDA neurons from E15.5 into adulthood support this theory (Chung et al., 2010; Di Salvio et al., 2009). The markers GIRK2 and Calbindin are widespread in the developing VM, and only become predominantly refined to subtypes of mesDA neurons from P18 onwards (Andersson et al., 2006a). Thus is subtype something that is acquired solely after target innervation? Or is it predetermined at the cellular level and these neurons are specified to respond to different signals and innervate specific structures?

From the developmental studies summarised above, the only factor determining mesDA subtype is birth-date. It is worth noting that neurons born on E9.5 (*Shh*-lineage) contribute mostly to the SNc, at the time of labelling the majority of progenitors were within the *Lmx1a/Msx1/Foxa2/Corin*-expressing domain, the most medial aspect of the midline (Blaess et al., 2011). Other studies which sorted CORIN⁺ cells from the developing VM (E10.5), thus isolating the most medial progenitor population, observed that after transplantation these cells gave rise to both A9 and A10 neurons while the lateral population (CORIN-low fraction) gave rise only to TH⁺/GIRK2⁺ A9 neurons (Jonsson et al., 2009). This suggests that the first born A9-destined progenitors have migrated laterally already at E10.5, while the ones born later are fated to give rise to both A9 and A10. Perhaps more functional studies on isolating these temporal progenitors for molecular analysis may result in the identification of determinants of mesDA neuronal subtype.

Another previously underestimated characteristic of SN DAergic neurons is that a single neuron can innervate 2.7% of the total striatal volume, and can potentially synapse with up-to an estimated 75,000 medium spiny neurons in striatum (Matsuda et al., 2009). This elaborate neuronal network from a single neuron, is almost unfathomable, and further adds to the mystique of the mesDA system. This may also shed some insight into the infamous compensatory nature of the mesDA system and why patients who are diagnosed clinically with Parkinson's disease (PD), have been undergoing the disease process for several years with little or no manifestation of motor symptoms.

Parkinson's disease

Following its initial description in 1817, the "shaking palsy" as it was first called remained somewhat of a mystery for over 150 years (Parkinson, 2002). It is diagnosed primarily based on motor symptoms, including: akinesia (loss of voluntary movement), bradykinesia (slowness of voluntary movement), rigidity, postural imbalance and resting tremor (uncontrolled involuntary quiver-

ing). There is no known aetiology of the disease, since it is idiopathic, with the only strong predictor being age. For an excellent and extensive review on PD, its symptomatology and pathophysiology I shall refer the reader to (Foltynie et al., 2002).

Around the 1960's a number of studies began to shed some light on what was responsible for motor control within the CNS. The Nobel Prize-winning work of Arvid Carlsson showed that when animals were treated with drugs that resulted in a depletion of catecholamines, they suffered from severe akinesia. This was due to DA depletion and could be reversed only by supplementing with exogenous DA, and was postulated to be the effect of DA within the corpus striatum (Carlsson, 1959; Carlsson et al., 1957). There was evidence for cell loss within the midbrain of PD patients, but they could not identify what neuronal cell type was missing (Greenfield and Bosanquet, 1953). Discoveries that PD patients had diminished levels of DA within the striatum and SN, further confirmed the causal link between DA within the nigrostriatal pathway and PD (Bernheimer et al., 1973; Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1963). The observation that the DA precursor, L-DOPA, could alleviate Parkinsonian symptoms further strengthened the role that DAergic neurons played a key role in PD (Cotzias et al., 1967).

NEURONAL SUBTYPE VULNERABILITY IN PARKINSON'S DISEASE

Since there are such diverse functions, target innervation and protein expression of mesDA neurons are there differences in what cells are part of the disease process? One of the first insights into selective vulnerability was the observation that melanised DAergic neurons were lost during PD (Hirsch et al., 1988). The melanised DAergic neurons are primarily contained in the SNc, and the loss of TH⁺ neurons in this area was 77% in PD cases as compared to controls. Hence SNc neurons seem to be more sensitive to the disease process than VTA neurons. This is also mirrored in the finding that Calbindin⁻ regions of the SNc (the ventral tier) are more vulnerable to PD, while Calbindin⁺ neurons are resistant to the disease process (Damier et al., 1999; Ito et al., 1992). Building on this, it is also reported that OTX2⁺ neurons, which highly co-express Calbindin, are more resistant to disease process and also to neurotoxic insults in rodent models of PD (Di Salvio et al., 2010). Deleting OTX2 in all DAergic neurons increases vulnerability to the neurotoxin MPTP, while conversely over-expressing it in all DAergic neurons confers protection. This finding also correlates with the putative regulation of glyco-DAT production by OTX2, since glyco-DAT⁺ DAergic neurons are more sensitive to neurotoxin lesion in rodents and monkey, but are

also the population lost in PD patients (Afonso–Oramas et al., 2009; Di Salvio et al., 2010). Studies on aphakia mice (Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003), a naturally occurring PITX3 mutant, and also PITX3–GFP knock–in transgenic mice (Maxwell et al., 2005; Zhao et al., 2004) reveal that loss of the transcription factor PITX3 in all mesDA neurons, results in a selective loss of DAergic neurons in the SN. Further analysis reveals that it is primarily a loss of A9 GIRK2⁺/TH⁺ DAergic neurons (Grealish et al., 2010). Despite the lack of PITX3 expression, most of the A10 Calbindin⁺/TH⁺ neurons survive, again revealing an inherent vulnerability of the A9 neurons. Thus the SNc neurons that are more vulnerable to the PD disease process and also neurotoxins modelling PD, have a reduced capacity to buffer intracellular Ca²⁺ and also have more active DA re–uptake mechanisms, potentially leading to increased generation of reactive oxygen species and thus cell stress (for review see Chan et al., 2010).

CELL REPLACEMENT THERAPY FOR PARKINSON'S DISEASE

The first studies showing that developing neural tissue could be placed into the adult CNS, survive and innervate (Bjorklund et al., 1976; Stenevi et al., 1976), prompted further research into the mesDA neuron system with the first examples of VM transplantation and functional effects reported by two groups independently (Bjorklund and Stenevi, 1979; Perlow et al., 1979). Not long after the first clinical trials of fetal VM being transplanted into Parkinsonian patients were performed in Sweden (Lindvall et al., 1990; Lindvall et al., 1988).

From the very first trials it was clear that transplantation of fetal VM into the striatum (more specifically the putamen) of PD patients provided an increased efficacy of L–DOPA (decreased off–state), less rigidity and an increase in ¹⁸F–DOPA uptake in the transplanted putamen (Lindvall et al., 1990). These observations were maintained in further open–labelled trials (Hagell et al., 1999; Li et al., 2008; Piccini et al., 1999; Piccini et al., 2000). However two sham–surgery placebo controlled, double–blind trials performed in the US showed limited improvements in motor function rating scales, despite PET–imaging indicating graft function (Freed et al., 2001; Olanow et al., 2003). The US trials also reported the appearance of severe side–effects, termed graft–induced dyskinesias, in 10–56% of patients transplanted depending on the trial (Ma et al., 2002; Olanow et al., 2003). This had not been previously reported in the European trials, but the US data prompted the re–examining of patients and consequently graft–induced dyskinesias were also observed in patients of the European trials (Hagell et al., 2002).

Although there was a number of procedural differences attributed to the conflicting results from the European and American trials (they will not be discussed here and thus I refer the reader to two extensive reviews see (Lane et al., 2010; Winkler et al., 2005), it can be agreed that if the primary outcome measures and length of observation of the American trials were slightly modified then positive data supporting the functionality of transplantation in PD patients could have been obtained (Freed et al., 2004; Ma et al., 2010).

Transplants can survive in patients for at least 14 years and still provide efficient functional benefit (Kordower et al., 2008; Li et al., 2008; Mendez et al., 2008). It has also been noted that the subtypes of mesDA neurons observed *in situ* in the adult midbrain are present within transplants with a distinct ring of A9, GIRK2⁺, neurons at the edge of the grafts and the A10, Calbindin⁺, neurons in the core (Mendez et al., 2005). This observation was also reproduced in rodent models (Thompson et al., 2005) where it was observed that despite the cells' ectopic location, the A9 neurons showed target specificity for the striatum while the A10 neurons projected to the prefrontal cortex.

ALTERNATIVE CELL SOURCES

A consistent finding in fetal tissue transplants, irrespective of trial centre, is that the response of patients is highly variable. With more than 300 patients transplanted worldwide (Lindvall and Kokaia, 2009), there is still no conclusive answer to the cause or contributing factors to the success or failure of clinical efficacy of transplantation in PD patients.

This inherent variability is compounded by having an inconsistent tissue source, electively aborted fetal tissue. It also becomes unfeasible to perform transplants in a large number of patients, and unrealistic to expect this to become a more widely used therapy. Thus pluripotent stem cells, specifically human embryonic stem cells (hESCs), become a very attractive alternative, although not without their own significant limitations (for review see (Brundin et al., 2010). With the potential to generate any cell type within the human body, the main goal of this field is to specify hESCs towards a defined fate, without contamination of other cell types, particularly undifferentiated cells. There is a large drive within the cell replacement therapy field in PD, to generate bona fide mesDA neurons for transplantation. By understanding what intrinsic and extrinsic factors are involved in mesDA development, this can allow us to generate the desired cell fate *in vitro*.

Despite what cell source is used, it is imperative to address some unresolved issues in preclinical studies in order to allow us to better predict positive outcome after transplantation. Especially with the aim of transplanting cells derived

from pluripotent stem cells, where the cell source has the potential to produce any somatic cell type in the body. We need to identify what critical characteristics a cell preparation needs to possess in order to survive in the adult brain, acquire the correct mesDA phenotype, provide appropriate innervation to the striatum, release dopamine in a physiological manner and also elicit relevant behavioural outcomes.

AIMS

AIMS OF THIS THESIS

The overall goal of this thesis is to provide new insights in to what factors mediate functional recovery after transplantation in rodent models of Parkinson's disease. The aims achieved here provide several considerations when attempting to generate mesDA neurons for transplantation from embryonic stem cells, and translate rodent transplantation studies to the clinical setting. To this end a number of specific aims were addressed:

- 1) To provide a better preclinical mouse model of Parkinson's disease. With the ultimate goal of contributing a solid platform for studies utilising transgenic mouse tissue or mouse embryonic stem cells as a cell source for transplantation.
- 2) To readdress the growth capacity of primary fetal mesDA neurons transplanted in a homotopic location into the adult substantia nigra of a rodent model of Parkinson's disease.
- 3) To investigate what subtype of mesDA neuron may be more efficient at providing functional recovery upon transplantation.
- 4) To assess the efficacy of human embryonic stem cell-derived mesDA precursors to differentiate into the correct phenotype and integrate in the adult rodent brain, with a direct comparison with primary human fetal tissue.

RESULTS AND COMMENTS

RESULTS AND COMMENTS

Paper №1

The use of mice as a preclinical model for PD has primarily been restricted to bilateral MPTP lesions. A number of disadvantages are associated with mice including spontaneous recovery, and limited behavioural analyses (for review see (Sedelis et al., 2000). Additionally, the unilateral 6-OHDA mouse model was not met with much success or favour in the past, and the primary cause of this was most likely due to a lack of standardisation of the lesion paradigms and behavioural analyses compared to more established rat models. In 2004 a study establishing a dyskinesia model in mice was published, describing two different lesion paradigms; MFB or intrastriatal lesion (Lundblad et al., 2004). However this was met with significant difficulties such as a high mortality rate (82%), namely with the MFB lesion, or variable lesion success. An earlier study (Parish et al., 2001) had an alternative but promising lesion paradigm (intranigral toxin injection) although the study was focussed on the effects of DA-receptor knockout on axonal terminals and thus did not provide a detailed characterisation of the lesion and the resultant deficits.

Thus, the principal aim of Paper №1 was to provide an extensive characterisation of the histological and behavioural impairments following a 6-OHDA lesion in mice. We met the same challenges in survival as previous studies did with MFB lesions, and thus decided to focus on the intranigral lesion paradigm, since >95% of mice survive this lesion without the need for special post-operative care.

Following an intranigral 6-OHDA lesion, mice displayed a range of DAergic denervation of the striatum from 33–92% lost compared to the intact side (Fig. 3A). This paper also describes a new behavioural task that had never before been applied to mice, the corridor task. This task showed a high correlation between deficits and striatal denervation (Fig. 3B). This new task proved to be equally as indicative of mesDA neuron degeneration as the “classical” tests used, such as drug-induced rotation. A factor in the utilisation of an animal model is being able to predict the extent of damage to the mesDA system in a living animal, using behavioural tests, in order to examine the effects of novel neuro-reparative or neuroprotective strategies.

The intranigral 6-OHDA lesion produces varying extents of damage to the nigrostriatal projections, as mentioned above, but can these be delineated using behavioural tests rather than post-mortem analyses? Figure 4 demon-

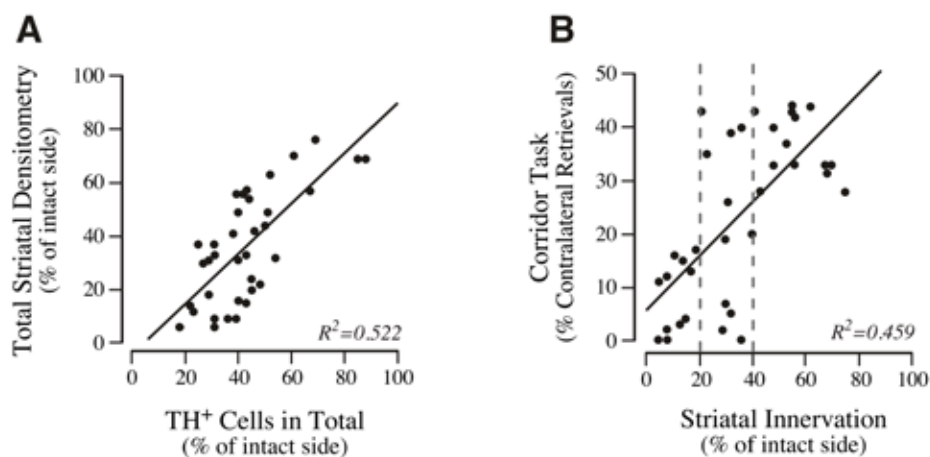


Figure 3. Validation and sensitivity of a novel behavioural test in mice. The intranigral 6-OHDA lesion induces a range of cell loss in the midbrain. There is also a direct relationship between DAergic cell and terminal loss in mice (A). The newly described corridor task is capable of measuring deficits across a wide range of striatal denervation (B). (Modified from Paper №1).

strates that almost all tests used in this study can distinguish lesioned mice from intact mice, but it is only the corridor test and apomorphine-induced rotation that can stratify mice based on the severity of lesion (Fig. 4A, B). Therefore we proposed a set of criteria that could be used to select severely lesioned mice only through behavioural analysis. The overall aim of this work is to provide a platform for further studies using transgenic mice as hosts, such as dyskinesia studies, or transplantation studies using alternative cell sources from mice (Caiazzo et al., 2011; Friling et al., 2009; Hedlund et al., 2008; Jonsson et al., 2009; Kim et al., 2011).

Since the publication of this study, other groups have since utilised the corridor task and intranigral lesions in mice with success. An extensive comparison between all three lesion paradigms in mice (MFB, intrastriatal and intranigral), and also a larger number of behavioural tests were performed (Heuer, 2012; Smith et al., 2012). Here the authors described that all three lesion paradigms produced a similar average deficit in the corridor test (~20%), and that the intranigral 6-OHDA lesion model was efficient for the study of L-DOPA-induced dyskinesias. It was also reported that the corridor task proved to be one of the most sensitive tests for predicting DAergic neuronal cell loss (Heuer, 2012), with particular utility due to the fact that it is non-pharmacological.

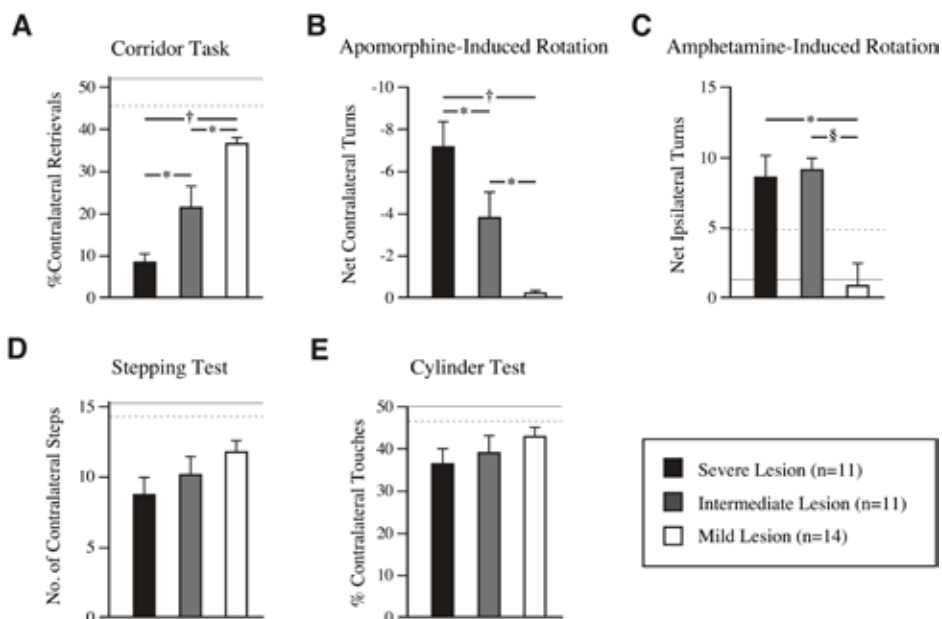


Figure 4. Validation and sensitivity of a novel behavioural test in mice. The corridor (A) and apomorphine-induced rotations (B) are capable of identifying mice with various extents of lesion, while other tests, such as amphetamine-induced rotation (C), stepping (D) and cylinder tests (E) can merely distinguish lesioned mice from intact mice, regardless of severity. The solid line represents performance of intact mice, while the dashed line represents S.E.M. (Modified from Paper №1).

Paper №2

The first attempts of transplanting fetal VM tissue into the adult rodent brain involved placing the tissue piece next to its primary target, the striatum, by either placing it in the overlying cortex (Bjorklund and Stenevi, 1979) or medial in the lateral ventricle (Perlow et al., 1979). Following this, the first study utilising grafts placed directly into the target structure (i.e. using cell suspensions), quickly followed and this has since become the standard approach (Bjorklund et al., 1980). The concept of placing the cells in a homotopic location (directly into the SNc) failed to yield favourable results, with the only evidence of DAergic axonal extension into the neighbouring SNr, and nothing observed in the stri-

tum (Nikkhah et al., 1994a). Additional studies attempted different approaches, such as bridge-grafts (Mendez et al., 1996; Mukhida et al., 2001; Wang et al., 1996; Zhou et al., 1996), but none of these were met with much success. A study by Bentlage and colleagues, investigated the possibility of the adult brain being less permissive for growth of new axons over long distances (Bentlage et al., 1999). They only observed DAergic fibres reaching the striatum if rat pups were transplanted intranigrally before 10 days of age, and none if they were transplanted later or as adults. Studies using a xenogenic approach demonstrated that transplanted human fetal tissue, detected using a species-specific neurofilament antibody, could extend axons that reached striatum (Wictorin et al., 1992). It was postulated that the non-permissive environment of the adult brain was circumvented in a xenograft paradigm. This was further confirmed with other species and cell types (Isacson et al., 1995; Wictorin et al., 1990). However the question remained if allografts of rodent tissue could extend axons across long distances, in a directed manner towards specific target structures in the adult brain.

One of the factors common to all previous intranigral allograft studies is the difficulty to differentiate between host and grafted neurons. Thus the question of any observed axons seen being truly graft-derived has always been an issue. In Paper №2 we set out to readdress if intranigral VM transplants can extend axons across long distances, to appropriate targets in the adult mouse brain. To this end, we utilised a transgenic reporter mouse that expressed GFP under the TH promoter (Sawamoto et al., 2001) in order to clearly visualise graft-derived DAergic neurons. In order to place discreet grafts within the mouse SN, we used a microtransplantation technique (Nikkhah et al., 1994b). We observed that only grafts located within the SNc extended GFP⁺ axons in a polar fashion towards the forebrain and terminated primarily in the striatum (Fig. 5a). We also observed innervation of other A9 and A10 target structures, such as amygdala, septum, NAc and frontal cortex. This occurred more readily in a 6-OHDA lesioned host, thus implicating a requirement for new, replacement DAergic innervation.

Close observation of axonal morphology showed that before reaching their target structure, the DAergic axons remained as thick fascicles and upon reaching the striatum formed a fine network of varicosities (Fig. 5B, C). This was clearly observed at the border between the globus pallidus and striatum (Fig. 6B). Thus newly transplanted neurons could follow a program, either intrinsically or extrinsically determined, that allowed them to innervate a target structure and terminate appropriately. When using retrograde tracing to prove bona fide graft-derived innervation of striatum, it was noted that the majority of labelled transplanted neurons were GIRK2⁺/GFP⁺ neurons of the A9 subtype.

Although GFP⁺ fibres could be readily observed in striatum, the level of innervation was not particularly high and we sought to boost this by utilising a

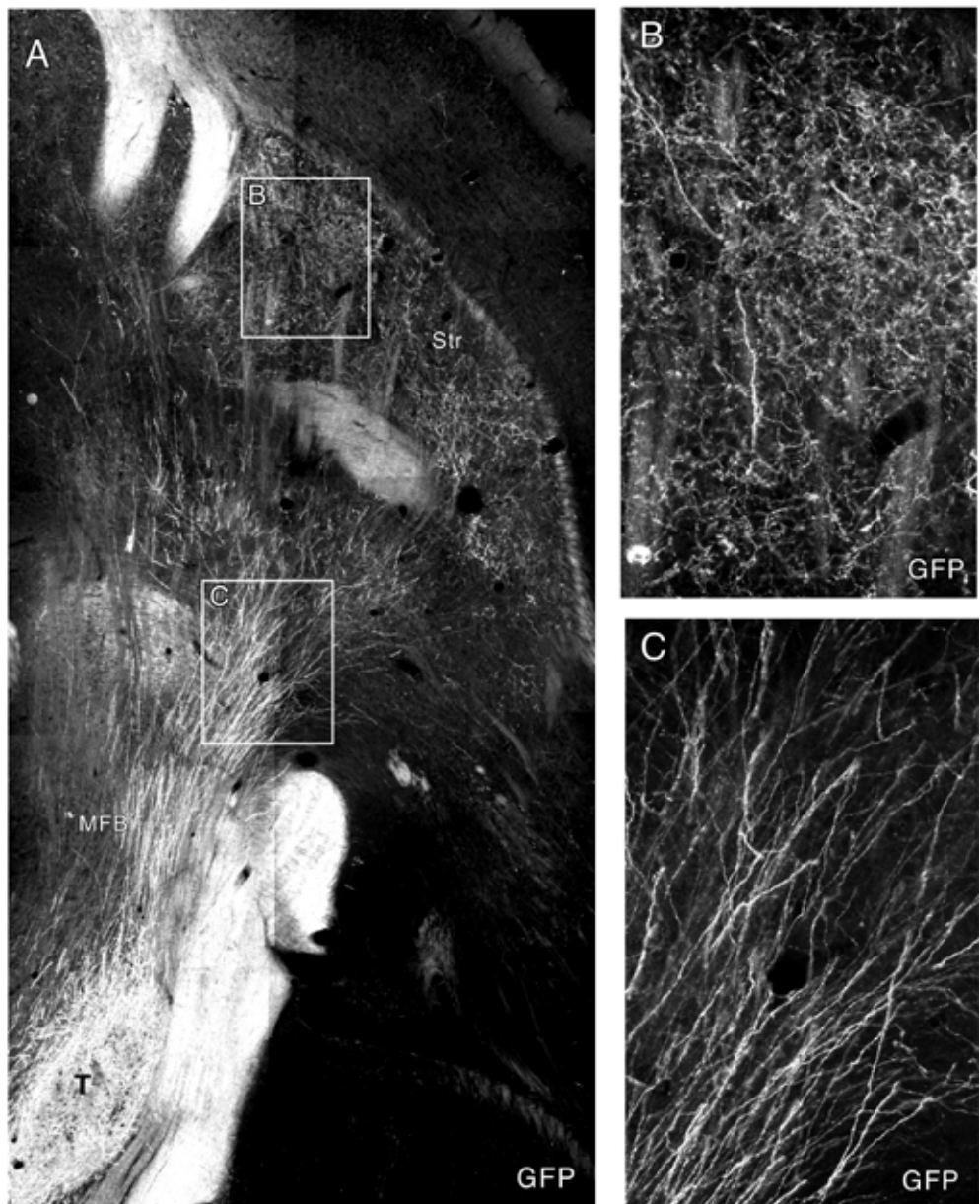


Figure 5. GFP⁺ VM neurons transplanted in the substantia nigra appropriately innervate forebrain structures. A horizontal section imaged in darkfield showing the GFP⁺ DAergic neurons transplanted in the SNc (A), extend axons along the medial forebrain bundle (C) towards their main target structure, the striatum (B), where they terminate. MFB=medial forebrain bundle, Str=striatum, T=transplant. (Modified from Paper №2).

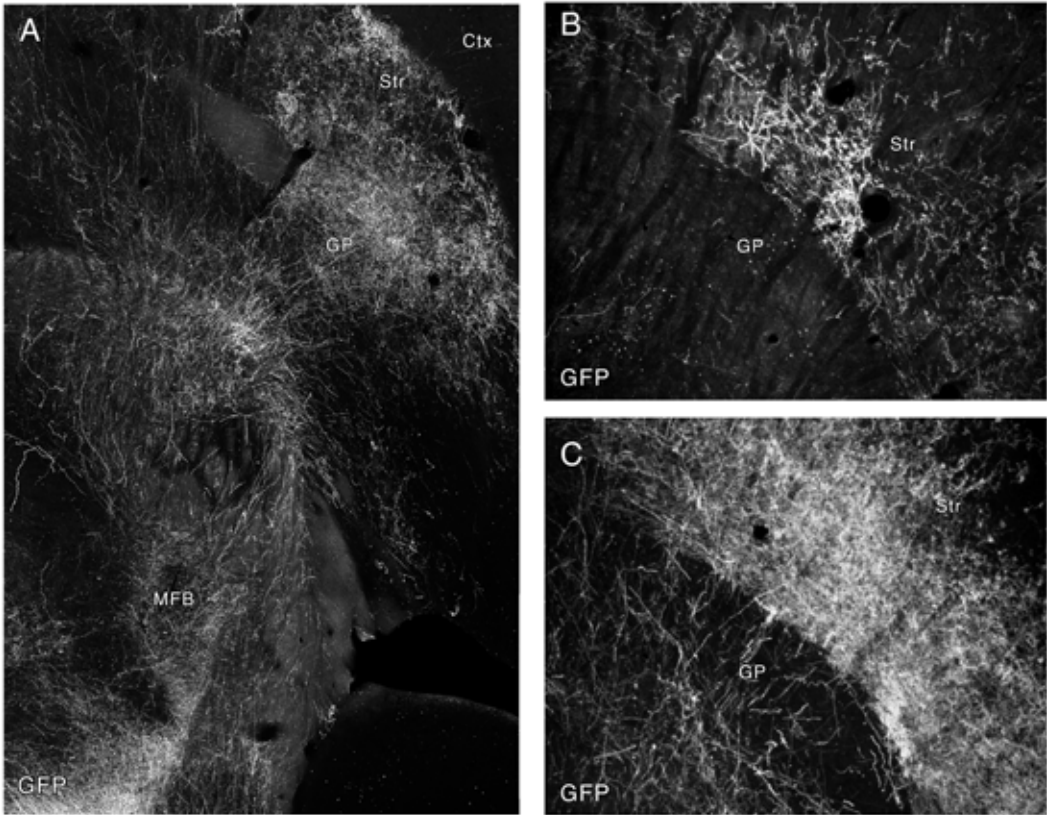


Figure 6. The effect of over-expression of GDNF in the striatum on axonal outgrowth of transplanted dopamine neurons. A horizontal section from an animal that received an intranigral graft, plus intrastriatal AAV-GDNF, displays significant DAergic axonal outgrowth (A). The striato-pallidal border of an animal with intranigral graft only (B), compared with an animal that also received AAV-GDNF in the striatum (C). Ctx=cortex, GP=globus pallidus, MFB=medial forebrain bundle, Str= striatum, T=transplant. (Modified from Paper №2).

well known neurotrophic factor for DAergic neurons, glial cell-derived neurotrophic factor (GDNF). We over-expressed GDNF using an AAV vector injected into the striatum, with the hope to elicit a more potent chemoattractive signal within the target forebrain structure. When this was performed concomitantly with intranigral transplantation, a noteworthy increase in both cell survival and axonal outgrowth was observed after 16 weeks (Fig. 6A). This can be more readily appreciated with a juxtaposition of examples with (Fig. 6C) and without (Fig. 6B) GDNF over-expression. These animals also exhibited a more significant reduction in amphetamine-induced rotations than animals that received an intranigral transplant alone.

In Paper №2 we conclusively showed that VM grafted into the adult SNc has the potential to innervate the striatum and other target structures over 6mm away, and also provide functional improvement. These observations were independently replicated by another group using a similar approach (Gaillard et al., 2009). They also noted that developing olfactory bulb TH⁺ neurons were incapable of extending axons in a similar fashion, thus this effect is specific for mesDA neurons.

Paper №3

It has previously been described that mesDA neurons rather than any DAergic or catecholaminergic neuronal type, are the most efficient at providing extensive reinnervation of striatum and significant functional benefits (Hudson et al., 1994; Zuddas et al., 1991). The heterogeneity of the mesDA system, as described in the previous section, is something that is maintained after transplantation. The transplanted developing VM tissue differentiates in the adult striatum and generates the subtypes that are present in the adult midbrain, namely the A9 and A10 subtypes as detected by the expression of GIRK2, Calbindin, Raldh1 and Cholecystokinin within the transplants (Haque et al., 1997; Mendez et al., 2005; Schultzberg et al., 1984; Thompson et al., 2005; Tornqvist et al., 2001).

As well as expression markers defining the subtypes, the specificity of target innervation is also maintained, despite the ectopic location of the cells. Tornqvist and colleagues noted that Calbindin⁺/TH⁺ neurons of VM grafts never co-labelled with Fluorogold injected into the host striatum (Tornqvist et al., 2001). This finding was further supported by the elegant study of Thompson and colleagues where retrograde tracers were injected into the dorsolateral striatum or frontal cortex of the transplanted hemisphere (Thompson et al., 2005). Only A9, GIRK2⁺/TH⁺ neurons co-labelled with tracer injected to the dorsolateral striatum, while the A10, Calbindin⁺/TH⁺ neurons innervated the frontal cortex, substantiating the findings of Tornqvist et al. It was also observed that the A9 neurons were typically located in the periphery of the graft, while the A10 neurons were contained in the graft core (Thompson et al., 2005).

Although both subtypes of mesDA are present in VM transplants, do they have a differential role? Something that has long been questioned is if the A9 neurons that die during PD progression are the neurons most needed for functional recovery after transplantation. In Paper №3 the primary aim of the study was to assess if A9 neurons are required for functional recovery after transplantation in a rat model of PD.

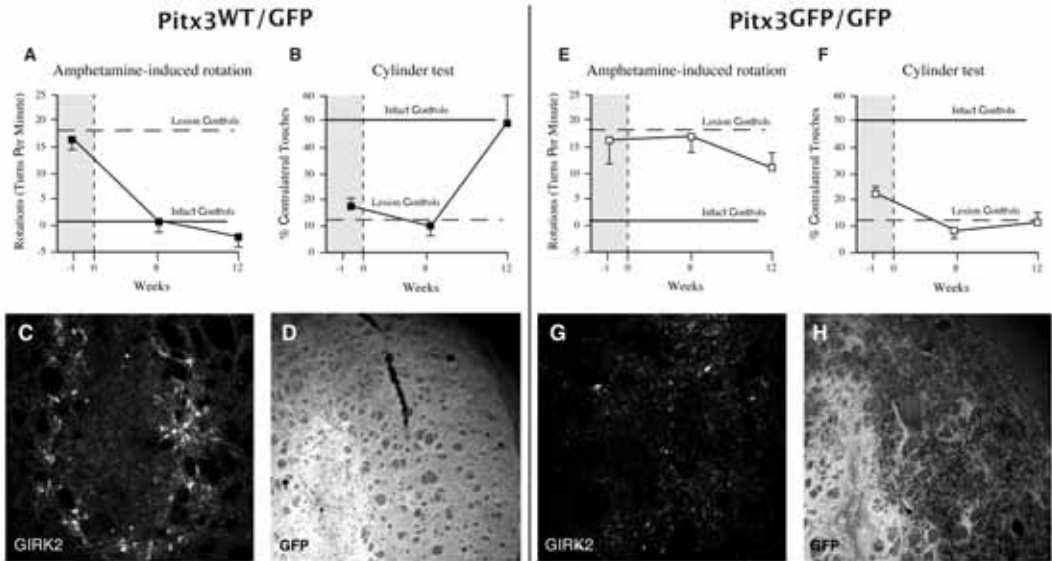


Figure 7. An overview of grafts derived from both genotypes of the Pitx3–GFP mice. Rats grafted with Pitx3^{GFP/WT} tissue showed a normalisation of amphetamine-induced rotations (A) and forelimb use (B) while the animals that received Pitx3^{GFP/GFP} tissue showed no recovery (E,F). Analysis of the grafts post-mortem revealed that the Pitx3^{GFP/WT} grafts contained show a normal complement of GIRK2⁺ A9 neurons (C) while the Pitx3^{GFP/GFP} grafts had a distinct lack (G). When analysing graft-derived innervation using GFP as a surrogate marker, only grafts that contained A9 neurons (D) provided dense innervation, while the Pitx3^{GFP/GFP} grafts did not (H), reflecting the groups behavioural performance. (Modified from Paper №3).

To this end we utilised a mouse model of PD, where there is a spontaneous loss of A9 neurons due to the absence of both alleles of the transcription factor PITX3, after being replaced by GFP (Maxwell et al., 2005). Thus we transplanted cells of the developing VM from the homozygous embryos, Pitx3^{GFP/GFP} that should yield grafts lacking A9 neurons, and the heterozygous control littermates, Pitx3^{GFP/WT}, that would represent the standard heterogeneous graft with a mixture of A9 and A10.

The rats that received a transplant of Pitx3^{GFP/GFP} tissue, showed no functional recovery as assessed by the amphetamine-induced rotation (Fig. 7E) and cylinder tests (Fig. 7F). Conversely, the standard Pitx3^{GFP/WT} grafted animals returned to normal (Fig. 7A, B). Both transplanted groups had similar numbers of surviving TH⁺ neurons, but when analysing the grafts for subtypes of mesDA neurons, we observed that the Pitx3^{GFP/WT} grafts had 65% GIRK2⁺ neurons and 26% Calbindin⁺, while the Pitx3^{GFP/GFP} had 15% GIRK2⁺ and 68% Calbindin⁺ (GIRK2 is depicted alone in Fig. 7C, G). Thus, there was a complete shift in the proportion of A9/

A10 neurons present in the grafts; and the Pitx3^{GFP/GFP} transplants contained a majority of A10 neurons. When assessing the graft-derived innervation in the dorsolateral striatum (the primary target of A9 neurons), the Pitx3^{GFP/GFP} grafts exhibited a distinct failure to innervate this area (Fig. 7H), while the Pitx3^{GFP/WT} grafts provided extensive innervation (Fig. 7D). As a consequence, the Pitx3^{GFP/WT} grafts were able to normalise expression of c-Fos, indicating functional innervation with the striatum, while the Pitx3^{GFP/GFP} grafts did not.

This body of work demonstrated that after transplantation A9 neurons are the population that reinnervate dorsolateral striatum, normalise medium spiny neuron activity and as a consequence, provide recovery in motor behaviours. The mere presence of DAergic neurons was not enough to provide functional benefit, as previously described using hypothalamic DAergic neurons (Haque et al., 1997; Zuddas et al., 1991), although in the current study the DAergic neurons were mesDA neurons, but of the A10 subtype. These neurons clearly displayed an inability to provide release of DA to induce a reduction in amphetamine-induced rotation, or normalisation of c-Fos expression, due to their lack of innervation of striatum (Thompson et al., 2005; Tornqvist et al., 2001). This study not only highlights the importance of mesDA neuron subtype in graft-derived functional recovery, but also that reinnervation of relevant structures is what underlies functional benefit.

This study has implications for the understanding of the mechanisms underlying functional recovery after transplantation, and also impacts on the field of generating DAergic neurons from alternative cell sources. From primary fetal tissue studies it is evident that any type of DAergic neuron is not suitable, and that the cells used for grafting in PD must be of a mesDA fate. It is also clear that A9 neurons are more efficient at mediating graft-induced recovery. Therefore *in vitro* protocols for the generation of mesDA neurons from pluripotent cells should be aimed at specifying a subtype of mesDA neurons. Given that we do not know how mesDA subtype is defined during development, this is a very difficult task. We also do not know if A10 neurons are obsolete or if they play a role in supporting A9 neurons, or even mediating non-motor effects of the graft. Therefore relevant differentiation protocols for pluripotent cells that would be interesting for the field of cell replacement therapy in PD should have the potential to form A9 mesDA neurons.

Paper №4

The generation of mesDA neurons from pluripotent cell sources, such as hESCs is a topic that has been greatly pursued in the last decade (Cho et al., 2008; Perrier et al., 2004; Roy et al., 2006; Yan et al., 2005). This pursuit has coincided with a greater understanding of how mesDA neurons are specified during development (Andersson et al., 2006b; Bonilla et al., 2008; Hebsgaard et al., 2009). Therefore, the focus now is to move towards more developmentally and physiologically relevant differentiation protocols (Chambers et al., 2009), most notably moving away from undefined feeder-layer methods (Buytaert-Hoefen et al., 2004). In the specific case of mesDA neurons this is also true by attempting to generate genuine mesDA progenitors through a transitional floor plate fate (Cooper et al., 2010; Fasano et al., 2010) rather than through a PAX6⁺ fate (Chambers et al., 2009; Koch et al., 2009; Perrier et al., 2004). Although these recent advancements in hESC neural and mesDA differentiation have proved promising, there has been no evidence that these floor plate derived progenitors can survive transplantation (Cooper et al., 2010; Fasano et al., 2010).

The aim of Paper №4 was to generate authentic mesDA progenitors from hESCs and test their capacity to survive and integrate into the adult brain and provide functional recovery in a rat model of PD. The protocol used in this study combined dual inhibition of SMAD signalling, for efficient neuralisation (Chambers et al., 2009) with embryoid body formation (Nat et al., 2007). SHH was added to ventralise cells during differentiation in order to induce a floor plate fate. Chemical activation of WNT signalling to mimic the isthmic organiser was used to pattern rostrocaudally to a VM-like fate. This was achieved through a glycogen synthase kinase3 (GSK3) inhibitor, which can free β -catenin and thus activate canonical WNT signalling. This in high a number of neural progenitors that co-express FOXA2 and LMX1A, both hallmarks of a mesDA fate reminiscent of the developing human VM. These neural progenitors were specified from an early stage and already expressed a number of characteristic mesDA markers after only 10 days of differentiation *in vitro*, as determined by an extensive gene expression analysis comparing hESC-derived progenitors with progenitors in the developing human neural tube.

We sought to assess if this population could survive transplantation and maintain the *in vitro* acquired identity. We found that hESC-derived neural progenitors analysed 6 weeks after transplantation were neural enriched, generated a large number of TH⁺ neurons and did not form any overgrowths. This is in contrast to other studies that have reported that hESCs transplanted this early reproducibly generate overgrowths (Aubry et al., 2008).

We sought to directly compare the phenotypic and morphological characteristics of these progenitors after transplantation with the only clinical cell source that has been shown to provide functional restoration to patients, human fetal VM. Rats that received transplants of human fetal VM tissue were sacrificed after 6 weeks and used as a standard for direct comparison. We observed that both the hESC-derived and the fetal VM cells generated TH⁺ rich grafts that co-expressed markers of a mesDA fate, such as: EN1, FOXA2, LMX1A and NURR1 (Fig. 8C). Grafts of both cell sources showed limited innervation of the host striatum. We also observed patches of NESTIN expression, a marker of immature neurons and also detected evidence of proliferation, despite no appearance of overgrowths.

To further test the potential of this promising cell source, we transplanted rats that were allowed to survive for a longer time point to truly evaluate any possibility of tumour formation, and more importantly to assess the maturation and function of the grafts. We also transplanted a control cell type, a rostral patterned fate that did not yield TH⁺ neurons (Fig. 8B). After 18 weeks, hESC-VM cells generated TH-rich grafts (Fig. 8A) that co-expressed markers of a mesDA fate (Fig. 8E). The VM-patterned cells released significantly more DA than forebrain-patterned grafts (Fig. 8F). These observations were in line with earlier studies using human fetal tissue (Stromberg et al., 1989). The VM grafts showed less proliferation than at 6 weeks, suggesting an ongoing maturation between 6 and 18 weeks. The DAergic neurons derived from hESCs were able to provide extensive TH⁺ innervation of the host striatum (Fig. 8C, D). We also observed efficient recovery in amphetamine-induced rotations from 12 weeks onwards only in animals receiving VM-patterned cells (Fig. 8G). This time course is in agreement with previous reports utilising human fetal VM tissue (Brundin et al., 1988; Clarke et al., 1988). Rats grafted with forebrain-patterned cells failed to make more paw touches in the cylinder test when compared to the lesion control rats. However the VM-patterned grafts were able to restore function to a significant level above the lesion controls, thereby normalising the lateralised bias in paw usage (Fig. 8H).

This study demonstrated that hESC-derived neural progenitors, differentiated through an intermediate FOXA2⁺ fate, are capable of surviving transplantation in the adult brain, and efficiently give rise to TH⁺ mesDA neurons that can release DA *in vivo*, appropriately innervate the host striatum and provide functional benefit in motor behaviours. This opens the discussion if bona fide human mesDA neurons, generated through a floor plate fate, are more efficient for functional recovery in PD. We know from rodent studies that mesDA TH⁺ neurons, particularly the A9 nigral neurons are more efficient than other DAergic populations in innervating the host striatum and providing reductions in amphetamine-induced

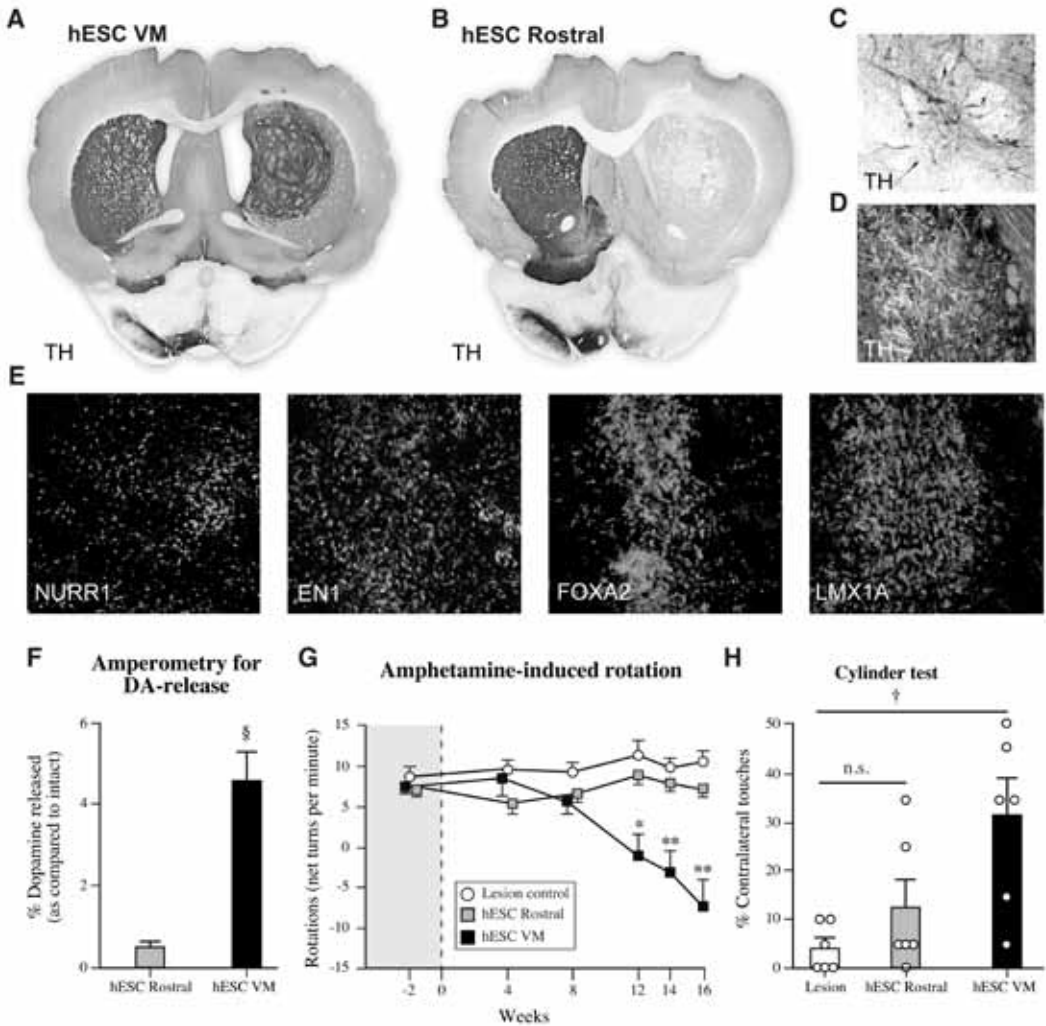


Figure 8. Survival of hESC-derived VM-like progenitors in the adult rat brain and resultant functional outcomes. A coronal overview of a 6-OHDA-lesioned rat, 18 weeks after transplantation of hESC-derived VM progenitors (A) or hESC-derived rostral (forebrain-like) cells (B). Grafts of hESC-derived VM cells extended TH⁺ axons into the host striatum and provided extensive innervation (C and D). The transplanted cells co-expressed markers of a true mesDA fate, such as NURR1, EN1, FOXA2 and LMX1A (E). The average potassium-evoked DA release from grafted hESCs was significantly higher in VM-patterned cells than the control rostral fate containing no TH⁺ neurons (F). Rats receiving hESCs patterned to a VM fate displayed significant recovery of amphetamine-induced rotations (G) and forelimb use in the cylinder test (H). (Modified from Paper №4).

rotations (Grealish et al., 2010; Hudson et al., 1994; Zuddas et al., 1991). Does the same hold true for human mesDA neurons?

A very recent paper, published during the preparation of this thesis, has independently produced similar data utilising a different culture protocol (Kriks et al., 2011). Using a previously published protocol to generate floor plate progenitors (Fasano et al., 2010) combined with GSK3-inhibition, Kriks and colleagues described more efficient generation of mesDA-like progenitors when directly compared to their previous PAX6⁺ protocol (Perrier et al., 2004). Transplantation of the floor plate derived cells gave rise to more TH⁺/FOXA2⁺ neurons than the PAX6⁺ protocol. An *in vitro* study describing another protocol for generating TH⁺ mesDA neurons through a floor plate fate observed these to be akin to VM TH⁺ neurons, as opposed to a Pax6⁺fate that appeared to give rise to diencephalic TH⁺ neurons (Cooper et al., 2010).

Both Paper №4 and the study by Kriks et al. independently prove that DAergic neurons generated from hESCs, differentiated through a floor plate fate, can efficiently survive transplantation and yield to true mesDA neurons that survive transplantation *in vivo*, and have clear functional efficacy. The only question that remains is how do these cells compare directly, in terms of functionality and innervation, with human fetal VM transplants.

CONCLUSIONS

CONCLUDING REMARKS AND PROSPECTS

Attempting to repair the brain is a complex and arduous task. Our knowledge of neurobiology and neuropathophysiology is constantly expanding and consequently, the goal of brain repair is getting ever closer. In the context of PD, some key questions still remain elusive: how and when do mesDA neurons die? When is the optimal window of therapeutic intervention? Can we identify patients within this window? The answers to these questions will undoubtedly shape approaches to novel and emerging therapeutic strategies, such as cell and gene therapies.

The preclinical advancements reported in this thesis help to address several issues such as; where should the neurons be placed, which type of neurons are responsible for recovery, and what cells should be used. It is difficult to envisage cell replacement therapy as a viable option for PD without a logistically accessible and consistent cell source. Although aborted human foetal tissue has been demonstrated to provide significant clinical benefit, it is inherently unreliable, difficult to standardise and is unsuitable for large-scale application. The ethical implications associated with such tissue are indeed contentious. The potential of alternative cell sources is thought to circumvent such concerns. Although research using human embryonic stem cells raises concerns similar to those associated with fetal tissue, they represent a most promising cell source. Recent advancements in stem cell biology, such as the advent of induced pluripotent stem cells (converting adult fibroblasts into stem cells) represents a potential avenue that could avoid these ethical concerns. Pluripotent stem cell sources are advantageous due to their potential to generate any cell type in the human body, and thus be used for numerous diseases. Conversely, this is also their disadvantage if undesired cell types form after transplantation. Ultimately, it is imperative that whatever cell type is used in patients after transplantation, will differentiate to the required fate and does not have the potential to form tumours.

In order to make significant advancements in cell replacement therapy for PD, we need to be able to systematically generate neural progenitors from alternative cell sources that can differentiate into genuine mesDA neurons, efficiently integrate in the host brain forming efferent and afferent connections and release DA in a physiological manner. Recent progress in this field suggests that this indeed may be achievable.

MATERIALS AND METHODS

MATERIALS AND METHODS

GENERAL

Throughout this thesis rats and mice have been used for experimentation (both surgery and behaviour), thus I will discuss the considerations for selected procedures using both species in parallel. For other procedures conducted on a selected basis, I will kindly refer you to the respective papers for a description of the methodology.

All animals were kept under a 12-hour light/dark cycle starting at 7 a.m. Animals had free access to food and water at all times, unless under food restriction for behavioural testing. Housing of the animals and all experimental procedures were conducted with the permission and approval of the Ethical committee for the use of laboratory animals at Lund University.

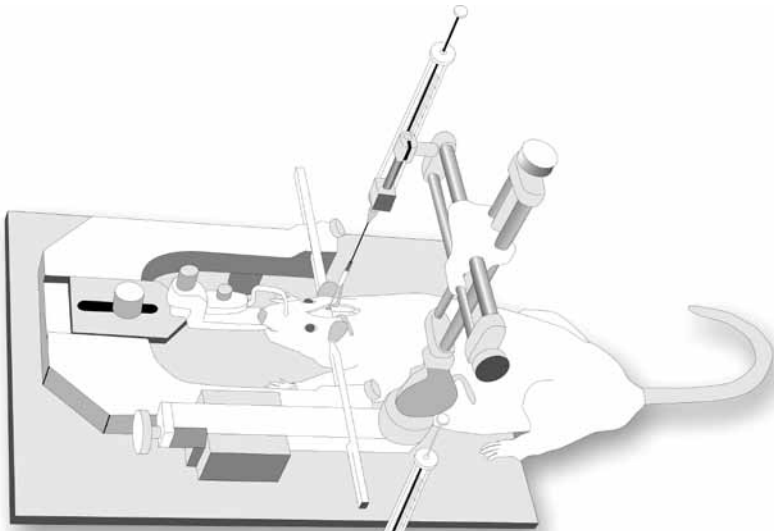
RATS

Female Sprague Dawley rats (Charles River, Germany) weighing between 225–250g at the beginning of each experiment were used as adult hosts. In some experiments neonatal rat pups were birthed from time-mated pregnant female rats, again Sprague Dawley.

For all surgical procedures in rats anaesthesia was achieved through i.p. injection, 6ml/kg, of a 20:1 solution of the μ -opioid agonist, fentanylcitrate (Fentanyl) [300mg/kg] and the $\alpha 2$ -agonist, medetomidine hydrochloride (Dormitor) [0.3mg/kg]. After surgery, anaesthesia was reversed by an s.c. injection, 1ml/kg, of the $\alpha 2$ -antagonist, atipamezole (Antisedan) [0.3mg/kg], while post-operative analgesia was administered by an s.c. injection, 1.5ml/kg, of the μ - κ -agonist, buprenorphine (Temgesic) [45 μ g/kg]. All drugs are diluted in sterile saline, with the exception for atipamezole (Antisedan), which was diluted in sterile water.

For neonatal rats, hypothermia-induced anaesthesia was used by burying each pup under ice for 7 minutes, and then being placed on the neonatal rat adaptor while maintaining a cool ambient temperature (-4°C) by dropping pieces of dry-ice into 70% EtOH in the trough at the front of the adaptor (Fig. 9B). Both Kopf and Stoelting stereotaxic frames were used for all surgical procedures (Fig. 9A). While for rat pups, a mouse/neonatal rat adaptor (Stoelting) was attached to the stereotaxic frame (Fig. 9B). For MFB lesions, a 10 μ l Hamilton syringe was used with a 26 gauge needle as standard (Fig. 10A). When performing any procedure entering the striatum, toxin lesion or transplantation, a 5 μ l Hamilton syringe with a 22 gauge needle fitted with a glass capillary was utilised in order to minimise unspecific tissue damage (Fig. 10B).

A



B

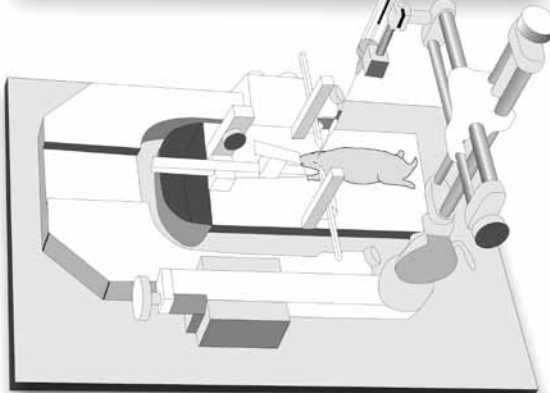


Figure 9. Stereotactic frames used. A depiction of a rat in a stereotactic frame during intracerebral surgery (A). The same stereotactic frame but with a neonatal rat/mouse adaptor for smaller procedures (B).

MICE

Female NMRI mice (Charles River, Germany) 8–10 weeks old at the beginning of each experiment were used as adult hosts. Although C57BL/6 are the most popular strain of choice, these mice are not the easiest to handle. The NMRI mice prove to be more robust when recovering from surgical procedures (typically multiple times with transplantation studies), they also proved to be much calmer during handling, resulting in easier behavioural assessment.

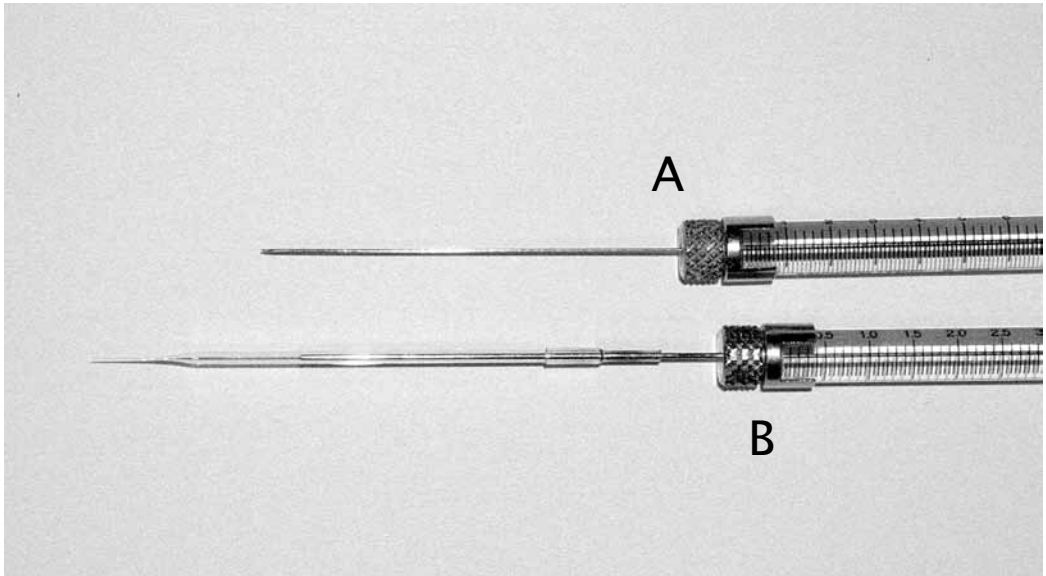


Figure 10. Juxtaposition of instruments used for intracerebral procedures. The standard Hamilton syringe and needle used for standard lesions in rats (A). For more delicate procedures, such as in mice or pups, a glass capillary is attached (B).

For all surgical procedures anaesthesia was induced and maintained using 2% isoflurane delivered in a mix of air and N_2O at a 4:1 ratio. Both Kopf and Stoelting stereotaxic frames were used for all surgical procedures with a mouse/neonatal rat adaptor (Stoelting) attached (Fig. 9B). For all stereotaxic intracerebral injections in mice, regardless of substance injected, a 5 μ l Hamilton syringe a 22 gauge needle fitted with a glass capillary was utilised in order to minimise artefact damage and also allow for more precise targeting in smaller brain structures as compared to the rat (Fig. 10B).

TRANSGENIC MICE

Transgenic mice have been used as donor tissue for transplantation and two strains of transgenic reporter mice are reported in this thesis. The TH-GFP transgenic mouse from Prof. Hideyuki Okano, Japan (Sawamoto et al., 2001) and the Pitx3-GFP knock-in mouse from Prof. Meng Li, UK (Zhao et al., 2004).

Male transgenic mice were paired together with wild-type female NMRI mice for at least 3–4 consecutive days or until a vaginal plug was observed. The morning a plug was detected (typically around 09:00 a.m.) was designated as embryonic day (E) 0.5. NMRI dams were used for this purpose as they carry significantly larger litters, typically >13 embryos at E12.5, rather than C57BL/6 dams, who typically carry ≤ 11 embryos at this stage (based on personal observations). Since the stud mouse is heterozygous, and both transgenic strains typically follow Mendelian inheritance, roughly 50% of embryos per litter were transgenic, and identified based on endogenous GFP fluorescence under a dissection microscope (Leica) equipped with a fluorescent lamp.

HUMAN FETAL TISSUE

Human fetal tissue was collected with consent of the donor from legal, elective abortions, primarily medical, conducted at the Gynaecology Department in Lund University Hospital. Fetuses were collected with the approval of the Swedish National Board of Health and Welfare. Gestational age was determined by measuring the crown–rump length (CRL) and referring to the O’Rahilly embryo staging (O’Rahilly and Muller, 2000).

6-HYDROXYDOPAMINE LESIONS

6-hydroxydopamine (6-OHDA) lesion model is the most established pre-clinical model of PD, and has been in use since the late 60’s (Ungerstedt, 1968). 6-OHDA is a hydroxylated form of DA, and thus it has a high affinity for DAT. 6-OHDA is unable to cross the blood–brain barrier and thus must be administered centrally in order to have an effect. When injected into the brain parenchyma, it is taken up by DAT and transported into the cytoplasm of DAergic neurons where it is readily oxidised. 6-OHDA causes oxidative stress and also inhibits mitochondrial respiration, to which the DAergic neurons are particularly vulnerable to, thus resulting in cell death (Glinka et al., 1997).

One great advantage of this lesion model is that one can inject increasing amounts of toxin into different targets and produce a variety of lesions. The most widely used, and somewhat classic model is a complete lesion of the ascending DA system by injecting into the MFB (Ungerstedt, 1968), thus destroying both the A9 and A10 cell bodies and terminal fields. The more recent partial lesion, involves injecting 6-OHDA into the terminal field, typically the dorso-lateral striatum and results in cell loss of the A9 nucleus (Ichitani et al., 1991; Kirik et al., 1998). There is a wealth of studies performed using these various lesions

in rats but less so in mice. It is only recently that mouse 6-OHDA lesion studies have undergone resurgence (Francardo et al., 2010; Iancu et al., 2005; Lundblad et al., 2004; Parish et al., 2001).

6-OHDA-HBr (Sigma Aldrich), is aliquoted and stored in the -20°C freezer in individual Eppendorfs and kept in containers with dessicant. When an aliquot is needed, it is removed from the freezer, diluted in a solution of 0.02% ascorbate in sterile saline, and then stored in the dark on ice. When calculating doses of 6-OHDA we only consider the active form, and thus correct the dose for the bromide salt. Thus a correction factor of 0.82 is applied, since the sample is typically 82% pure 6-OHDA base. The ascorbate and darkness helps prevent the rapid oxidation of the 6-OHDA, thus maintaining its toxicity after injection. A single aliquot is only kept for a maximum of 2 hours after dilution in order to ensure toxicity.

For a summary of lesion coordinates for both species see Table 2.

TISSUE DISSECTION

The VM was dissected out of freshly harvested fetuses, of either murine or human origin. The tissue was collected in the following ways:

- For mice, the time-mated dams were sacrificed by cervical dislocation and the uterine horns containing the fetuses were removed and placed into ice-cold L-15 (Gibco) medium until dissection.
- Human fetuses were collected in ice-cold hibernation medium (Apoteks-bolaget), and transported from the Gynaecology Clinic on ice. Fetuses were placed into fresh hibernation medium, photographed, measured and morphologically documented.

In all cases dissections were carried out under a dissection light-microscope (Leica) using sterile forceps and micro-scissors (Fine Science Tools Inc.) in a sterile Petri-dish containing a fresh volume of the solution they were collected in. In the case of GFP-reporter mice, the fluorescent lamp and GFP filter were utilised in order to identify the transgenic fetuses from their wild-type littermates. The dissection was performed as demonstrated in Figure 11A-C).

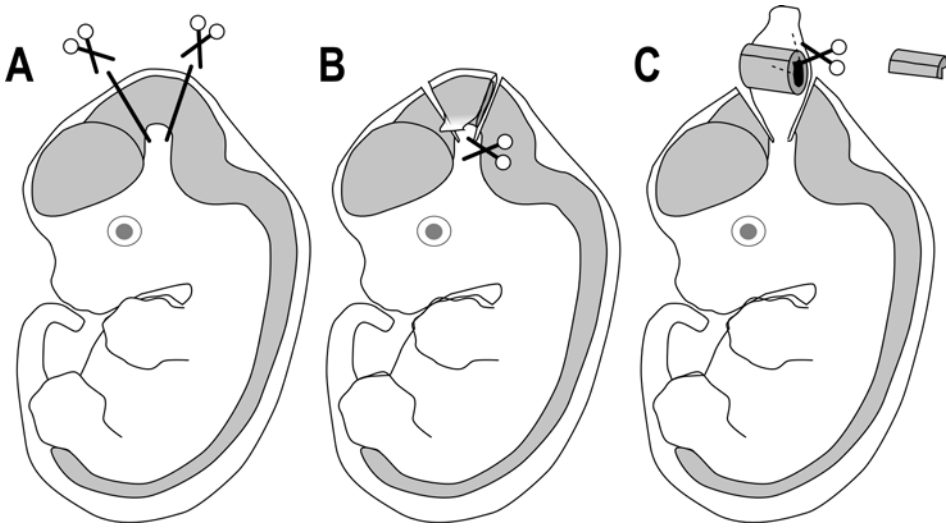


Figure 11. Dissection of the ventral mesencephalon from the embryo. A vertical incision posterior to the meso-diencephalon border is made followed by a (roughly) 30° incision posterior to define the mesencephalon (A). The surrounding tissue, developing skin and skull, is removed and the neural tube is freed (B). Then the most ventral aspect of the mesencephalon is dissected in order to achieve the classic “butterfly” shape (C).

CELL PREPARATION FOR TRANSPLANTATION

Dissected VMs were collected in sterile 1.5ml Eppendorf tubes containing the dissection medium and stored on ice until the dissociation. VM pieces were washed once with sterile HBSS (without Mg^{2+} or Ca^{2+}) containing 0.05% DNase (Sigma Aldrich) hereon referred to as “wash buffer”. The first solution was removed and replaced with wash buffer containing 0.1% trypsin (Sigma Aldrich), in order to enzymatically digest the tissue pieces. This preparation was then placed in a 37°C incubator for 20 minutes. Trypsin was removed and the tissue pieces washed four times in wash buffer, in order to remove all traces of the enzyme. The tissue was gently dissociated in a known volume of wash buffer (approximately 200µl per 20 E12.5 VM pieces, or varying volumes, typically 20µl, depending on number and age of human fetuses). Mechanical dissociation was achieved by first using a 1000µl pipette-tip, and then followed with a 200µl pipette-tip. The tissue pieces were gently dissociated, with great care taken to avoid air bubbles, until a milky solution was obtained with no obvious tissue pieces. The concentration of the suspension and cell viability was estimated at this step using trypan-blue exclusion, and the rest of the cell preparation was

Table 2. Summary of intracerebral coordinates. Summary of all surgical procedures performed on adult rats, rat pups and adult mice with respective coordinates, volumes and concentrations.

Species	Surgery Type	Coordinates in mm (from bregma)				Concentration	Volume
		A/P	M/L	D/V	Tooth Bar		
RATS	MFB Lesion	-4.4	-1.2	-7.8	-2.4	3.5µg/µl	4µl
	4-Site Striatal Terminal Lesion	+1.3	-2.6	-5.0	0.0	3.5µg/µl	2µl
		+0.4	-3.2	-5.0	0.0	3.5µg/µl	2µl
		-0.4	-4.2	-5.0	0.0	3.5µg/µl	2µl
		-1.3	-4.5	-5.0	0.0	3.5µg/µl	2µl
		+1.2	-2.6	-4.5	-2.4	75,000€ /µl	2µl
	Striatal Transplant	+0.5	-3.0	-4.5	-2.4	75,000€ /µl	2µl
RAT PUPS	Ventricular Lesion	+0.5	-1.1	-2.9	Flat	10µg/µl	5µl
	Striatal Transplant	+0.7	-1.9	-2.9	Flat	50,000€ /µl	1µl
MICE	Nigral Lesion	-3.0	-1.2	-4.5	Flat	1.6µg/µl	1.5µl
	Striatal Vector	+1.0	-2.3	-3.2	Flat	n/a	0.3µl
		+1.0	-2.3	-2.2	Flat	n/a	0.3µl
	Nigral Transplant	-3.2	-1.4	-4.2	Flat	100,000€ /µl	1.5µl
	Striatal Transplant	+1.0	-2.3	-3.2	Flat	150,000€ /µl	1µl
+1.0		-2.3	-2.2	Flat	150,000€ /µl	1µl	

centrifuged at 600g for 5 minutes, and then the pellet was re-suspended in wash buffer to the desired concentration for transplantation. The cell preparation typically did not exceed 200,000 cells/µl to avoid clogging of the glass capillary. Once the cell preparation was ready for transplantation, it was kept on ice for the entire procedure, and gently mixed, to ensure a homogenous density, with a sterile pipette tip before each intracerebral injection. All procedures were performed ideally within 4–5 hours of making the cell preparation. For a summary of transplantation coordinates for both species see Table 2.

BEHAVIOUR

All the behavioural tests used in the 6-OHDA rodent models exploit the unilateral nature of the lesion. Thus each animal has a “normal” side and a Parkinsonian side of both the brain and the body. We routinely inject 6-OHDA

into the right hemisphere, and thus observe motor deficits on the left-hand side of the body. Since the corticospinal tract, both lateral and anterior tracts, cross over to the contralateral side of the body, thus the left hemisphere controls the right-hand side of the body and *visa versa*.

Therefore we use tests that expose this lateral-bias within each animal and can observe the normal side being directly compared to the Parkinsonian side. Since both rats and mice have been used throughout this thesis using similar behavioural tests, I will provide a brief description of each test, followed by the procedural differences and considerations for performing said test using each species.

DRUG-INDUCED ROTATION

Drug-induced rotation is based on DA-releasing agents, amphetamine, or DA-receptor agonists, *e.g.* apomorphine. This test was first described by Ungerstedt and colleagues, who also described an apparatus, the rotometer, for automated scoring of the circling movements (Ungerstedt and Arbuthnott, 1970). The equipment has largely gone unchanged except for the addition of electronic assessment and readout. Here the rodent is placed in a plastic bowl and a harness is attached around the thorax, which is connected to an automated counter, that in turn signals to a computer where all the clockwise and counter-clockwise turns made by the animal, over a defined period of time, are recorded and the net number of turns per minute, in a clockwise direction, are automatically calculated. This test is easy to implement and interpret test. Multiple animals can be recorded simultaneously in the same session, thus making it relatively high-throughput and popular to use.

Amphetamine is a monoamine-releasing agent that causes the release of DA from stored vesicles and also blocks the activity of DAT, thus causing a massive, sustained release events of DA at the synapse (Sulzer et al., 1995). Since our 6-OHDA model is unilateral, if the lesion is successful, the release of DA should only occur in one hemisphere, and the animal will have an overwhelming motoric response unilaterally and thus the animal will rotate towards the side of the lesion, clockwise. Simplistically, amphetamine-induced rotation is a measure of the absent DA-release in the lesioned hemisphere. Hence, if the animal receives a transplant containing cells that are capable of releasing DA, then they should normalise the hemispheric imbalance of DA, and the animal should turn equally in both directions.

Apomorphine-induced rotation acts via another mechanism known as denervation supersensitivity, whereby after the 6-OHDA lesion the post-synaptic DA receptors on the medium spiny neurons of the striatum attempt to compensate

Table 3. Drugs used in rotation test. The table summarises the drug doses, injection volumes and test periods for drug-induced rotation tests used in this thesis for both species.

RATS	Amphetamine	Apomorphine
Injection volume	1ml/kg	1ml/kg
Dose	2.5mg/kg	0.05mg/kg
Time recorded	90 minutes	60 minutes
MICE		
Injection volume	10ml/kg	10ml/kg
Dose	5mg/kg	0.1mg/kg
Time recorded	40 minutes	40 minutes

for the loss of DAergic innervation and become hyper-sensitive to DA-activation. When the animals are exposed to a DA receptor agonist, the medium spiny neurons have a supraphysiological reaction and thus motor activation occurs on the side of the lesion, resulting in rotation away from the side of the lesion, counter-clockwise.

For both species, d-amphetamine is diluted in sterile saline. Here a full volume for rotating all the animals is prepared at once, since amphetamine is very stable in solution. Apomorphine hydrochloride is diluted in a solution of 0.02% ascorbate diluted in sterile saline. As with 6-OHDA, apomorphine can be rapidly oxidised and thus similar cautionary steps are taken to ensure the drug's physiological effect. A single aliquot is diluted and kept in the darkness at 4°C when not in use, for a maximum of 2 hours. In the case of apomorphine only, to ensure that the denervation supersensitivity has reached a somewhat maximal threshold, before the first apomorphine-induced rotation the animals are given a single priming dose both on four and two days before the rotation session.

The same automated rotometer system (AccuScan Instruments, Columbus, OH, USA) is used for both species. For a given session, each animal is placed into its individual rotometer bowl for at least 5 minutes in order to habituate to the environment before recording commences. Upon injection of the drug, the animal is placed back into its bowl and the harness attached. The recording is not begun until 5 minutes have elapsed, in order to ensure that no turns due to struggling with the harness are recorded, also this phase allows for an increased bioavailability of the drug during the recording session.

Separate harnesses for mice, with a smaller fastening length are used to ensure that mice do not escape during the test.

PERFUSION AND HISTOLOGY

Rodents were given a terminal anaesthetic dose of sodium pentobarbital i.p. (see Table 4). Once the animal is anaesthetised, a bilateral incision from the base of the sternum along the lower ribs is made, with care taken to leave the diaphragm intact (this allows for better visualisation of the thorax as the abdominal viscera are contained). The incision is continued along the midaxillary line until the chest cavity is completely exposed. The descending thoracic aorta is then cross-clamped, to avoid perfusion inferior the diaphragm. A perfusion cannula (metal feeding cannulae, see table 4) is then inserted through the left ventricle, and into the ascending aorta, in the case of rats, or the left atrium, in mice, and then cross-clamped. A small incision is made in the right atrium, in order for the blood and perfusate to exit. Then room-temperature 0.9% saline is perfused through first, in order to flush out all of the blood, until the perfusate exiting the right atrium runs clear, and also the forepaws and tongue are whitish in appearance. The flow is then switched to ice-cold 4% paraformaldehyde, in 0.1M phosphate-buffered saline (PBS), and then allowed to run for a set volume (see table 4).

The 4% PFA is always prepared fresh, used less than three days from preparation, filtered for particles and is at a physiological pH, 7.4 ± 0.2 , at 4°C .

After perfusion, the brain was removed from the skull, with particular attention being made removing the meninges, and placed into a volume of 4% PFA for 2 hours post-fixation. Following this, the brain was transferred into 20ml of 25% sucrose solution in 0.1M PBS, and stored overnight on a shaker, or at 4°C for at least 48 hours, until the brains have sunk to the bottom. This cryoprotects the brain, removing all water that would crystallise upon freezing and thus destroy the tissue.

All adult brains were sectioned on a freezing microtome with a section thickness of $35\mu\text{m}$, and collected in a one in 6, or 8, series. Typically brains were sectioned in the coronal plane, but for certain projects (namely Paper №2) brains were also sectioned in the horizontal plane. This was achieved by lying the brain supine, and orienting it so that the dorsal part of the cerebellum was also touching the chuck. This creates horizontal plane for the ventral surface of the whole brain.

The protocol for immunohistochemistry is detailed in Paper №1 and №3, thus in this section I will provide a list of primary antibodies used throughout this thesis, regardless of publications, with comments on the use of each, and also some considerations when performing this procedure (see Table 5).

Table 4. Volumes used during perfusion. Detailed here are the drugs, cannulae, volumes and perfusion rates used to perfusion rats and mice with paraformaldehyde.

	RATS	MICE
Pentobarbital dose/	330mg/kg	600mg/kg
Volume	6ml/kg	10ml/kg
Perfusion needle	16G, 50mm length with a 3.0mm round tip	24G, 25mm length with a 1.25mm round tip
Pump speed	50ml/min	13–15ml/min
Volume of PFA	250ml	150ml

HISTOLOGICAL QUANTIFICATION

For quantification of the number of surviving cells present in a graft a total estimation method was used. Where images were captured in 20X magnification, in two focal planes (the top and bottom of the section). A montage of the whole graft for a given section was created for each focal plane and then overlaid, and then all cells that appeared in a given focal plane were labelled in a software program (Canvas, v10) in order to ensure that the same cell was not counted twice. This was done for the entire structure of the transplant, thus all surviving cells within the series were counted. The Abercrombie correction was applied, to remove errors in double-counting of individual cells, and the value multiplied by the series number in order to provide an estimation of the entire graft.

There can be some contention on how best to estimate of number surviving cells after transplantation. Despite some misconceptions, it is difficult to apply optical fractionator-based stereology to transplants for a number of reasons:

In order to have a reasonable sampling, either very large grafts need to be obtained, or the sampling frequency needs to be quite large, such as a 1:3 series. If one tries to apply a “standard” stereological approach to the transplants, as they are processed in the manner described in the section above, then one needs to have a high fraction thereby counting almost 100% of the area

Table 5. Primary antibodies used. Details of the species, purchasing company and working dilution for specific antibodies.

ANTIBODY	HOST	COMPANY	Cat. No.	DILUTION
AADC	Rabbit	Millipore	Ab1569	1:500
Calbindin	Mouse	Sigma	C9840	1:1,000
Calbindin	Rabbit	Swant	cb38	1:1,000
cFos	Rabbit	Calbiochem	Ab-2	1:5,000
ChAT	Goat	Chemicon	Ab144P	1:500
En1	Mouse	Hybridoma Bank	-	1:50
FoxA2 (HNF3B)	Goat	Santa Cruz	sc6554	1:600
GABA	Rabbit	Sigma	A2052	1:500
GFP	Chicken	Abcam	Ab13970	1:1,000
GFP	Rabbit	Abcam	Ab290	1:5,000
Girk2	Rabbit	Alomone Labs	APC0061	1:100
vGLUT1	Rabbit	Synaptic Systems	-	1:100
HuNu	Mouse	Millipore	MAB1281	1:200
Lmx1a	Rabbit	Millipore	Ab10533	1:5,000
M2/M6	Rat	Hybridoma Bank	-	1:50
Nestin	Rabbit	Chemicon	Ab5922	1:200
hNCAM	Mouse	Santa Cruz	sc106/Eric-1	1:100
Nurr1	Rabbit	Santa Cruz	sc990	1:1,000
PCNA	Mouse	Abcam	Ab29	1:500
Pitx3	Rabbit	Zymed	38-2850	1:200
TH	Mouse	Chemicon	MAB 318	1:2,000
TH	Rabbit	Chemicon	Ab152	1:1,000
TH	Rabbit	Pelfreeze	P40101-0	1:1,000

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