Cell Replacement Therapy for Parkinson's Disease: Potential for Circuitry Repair

Cardoso, Tiago

2018

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Cardoso, T. (2018). Cell Replacement Therapy for Parkinson's Disease: Potential for Circuitry Repair Lund: Lund University, Faculty of Medicine

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Academic dissertation

Cell Replacement Therapy for Parkinson’s Disease: Potential for Circuitry Repair

Tiago Cardoso

2018

With approval of the Faculty of Medicine of Lund University this thesis will be defended at 09:00h on November 30th, 2018 in Belfragesalen Biomedical Center, Lund, Sweden

Faculty Opponent:

Viviane Tabar, MD
Chair, Department of Neurosurgery
Memorial Sloan Kettering Cancer Center, New York, USA
The derivation of dopamine neurons from human embryonic stem cells (hESCs) now offers a promising alternative to fetal tissue for cell replacement therapy (CRT) in Parkinson’s disease (PD). Using the appropriate chemical cues in vitro, hESCs can be patterned towards bona fide ventral midbrain (VM) DA neurons that survive, reinnervate, release DA and provide functional recovery when transplanted into rodent and non-human primate models of PD. However, the extent to which transplanted neurons integrate into the damaged host circuitry, which is necessary for regulated DA release, and hence to elicit a more complete circuitry repair, remains unknown.

In this thesis, the potential for transplanted hESC-derived neurons to repair damaged circuitry in parkinsonian rats, as assessed by synaptic integration and targeted axonal outgrowth, was investigated. The role of graft- and host-dependent variables on synaptic integration and innervation were investigated in view of a better understanding of transplant biology, with potential to optimize the functionality of the graft.

We established a modified rabies-based tracing methodology that allows for the identification of monosynaptic inputs to a defined starter population in order to assess host-to-graft and graft-to-host synaptic integration in a cell transplantation model. In Paper I, we investigated the integration of hESC-derived neurons, and in Paper II during in vivo reprogramming, to investigate the integration of in situ converted neurons into pre-existing circuitry. Subsequently in Paper III, this methodology was utilized to reveal that transplanted hESC-derived VM neurons receive the correct set of presynaptic inputs from the host circuitry when placed in their homotopic location within the substantia nigra, while the profile of axonal outgrowth showed that transplanted neurons innervate across long-distances in a target-specific manner towards appropriate forebrain targets. Moreover, functional recovery as assessed by amphetamine-induced rotations matched the presence and timing of the arrival of graft-derived dopaminergic innervation in the dorsolateral striatum. Finally, in Paper IV, the role of graft neuronal phenotype and host environment on synaptic integration and graft-derived axonal outgrowth was investigated. These results show that graft-derived innervation is determined by cell intrinsic factors, as only the correct hESC-derived VM neurons innervate the appropriate forebrain DA target regions. On the other hand, monosynaptic tracing showed that the pattern of integration is dependent on graft placement, as monosynaptic inputs to infrastralatal and intranigral grafts differed. Nonetheless, a certain level of anatomical and phenotypic overlap in presynaptic inputs to both ectopic and homotopic hESC-derived VM grafts was detected, suggesting that ectopically placed grafts may be modulated by functionally relevant structures involved in motor control, supporting the validity of this grafting paradigm in the clinic. Finally, transplantation of hESC-derived neurons in intact or 6-OHDA-lesioned animals revealed that the globus pallidus is differentially connected to transplanted neurons, identifying this structure as a possible important modulator of graft function in the DA-depleted parkinsonian brain.

Overall, the results of this thesis suggest that transplanted hESC-derived VM DA neurons have the capacity to achieve a more complete repair of the damaged host circuitry beyond simple DA neuron replacement. As such, they support the validity of ectopic grafting into the lesioned brain as a valid strategy for CRT in PD. Moreover, this work identifies host nuclei that may play an important role in graft modulation, hence prompting further functional experimentation.
Cell Replacement Therapy for Parkinson’s Disease: Potential for Circuitry Repair

Tiago Cardoso

2018

Developmental and Regenerative Neurobiology
Department of Experimental Medical Science
Faculty of Medicine
Lund University
“Science is a way of thinking much more than it is a body of knowledge.”

-Carl Sagan

“There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another theory which states that this has already happened.”

-Douglas Adams. The Restaurant at the End of the Universe
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*Manuscript under preparation*
ABSTRACT

The derivation of dopamine neurons from human embryonic stem cells (hESCs) now offers a promising alternative to fetal tissue for cell replacement therapy (CRT) in Parkinson’s disease (PD). Using the appropriate chemical cues \textit{in vitro}, hESCs can be patterned towards \textit{bona-fide} ventral midbrain (VM) DA neurons that survive, reinnervate, release DA and provide functional recovery when transplanted into rodent and non-human primate models of PD. However, the extent to which transplanted neurons integrate into the damaged host circuitry, which is necessary for regulated DA release, and hence to elicit a more complete circuitry repair, remains unknown.

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Overall, the results of this thesis suggest that transplanted hESC-derived VM DA neurons have the capacity to achieve a more complete repair of the damaged host circuitry beyond simple DA neuron replacement. As such, they support the validity of ectopic grafting into the lesioned brain as a valid strategy for CRT in PD. Moreover, this work identifies host nuclei that may play an important role in graft modulation, hence prompting further functional experimentation.
Parkinson’s disease (PD) is the second most common neurodegenerative disease worldwide, affecting approximately 1% of people over the age of 60. The symptoms of the disease include rigidity, tremor, slowness of movement and postural instability. However, other non-motor symptoms such as dementia and depression are also common in late stages of PD. While the root causes of the disease are still a matter of debate and investigation, it is known that the motor symptoms are caused by the degeneration of a specific type of neuron (brain cells) that produce dopamine (DA, a neurotransmitter) in the brain. The most common therapeutic approaches available to date are pharmacological, consisting of the usage of medication aimed at repeatedly replenishing DA in the brain to alleviate the motor symptoms. However, as the disease progresses, the efficacy of these medications wane, and debilitating secondary effects including dyskinesias (involuntary movements) often appear. The main caveat of these pharmacological approaches is that they do not provide a finely-tuned and localized release of DA in specific regions of the brain, as would occur in a healthy individual. In light of this, several other therapeutic alternatives aimed at repairing the diseased brain in order to provide a more sustained symptomatic relief have been proposed and tested over the years.

One proposed approach to brain repair for PD is cell replacement therapy, which is the particular interest of this thesis. This strategy postulates that the damaged and lost DA neurons in the brain of PD patients can be replaced with an external source of healthy and fully functional DA neurons by means of transplantation. Early clinical trials using DA neurons harvested from human fetal brain have shown that these cells can survive up to 25 years in the brain of PD patients and can alleviate motor symptoms to a remarkable extent, in some cases. However, the utilization of the fetal human brain as a reliable and reproducible source of transplantable tissue poses several ethical and practical obstacles. Therefore, if this therapy is to be applied at a full clinical scale, alternative cell sources must be considered. In particular, stem cells are the most promising and practical alternative available to date, and are currently on the brink of clinical trial testing. These cells have the capacity to self-renew indefinitely, and to differentiate into any cell type in the body, including DA neurons, when provided with appropriate chemical cues.

The overall aim of this thesis was to investigate the full potential of DA neurons derived from human stem cells to fully repair the damaged brain after transplantation into a rat model of PD. For that purpose, I applied a novel technology that allowed me to evaluate if and how transplanted DA neurons connect with the pre-existing host neurons after transplantation into the rat brain. Using this methodology, I analysed if the transplanted DA neurons connect with the correct type of host neurons after transplantation, thereby evaluating if they are able to reconstruct the damaged neuronal network in a biologically correct manner. I also identified variables that may affect the capacity for transplanted neurons to integrate into the rat neuronal network, with a view to better understand the way that these cells work in the brain. Ultimately, the observations herein provide a valuable framework for optimizing the functionality of these cells in upcoming clinical trials with PD patients.

En potentiell strategi för reparering av hjärnan vid Parkinsons sjukdom är cellterapi, vilket är av särskilt intresse för denna avhandling. Cellterapi förutsätter att de skadade och förlorade dopamin nervcellerna kan ersättas med celler från en extern källa av friska och funktionella dopamin nervceller genom transplantation till patientens hjärna. Kliniska prövningar i vilka man användt dopamin nervcel- ler från humana fosterhjärnor har visat att de transplanterade cellerna kan överleva i upp till 25 år i hjärnan hos en patient och att de i vissa av fallen lindrar de motorisk symptomen drastiskt. Användan- det av humana fosterhjärnor som källa till celler för transplantation är dock förknippat med både etiska och praktiska svårigheter. Om behandlingen ska kunna tillämpas kliniskt i stor skala så kommer det därför att behövas en alternativ källa till celler. Stamceller är en mycket lovande alternativ cellkälla och användandet av dessa celler börjar närmast sig kliniska prövningar. Stamceller karaktäriseras av att de har förmågan att differentiera (mogna) till vilken celltyp som helst i kroppen om de ges de rätta kemiska signalerna, däribland dopamin-celler.

Det övergripande målet med denna avhandling var att undersöka den fulla förmågan hos dopamin nervcellerna som producerats från stamceller att reparera den skadade hjärnan efter transplantation i en rätt-modell av Parkinsons sjukdom. I detta syfte så använde jag en ny teknik som gjorde det möjligt att utvärdera om och hur transplanterade dopamin nervceller kopplar samman med rättens egna nervceller. Genom att använda denna metod kunde jag analysera om de transplanterade dopamin nervcellerna kopplade samman med värddjurets nervceller av rätt identitet och därmed utvärdera om de var kapabla att återskapa de skadade neurala nätverket på ett korrekt sätt. Jag identifierade även variabler som kan påverka de transplanterade nervcellernas kapacitet att integreras i rättens neurala...
nätverk, med hopp om att bättre förstå hur dessa celler fungerar i hjärnan. Observationerna i denna avhandling erbjuder ett värdefullt ramverk för optimeringen av dessa celler i kommande kliniska prövningar med Parkinson patienter.
RESUMO EM PORTUGUÊS

A doença de Parkinson é uma perturbação crónica do sistema nervoso central que afeta cerca de 1% da população acima dos 60 anos de idade. Os sintomas são principalmente motores, e incluem tremor, rigidez e lentidão de movimentos. Mas à medida que a doença progride, outras complicações não-motoras surgem, incluindo demência, depressão, ansiedade e problemas sensoriais e emocionais. Embora as causas originais da doença não sejam totalmente conhecidas – e provavelmente envolvem tanto factores genéticos como ambientais – sabe-se que os sintomas motores são causados pela morte de um tipo específico de neurónios (células cerebrais) que se especializam na produção do neurotransmissor dopamina (neurónios dopaminérgicos). Não existe cura para a doença de Parkinson, e os tratamentos mais comuns consistem no aliviar dos sintomas motores e não-motores através do uso de medicação. Por exemplo, através da administração sistemática de medicação dopaminérgica, que leva ao aumento dos níveis de dopamina no cérebro. No entanto, à medida que doença progride, a eficácia destes medicamentos vai diminuindo e inclusive levam ao surgimento de efeitos secundários chamados discinesias (movimentos involuntários). O problema associado a este tipo de estratégias farmacológicas, é que elas não permitem uma entrega de dopamina de forma regulada e localizada ao local certo do cérebro como ocorreria num individuo saudável. Por essa razão, outro tipo de estratégias terapêuticas que têm como objetivo o efectivo reparo cerebral e um alívio sintomático mais eficiente, têm sido propostas e testadas ao longo dos anos.

Uma dessas estratégias é a chamada terapia de substituição celular, que foi a área de estudo da minha tese. Esta estratégia consiste em transplantar neurónios dopaminérgicos saudáveis e funcionais diretamente no cérebro de pacientes de Parkinson, assim restabelecendo os neurónios dopaminérgicos que degeneraram no decorrer da doença. Testes clínicos mostraram que neurónios dopaminérgicos obtidos de tecido fetal humano sobrevivem por várias décadas quando transplantados no cérebro de doentes de Parkinson e em alguns casos proporcionam um alívio sintomático notável. No entanto, a utilização de tecido fetal (obtido através de abortos electivos) levanta óbvios problemas éticos e práticos, tornando impraticável a sua aplicação à plena escala clínica. Assim sendo, são necessárias fontes alternativas de tecido celular para transplante. Uma das alternativas mais promissoras é a utilização de células estaminais. Estas células têm a capacidade de, em cultura, se multiplicarem indefinidamente e de poderem ser “transformadas” em qualquer tipo de célula do corpo humano, inclusive neurónios dopaminérgicos, dados os sinais químicos apropriados. Assim sendo, e em teoria, este tipo de células podem ser usadas para transplante em pacientes de Parkinson com vantagens práticas e éticas evidentes em comparação com tecido fetal.

O objetivo da minha tese e dos estudos aqui incluídos, foi o de investigar o potencial de neurónios dopaminérgicos derivados de células estaminais, de poderem providenciar um reparo mais completo do circuito cerebral “estragado”, quando transplantados em ratos-modelo de Parkinson. Com esse fim em vista, utilizei uma nova tecnologia que permite investigar o modo como os neurónios transplantados integram o circuito neuronal do hospedeiro. Utilizando esta tecnologia analisei se os neurónios
transplantados conectam com os neurónios correctos do hospedeiro, assim avaliando se são capazes de reconstruir de modo fidedigno o circuito neuronal “estragado”. E também identifiquei variáveis que influenciam negativamente e positivamente este fenómeno de modo a melhor compreender o modo como estes neurónios se comportam no cérebro dos ratos. Em conclusão, as descobertas aqui incluídas, providenciam uma base de conhecimento valiosa com o intuito de optimizar o potencial terapêutico da terapia de substituição celular em futuros testes clínicos em pacientes de Parkinson.
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<td>6-hydroxydopamine</td>
</tr>
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<td>A/P</td>
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<td>AAV</td>
<td>adeno-associated virus</td>
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<tr>
<td>BG</td>
<td>basal ganglia</td>
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<td>CNS</td>
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<td>CRT</td>
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<td>DAB</td>
<td>di-aminobenzidine</td>
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<td>DAT</td>
<td>dopamine transporter</td>
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<td>DBS</td>
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<td>FACS</td>
<td>fluorescent-activated cell sorting</td>
</tr>
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<td>FB</td>
<td>forebrain</td>
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<td>fibroblast growth factor 8</td>
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<tr>
<td>FRG</td>
<td>fluorescent gene reporter</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GIRK2</td>
<td>G-protein-regulated inward-rectifier potassium channel 2</td>
</tr>
<tr>
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<td>globus pallidus</td>
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<td>GSK</td>
<td>glycogen synthase 3</td>
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<td>hESCs</td>
<td>human embryonic stem cells</td>
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<td>iPSCs</td>
<td>induced pluripotent stem cells</td>
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<td>M/L</td>
<td>medial/lateral</td>
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<td>MFB</td>
<td>medial forebrain bundle</td>
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<td>MOR</td>
<td>µ-opioid receptor</td>
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<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<td>MSNs</td>
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<td>rabies glycoprotein</td>
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<tr>
<td>RPE</td>
<td>retinal pigmentary epithelial cells</td>
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<td>SHH</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SNc</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNr</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TSA</td>
<td>tyramide signal amplification</td>
</tr>
<tr>
<td>UPDRS</td>
<td>unified Parkinson’s disease rating scale</td>
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<td>VM</td>
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<td>VMAT</td>
<td>vesicular monoamine transporter</td>
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<td>VTA</td>
<td>ventral tegmental area</td>
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INTRODUCTION

The general aim of this thesis was to investigate the therapeutic potential of human embryonic stem cells (hESCs) as a cell replacement therapy for Parkinson’s disease (PD). In particular, to study the capacity of hESC-derived neurons to integrate, innervate and repair the damaged brain when transplanted in a rat model of PD, using monosynaptic circuit tracing technologies.

In this section I will provide a brief overview on PD itself (symptoms, etiology and therapeutic approaches), as well as the basic organization of the basal ganglia (BG) circuitry and how it is affected in PD. I will focus on cell replacement strategies as disease modifying therapies for PD, and mention alternative sources of transplantable tissue, with special focus on efforts to implement and develop protocols for neural differentiation and regional patterning of pluripotent stem cells. Finally, I will address currently-available technologies that can be applied to elucidate the function and potential for synaptic integration of transplanted neurons with host circuits.

Parkinson’s disease

Parkinson’s disease is the second most common neurodegenerative disease after Alzheimer’s disease, affecting 1% of the population above 60 years old, with the prevalence increasing with age (Dexter & Jenner, 2013). PD is primarily characterized as a movement disorder with motor symptoms including bradykinesia (slowness of movement), rigidity, tremor and postural instability (Jankovic, 2008). However, non-motor symptoms including dementia, depression, autonomic and sensory disorders are also a feature of the disease (Duncan et al., 2014).

The core clinical features of this disease were first described in 1817 by neurologist James Parkinson in his “An Essay on the Shaking Palsy” (Parkinson, 2002):

“Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured.”

More than a century later, Arvid Carlsson and colleagues observed the presence of dopamine (DA) in the mammalian brain (Carlsson, Lindqvist, Magnusson, & Waldeck, 1958), and found that administration of DOPA (a DA precursor) could reverse akinesia in reserpine-treated animals (Carlsson, 1959), thereby prompting the discovery of the cardinal pathological features of PD: loss of neurons of the substantia nigra and consequent DA depletion in the putamen (Ehringer & Hornykiewicz, 1960; Hornykiewicz, 1963). The observation that L-DOPA could alleviate motor symptoms in PD
patients further emphasized the causal link between the loss of DA in the brain and PD, paving the way for the first pharmacological approach for treating the disease (Cotzias, Van Woert, & Schiffer, 1967).

**Parkinson’s disease pathological and etiological features**

The etiological aspects of PD still remain elusive, but a complex interplay between environmental and genetic factors appear to play a role in the onset of PD (Dauer & Przedborski, 2003). Self-administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes DA depletion and Parkinsonian syndromes (Langston et al., 1999), and epidemiological studies have related the exposure to herbicides and pesticides (such as rotenone and paraquat) to a higher risk of developing PD (de Lau & Breteler, 2006). However, exposure to toxins alone is unlikely to fully explain the etiology of idiopathic PD. Mutations in several genes, including SCNA (α-synuclein gene), Parkin, LRRK2 and GBA have also been implicated with higher risk of developing PD and to initiate familial forms of the disease (Klein & Westenberger, 2012).

A cardinal pathological hallmark of PD is the widespread intraneuronal accumulation of eosinophilic round protein aggregates termed Lewi bodies in the brains of patients (Jellinger, 2012). The inclusions are primarily composed of insoluble forms of aggregated α-synuclein fibrils and ubiquitin (Spillantini et al., 1997). It is unclear if the accumulation of these intracellular inclusions are a coincident or causative factor in cellular dysfunction, but several cellular processes such as lysosomal and mitochondrial function, autophagy, vesicular homeostasis and microtubule transport seem to be disrupted by various forms of α-synuclein (reviewed in Rocha, De Miranda, & Sanders, 2018). Neurromelanin-rich dopaminergic neurons of the substantia nigra pars compacta (SNc) are particularly vulnerable to the disease process (Hirsch, Graybiel, & Agid, 1988). These neurons form the nigrostriatal pathway (NSP) and mainly project to the dorsolateral area of the putamen, where DA depletion is more pronounced in the brain of PD patients (Bernheimer, Birkmayer, Hornykiewicz, Jellinger, & Seitelberger, 1973). In contrast, medial DA neurons of the ventral tegmental area (VTA), which project to the caudate nucleus to form the mesolimbic pathway, are less vulnerable to the disease process (Uhl, Hedreen, & Price, 1985). The dopaminergic denervation of the putamen accounts for the motor impairments observed in PD (Dauer & Przedborski, 2003). However, the widespread nature of the α-synuclein pathology, involving the central, peripheral and autonomic nervous systems, coupled with the degeneration of other non-dopaminergic nuclei such as the locus coeruleus, raphe nucleus, basal nucleus of Meynert, amygdala and hippocampus, may account for the non-motor symptoms of the disease (Dexter & Jenner, 2013; Hall et al., 2014; Jellinger, 2012).

**Current therapeutic practices in Parkinson’s disease**

Currently-available therapeutic options for PD rely on symptomatic relief either by pharmacological or surgical treatment. The prescription of L-DOPA, monoamine oxidase type B inhibitors or DA agonists are the most common pharmacological options used to mitigate motor symptoms of the disease by increasing the levels of DA in the brain or by directly modulating DA receptors (Connolly & Lang, 2014; Fox et al., 2011), while standard neuropsychiatric drugs are frequently employed to address non-motor features of the disease (Connolly & Lang, 2014; Seppi et al., 2011). However, these pharmacological approaches are often associated with side effects and complications related to
non-physiological release of DA in the brain and off-target effects, which worsen with disease progression. Such side effects include motor fluctuations and dyskinesias (involuntary movements) that negatively impact the efficacy of the therapy and the quality of life of PD patients (Jankovic, 2005). Current pharmacological practices aimed at bypassing these complications have focused on implementing alternative routes for the systemic delivery of DA precursor drugs (Zibetti et al., 2014) and DA agonists (Garcia Ruiz et al., 2008). Alternatively, more invasive strategies are also in practice in the clinic such as deep brain stimulation (DBS), whereby an electrical stimulation of the subthalar nucleus (STN) or the internal segment of the globus pallidus (GPi) via surgically implanted electrodes, can effectively and safely relieve certain motor symptoms (Benabid, Chabardes, Mitrofanis, & Pollak, 2009; Fang & Tolleson, 2017).

Nonetheless, none of these therapeutic approaches tackle the underlying cause or the progression of the disease, nor do they represent disease-modifying strategies aimed at brain repair. In light of this, various alternatives have been suggested as potential disease-modifying therapeutic alternatives for PD. These include pharmacological modulation of cellular neurodegenerative processes, direct targeting of the α-synuclein protein, delivery of neurotrophic factors (either pharmacologically or via genetic therapy) to halt the disease progression and/or regenerate lost neurons, and transplantation of neuronal tissue in order to replenish the levels of DA in the diseased brain in a better-regulated, more physiological manner (reviewed in Lang & Espay, 2018).

Basal ganglia and pathophysiology in Parkinson’s disease

The BG is comprised of several subcortical nuclei involved in a variety of processes such as motor, associative and cognitive functions. In a simplified manner, the BG acts as the integration center of sensorimotor, associative and limbic information originating from the entire cortical mantle. The BG influences behavior by relaying the processed information to the thalamus via the BG output nuclei. In turn, the thalamus transmits the information back to the cortex forming a complex cortico-basal ganglia-thalamo-cortical loop with several levels of parallel topographical processing (Bolam, Hanley, Booth, & Bevan, 2000; Gerfen & Wilson, 1996). Recent elucidations of BG connectivity reveals a more complex picture of the anatomical organization of the system, involving direct input from the thalamus as well as the amygdala, hippocampus and dorsal raphe nucleus to the BG, and additional regions targeted by the BG output nuclei, including the superior colliculus, the pedunculopontine nucleus, the lateral habenula and the reticular formation (Bolam et al., 2000). For the purpose of this thesis, the circuits and nuclei involved in motor control, including the direct and indirect striatal pathways, and the modulating role of the midbrain dopaminergic neurons, as well as their involvement in PD, will be addressed. For a detailed description of the BG circuitry, topographical organization of cortical and thalamic input to the striatum in rodents and primates, I address the reader to the following reviews (Gerfen & Bolam, 2010; Y. Smith, Galvan, Raju, & Wichmann, 2010; Winn, Wilson, & Redgrave, 2010).
Basal ganglia organization and circuitry

The BG is organized into a dorsal division, mostly involved in controlling motor processes, and a ventral division associated with limbic functions. In rodents, the dorsal division is comprised of the striatum, the globus pallidus (GPe), the entopeduncular nucleus (EP), the STN, the SNC where dopaminergic neurons are located, and the substantia nigra pars reticulata (SNr) containing γ-aminobutyric acid (GABAergic) neurons. The ventral division of the BG also includes the dopaminergic VTA and the nucleus accumbens (NAc) (Bolam et al., 2000; see Table 1 for homologous nomenclature in primates).

The majority of glutamatergic inputs to the BG originate from the cortex and the thalamus, and have neurons in the striatum as the principal postsynaptic target. Cortical inputs to the striatum originate from most cortical areas and maintain a certain level of topographical organization (Alexander, DeLong, & Strick, 1986), with inputs to the dorsal striatum originating mostly from motor cortical areas (Kunzle, 1975). In turn, thalamic input to the striatum originates mainly from the parafascicular nucleus (Pf) and also show a level of topographical organization. However other intralaminar and non-intralaminar nuclei also project to both striatal projection and interneurons (Y. Smith et al., 2010). The striatum is the main input target region of the BG and is comprised almost entirely of inhibitory GABAergic medium spiny projection neurons (MSNs). MSNs account for 95% of the neurons in the striatum with the remaining population consisting of different GABAergic and cholinergic interneurons (Kawaguchi, Wilson, Augood, & Emson, 1995). MSNs can be classified into two distinct types related to the region of projection and neurochemical profile: the “direct” and the “indirect” pathways (Kawaguchi, Wilson, & Emson, 1990). MSNs of the “direct” pathway project directly to the output nuclei of the BG – the SNr and EP – and express the DA receptor D1, as well substance P and dynorphin. In contrast, the MSNs from the “indirect” pathway project indirectly to the output nuclei via the GPe and the STN, express the DA receptor D2 and the peptide enkephalin (Gerfen & Young, 1988). Stimulation of the “direct” MSNs leads to the inhibition of the output nuclei neurons (EP/SNr), which are themselves inhibitory GABAergic neurons. This in turn reduces the inhibitory action of the EP/SNr on excitatory thalamic neurons, ultimately leading to excitation of motor cortical regions and facilitation of movement. In contrast, the activation of “indirect” MSNs leads to the inhibition of the GPe, which in turn disinhibits the excitatory glutamatergic neurons of the STN. This causes excitation of the EP and consequent inhibition of the thalamus leading to reduced activity in the motor cortex, and ultimately to inhibition of movement (see Figure 2). Therefore, in the classical model of motor circuitry, the activation of the “direct” pathway facilitates initiation of movement, while the activation of the “indirect” pathway inhibits movement (Albin, Young, & Penney, 1989).

The striatum presents another level of topographical organization in rodent termed the patch and matrix that relates to differential input from the cortex and thalamus, but also segregated innervation of the dorsal/ventral portion of the substantia nigra (Gerfen, 1992). The patch and matrix compartments express different levels of neurochemical markers, with patch compartment being enriched in μ-opioid receptor (MOR) and acetylcholinesterase (Graybiel & Ragsdale, 1978; Herkenham & Pert, 1981), and patch compartment enriched in calbindin (Gerfen, 1985). More modern views on the topography of the stratum also describe the presence of scattered MOR+ neurons in the matrix compartment, which are termed “exo-patch” neurons (J. B. Smith et al., 2016). The origin of cortical input to the patch of the striatum is located in deep layer 5 cortical neurons, while superficial layer 5 cortical neurons project preferentially to the matrix compartment (Gerfen, 1989). Thalamic projections to
the patch and matrix compartment also originate from different thalamic nuclei (Berendse, Voorn, te Kortschot, & Groenewegen, 1988; Herkenham & Pert, 1981). Importantly, patch and matrix neurons in the striatum also project to different regions of the BG and hence control different aspects of the system. Projections from the matrix compartment of the striatum are directed to BG output nuclei such the EP and the GABAergic neurons of the SNr, while the patch compartment project to DA neurons in SNC therefore regulating the DA feedback to the striatum (see Figure 1).

The schematic presented here is an oversimplified model of the BG circuitry when considering the range of different subcortical inputs to the striatum (including raphe nucleus, amygdala), the different targets of the output nuclei (including superior colliculus, pedunculopontine nucleus), the reciprocity of connectivity between the different BG nuclei and the presence of a variety of interneuron populations (reviewed in Gerfen & Bolam, 2010). However, this simplified model of BG motor control is useful in understanding the basics behind initiation an inhibition of movement and the reciprocal control of DA action in the striatum and for interpreting the data in this thesis.

Nigrostriatal dopamine pathway and role in basal ganglia

The development of the Falck and Hillarp formaldehyde histofluorescence method back in the 1960s for detection of catecholamine neurons in brain tissue (Falck, Hillarp, Thieme, & Torp, 1982) prompted the first description of the distribution of DA and noradrenaline neurons in the mammalian brain (Carlsson, 1959). Dahlström and Fuxe described the presence of 12 different catecholaminergic nuclei in the rodent brain which they numbered from A1 to A12 (Dahlstrom & Fuxe, 1964). Further technological developments in histological techniques for the identification of enzymes involved in DA synthesis, such as tyrosine hydroxylase (TH) and proteins involved in DA transport and vesicular loading (DAT and VMAT), now permit the identification of DA neurons in the brain with great detail and confirm the original description of the spatial distribution of catecholaminergic nuclei in the brain. In particular, the A8 (retrorubral field), A9 (SNC) and the A10 (VTA) nuclei are the most important DA players in the BG circuitry, and provide dense axonal innervation to the striatum, forming the nigrostriatal and mesolimbic dopaminergic pathways, as well as the cortex forming the mesocortical pathway. Indeed, TH immunohistochemistry shows that the entirety of the

<table>
<thead>
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<th>Common nomenclature in rodents</th>
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<tr>
<td>Dorsal Striatum</td>
<td>Caudate/putamen unit (CPu)</td>
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<td>Ventral striatum</td>
<td>Nucleus accumbens (NAc)</td>
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<tr>
<td>Globus pallidus (GP)</td>
<td>External segment of globus pallidus (GPe)</td>
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<td>Entopeduncular nucleus (EP)</td>
<td>Internal segment of globus pallidus (GPI)</td>
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<td>Parafascicular nucleus (Pf)</td>
<td>Centromedial/parafascicular nucleus (CM/Pf)</td>
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Figure 1. Neuroanatomy of the rodent nigrostriatal and striatonigral pathway

Mesencephalic dopaminergic neurons projecting to the limbic, associative and motor striatum are segregated in a mediolateral manner.

Reciprocal striatonigral connections to the dopaminergic neurons of the substantia nigra pars compact originate from the patch compartment of the striatum. The matrix compartment projects to the GABAergic neurons of the substantia nigra pars reticulata.
The striatum receives dopaminergic input (Bjorklund & Dunnett, 2007). In the simplified motor/limbic dopaminergic circuitry model, the medial DA A10 cell group projects to the NAc and cortex to regulate limbic functions and forming the mesolimbic and mesocortical pathway. In contrast, the lateral A9 population innervates the dorsal and lateral regions of the striatum to control motor functions, comprising the NSP (see Figure 1). Another level of compartmental organization of the midbrain dopaminergic neurons is the division between the dorsal and ventral tier. The dorsal tier comprises of the A8 nucleus and the dorsal portion of the A9 and A10 nuclei, while the ventral tier contains the ventral portion of the A10 and A9 nuclei (Gerfen & Bolam, 2010). This compartmentalization can be differentiated based on subtype-specific marker expression and differential innervation of forebrain targets. Neurons of the dorsal tier express calbindin and project to limbic and cortical areas, as well as the matrix compartment of the dorsal striatum. Ventral tier neurons are calbindin-negative but express G-protein-regulated inward-rectifier potassium channel 2 (GIRK2) instead, and project mostly to the patch compartment of the striatum. However, this model describing the anatomical organization of the mesencephalic DA neurons and their projections is now accepted as being an oversimplification, as tract tracing studies have shown that DA neurons projecting to the same striatal region are somewhat intermingled in both A9 and A10 nuclei in rodents and primates (reviewed in Bjorklund & Dunnett, 2007). DA neurons in the A9 nuclei also receive a vast range of synaptic inputs from the BG, cortex and other midbrain and hindbrain populations. Using monosynaptic tracing to map retrograde connectivity specifically from DA neurons in the DAT-cre mouse, Watabe-Uchida and colleagues have shown that rostral cortical areas in motor and sensorimotor cortex, the patch compartment of the striatum, GPe, STN and several midbrain and hindbrain nuclei (including the superior colliculus, the periaqueductal grey and the dorsal raphe nucleus) provide extensive synaptic input to A9 DA neurons (Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012).

Basal ganglia circuits in PD

PD symptomatic features include bradykinesia, akinesia and rigidity. Therefore, PD can be characterized as a hypokinetic disorder, i.e., a movement disorder where the initiation and amplitude of movements are impaired. This is caused by an imbalance in the activity of the “direct” and “indirect” pathways as a consequence of DA denervation in the striatum. The absence of DA signaling via the D1 and D2 receptors leads to a decreased excitability of the MSNs of the “direct” pathway and increased excitability of the MSNs of the “indirect” pathway respectively. The excessive activity of the “indirect” pathway in the parkinsonian brain leads to excessive excitatory drive of STN neurons to the output nuclei of the BG (GPe and EP). As a consequence, the excessive activity of the output centers of the BG leads to an inhibition of the thalamocortical nuclei and motor cortical neurons, which explain the hypokinetic features observed in PD (Albin et al., 1989; DeLong, 1990; see Figure 2). In agreement with this model, blocking the activity of STN neurons via lesion or electrical stimulation leads to an amelioration of the bradykinesia and akinesia in non-human primates (Bergman, Wichmann, & DeLong, 1990) and in PD patients (Benabid et al., 2009).
Figure 2. Overview of the motor basal ganglia circuitry in the normal and parkinsonian brain

(A) In the normal brain, information from the thalamus and cortex is integrated in the striatum and then relayed via the direct and indirect pathway towards the output nuclei of the basal ganglia (EP and SNr). In turn, the information is returned to the thalamus and then motor cortex to ultimately influence the initiation of movement. The flow of information in striatum is finely-tuned by the dopaminergic neurons of the SNc, leading to the appropriate balance of activity in the direct and indirect pathways.

(B) In the parkinsonian brain, the absence of dopamine regulation in striatum causes the increased activation of the “indirect” pathway and, as a consequence, excessive activity of the output nuclei of the BG. Ultimately, this leads to an inhibition of the thalamocortical nuclei and motor cortical neurons, explaining the hypokinetic features of the disease. Darker lines represent increased activity; dashed lines represent decreased activity.
Cell replacement therapy for Parkinson’s disease

Current pharmacological and surgical approaches to treat PD are accompanied by several shortfalls and secondary complications (see above). Moreover, these approaches are solely targeted to alleviate motor and non-motor symptoms of the disease. Therefore, disease-modifying approaches aimed at regenerating lost neurons and repairing the damaged circuitry have been proposed and tested both in experimental models and in clinical trials. Among the experimental therapeutic approaches being considered for the treatment of PD, and of particular interest in this thesis, is cell replacement therapy. This therapeutic approach postulates that neurons lost in the diseased brain can be replaced by providing an external source of neuronal tissue via transplantation (Barker, Barrett, Mason, & Bjorklund, 2013; Barker, Drouin-Ouellet, & Parmar, 2015; Lindvall et al., 1990), or by reprogramming resident cells in the brain to the desired neuronal phenotype(s) (Torper & Gotz, 2017). In the context of PD, the transplantation of DA neurons from fetal or pluripotent stem cell origins (discussed later) directly into the caudate/putamen of PD patients, would reinnervate the structure to reinstate appropriate DA levels – providing a more physiological, controlled and targeted release of DA when compared to pharmacological DA approaches, and elicit a superior therapeutic benefit (Barker et al., 2015).

From early neural transplantation studies to clinical trials

The history of transplantation of tissue and cells into the brain can be dated as far back as the late 19th century. However, the studies that prompted the development of modern neural grafting strategies for PD were performed in Sweden in the 1970s. Coinciding with the development of novel histological techniques to identify catecholaminergic neurons (Falck et al., 1982), Olson and colleagues showed that monoaminergic tissue of rat fetal origin could be transplanted into the anterior chamber of the rat eye, survive and innervate the iris (Olson & Seiger, 1972). While providing invaluable insight in the development of the monoaminergic system (Olson & Seiger, 1973), these studies did not address functionality of the transplanted neurons in a disease context. Following the development of the 6-hydroxydopamine (6-OHDA) rat model of PD, which allows for the selective lesioning of the NSP (Ungerstedt & Arbuthnott, 1970), questions related to functionality and brain repair in a PD context could be addressed. Experiments in the late 1970s and early 1980s showed that tissue pieces of rat fetal ventral midbrain (VM) transplanted into cortical cavities could survive and reinnervate the adjacent striatum (Bjorklund, Dunnett, Stenevi, Lewis, & Iversen, 1980; Bjorklund & Stenevi, 1979; Bjorklund, Stenevi, Dunnett, & Iversen, 1981; Dunnett, Bjorklund, Stenevi, & Iversen, 1981a, 1981b; Perlow et al., 1979). The transplanted tissue was able to reverse drug-induced and spontaneous motor asymmetries in 6-OHDA lesioned rats (Bjorklund, Dunnett, et al., 1980; Bjorklund & Stenevi, 1979; Bjorklund et al., 1981; Dunnett et al., 1981a; Perlow et al., 1979) and even alleviate sensorimotor neglects (Bjorklund et al., 1981; Dunnett et al., 1981b). The reinnervation capacity and functionality of the graft was further enhanced by using a cell suspension from dissociated rat VM tissue placed directly into the striatal parenchyma (Bjorklund, Schmidt, & Stenevi, 1980; Dunnett, Bjorklund, Schmidt, Stenevi, & Iversen, 1983), which would become the standard transplantation method for subsequent clinical trials. Finally, the evidence that human VM tissue harvested from aborted fetuses aged 6.5 - 9 weeks could survive, reinnervate the host striatum and function when
transplanted to immunosuppressed 6-OHDA-lesioned rats (Brundin et al., 1986; Brundin et al., 1988; Clarke et al., 1988; Stromberg, Bygdeman, Goldstein, Seiger, & Olson, 1986) laid the foundations for the first clinical trials using human fetal VM tissue in PD patients.

In light of the exciting preclinical evidence of the efficacy and safety of human fetal VM tissue in vivo (Brundin et al., 1986; Brundin et al., 1988; Clarke et al., 1988; Stromberg et al., 1986), the first PD patients transplanted with VM tissue from aborted fetuses were performed in Lund, Sweden in the late 1980s (Lindvall et al., 1990; Lindvall et al., 1989). In the subsequent years, hundreds of patients were transplanted in the same center, and in other centers around the world (Barker et al., 2013). While the results from these open-label clinical trials were highly variable, transplants were seen to restore DA levels in striatum as assessed by 18F-dopa positron-emission tomography (PET) imaging, some patients showed clinical improvement and, in the best cases, were able to cease DA medication altogether (reviewed in Barker et al., 2013). Furthermore, transplants were shown to release DA and to functionally integrate into the host brain (Piccini et al., 1999; Piccini et al., 2000). Clinical benefit in certain transplanted patients was maintained for 20 years (Kefalopoulou et al., 2014), and post-mortem analysis revealed long-term survival of transplanted DA neurons with extensive innervation of host caudate-putamen (Hallett et al., 2014; Kordower, Chu, Hauser, Freeman, & Olanow, 2008; W. Li et al., 2016; Mendez et al., 2005). The promising observations from these open-label studies prompted the funding of two double-blind, placebo-controlled trials in the United States (Freed et al., 2001; Olanow et al., 2003). Disappointingly, the conclusions from both of these studies were negative, as they failed to meet their primary endpoints and some patients developed severe side-effects caused by the transplant (graft-induced dyskinesias). Of note however, several aspects related to trial design including subjectivity of the primary endpoint, short follow-up time, sub-optimal tissue preparation and transplantation procedures, and lack of immunosuppression may have negatively influenced the outcome of both studies (see Barker et al., 2013; Barker et al., 2015). Nonetheless, 18F-dopa PET imaging confirmed graft survival in patients from both studies, and younger patients (Freed et al., 2001), and patients in a less advanced stage of PD (Olanow et al., 2003) did show improvement in motor scores of the unified Parkinson’s disease rating scale (UPDRS; Barker et al., 2013). Moreover, graft-induced dyskinesias were closely linked to severe on-off fluctuations and existing levodopa-induced dyskinesias prior to transplantation, and were most likely caused by the presence of serotonergic neurons in the tissue preparation and/or uneven distribution of DA neurons (Hagell & Cenci, 2005; Politis et al., 2010). In conclusion, with proper patient selection and better-controlled fetal cell preparation, cell replacement therapy is a promising therapeutic strategy for treating certain motor aspects of PD. In light of this, a new multicenter clinical trial where refined patient selection, tissue preparation and trial design were taken into consideration, is currently ongoing (Barker et al., 2015; www.transeuro.org.uk).

Expandable and standardizable cell sources for cell replacement therapy in Parkinson’s disease

While human fetal VM tissue has shown safety and efficacy when transplanted into patients of PD, this cell source cannot be regarded as a viable option for future large-scale implementation into the clinical setting. Firstly, there are ethical issues related to the usage of tissue harvested from aborted fetuses, and second there are practical issues related to the low availability and difficulty to standardize the tissue preparation for application in the clinic. Therefore, alternative cell sources that can
overcome these hurdles need to be considered if the long-term benefits cell replacement therapy can provide patients is to become standard clinical practice for PD.

Several alternate cells have already been tested in patients as possible sources of transplantable tissue for PD, including autologous adrenal medullary tissue (Madrazo et al., 1987), xenografts of porcine VM tissue (Schumacher et al., 2000), autografts of carotid body cells (Minguez-Castellanos et al., 2007) and human retinal pigmented epithelial (RPE) cells (Gross et al., 2011). However, none of these trials were supported by sufficiently strong preclinical evidence and, as expected, did not provide any therapeutic benefit to PD patients (reviewed in Barker et al., 2015). Approaches that would follow the neurophysiological rationale behind the function of transplanted fetal VM tissue, would therefore be key for a successful implementation of a cell replacement strategy in PD. Pluripotent stem cells (PSCs) sources promise to achieve this standard, and include hESCs and induced pluripotent stem cells (iPSCs). These cells can be patterned in vitro towards the appropriate mesencephalic DA phenotype and transplanted into the diseased brain (Doi et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011). Alternatively, transplantable DA neurons could also be obtained from directly reprogrammed somatic cells (Caiazzo et al., 2011; Pfisterer et al., 2011). Direct conversion in vivo of residing glial cells to DA neurons is another alternate approach being proposed (see Figure 3; Rivetti di Val Cervo et al., 2017; Torper et al., 2015; Torper et al., 2013).

Deriving authentic mesencephalic dopamine neurons from pluripotent stem cells

Pluripotent stem cells are, by definition, self-renewable and able to differentiate into any somatic cell type. These characteristics make the cells highly promising for use in regenerative medicine. ESCs are derived from the inner mass of a blastocyst – a pre-implantation early stage embryo (Thomson et al., 1998), whereas iPSCs are derived from adult somatic cells reverted back to the pluripotent stage, by forced expression of transcription factors expressed in early development (K. Takahashi & Yamanaka, 2006). In theory, iPSCs can allow for patient-specific cell therapy, and do not carry the ethical concerns associated with hESCs.

The vast potential of PSCs promises to revolutionize the treatment of neurodegenerative diseases, including PD. Indeed, shortly after the very first derivation of hESCs by Thomson and colleagues in 1998 (Thomson et al., 1998), protocols for deriving neural progenitors from hESCs, based on embryoid body formation and feeder cells were quickly developed (Itskovitz-Eldor et al., 2000; Reubinoff et al., 2001; Zhang, Wernig, Duncan, Brustle, & Thomson, 2001). The first attempts to generate DA neurons from hESCs were based on protocols used at the time for mouse embryonic stem cells (Kawasaki et al., 2000; Kim et al., 2002) and then-current knowledge of cues involved in neuronal development. Therefore, these early protocols relied on the co-culture of hESCs with mouse stromal feeder cells such as PA6 and MS5, co-culture with midbrain astrocytes, or incubation with developmental cues such as fibroblast growth factor 8 (FGF8) and sonic hedgehog (SHH) (Perrier et al., 2004; Roy et al., 2006; Yan et al., 2005; Zhang et al., 2001). These protocols were efficient in generating TH expressing neurons in vitro that, in certain cases, survived in vivo and provided modest functional recovery when grafted to rat models of PD (Sonntag et al., 2007; Yang, Zhang, Oldenburg, Ayala, & Zhang, 2008). However, these protocols generated highly heterogenous cell products with only small levels of the desired mesencephalic DA neurons, and retained proliferative undifferentiated cells that in some cases, generated teratomas in vivo (Brederlau et al., 2006; Roy et al., 2006; Sonntag
et al., 2007). Nonetheless, these studies provided proof-of-principle that PSCs could be differentiated into DAergic neurons that survived and provided some level of functional recovery when transplanted into animal models of PD.

In 2007, Ono and colleagues made an important discovery on the origin and development of mesencephalic DA neurons. In a fate mapping experiment using fluorescence-activated cell sorting (FACS), they found that these neurons originate from the floor-plate of the developing neural tube – a region originally believed to be non-neurogenic – instead of the neuroepithelium (Ono et al., 2007). Shortly after, Bonilla and colleagues identified midbrain radial glia-like cells as being the progenitors of the mesencephalic DA neurons (Bonilla et al., 2008). These findings quickly prompted the establishment of protocols that recapitulate this development process in vitro, which could differentiate hESCs to floor-plate cells (Fasano, Chambers, Lee, Tomishima, & Studer, 2010). At the same time, a protocol to generate high yields of neuronal progenitors from PSCs, based on dual inhibition of SMAD signaling with Noggin and SB431442 was developed (Chambers et al., 2009). Both of these breakthroughs lead, finally, to the development of protocols to generate authentic mesencephalic DA neurons via a floor plate intermediate in vitro (Kirkeby et al., 2012; Kriks et al., 2011). These protocols track the developmental cues that lead to the generation of midbrain DA neurons in utero (reviewed in Arenas, Denham, & Villaescusa, 2015). In short, early activation of the canonical WNT pathway via precise inhibition of glycogen synthase 3 (GSK3), is crucial for inducing the appropriate anterior/posterior (A/P) fate. Ventral identity is then achieved by potent agonists of SHH signaling in culture (Kirkeby & Parmar, 2012). Both of these modulators produce VM-committed progenitors that express the specific midbrain floor-plate markers FOXA2, LMX1A, EN1 and CORIN (Kirkeby & Parmar, 2012). However, recent findings have identified that STN neurons share a similar developmental progression as mesencephalic DA neurons and similarly co-express most VM markers (Kee et al., 2017). In light of this, improved protocols that rely on more potent caudalization via FGF8, can now generate highly standardized and pure preparations of mesencephalic DA neurons (Kirkeby, Nolbrant, et al., 2017; Nolbrant, Heuer, Parmar, & Kirkeby, 2017).

Importantly, the development of floor-plate derived protocols to generate DA neurons (Kirkeby et al., 2012; Kriks et al., 2011), lead to improved survival and functionality of these cell preparations in vivo when compared to earlier strategies. The transplanted DA neurons survive to a great extent, innervate the host striatum, release DA, integrate into the host circuitry and provide functional recovery in different animal models of PD (Doi et al., 2014; Grealish et al., 2014; Grealish et al., 2015; Kikuchi et al., 2017; Kirkeby et al., 2012; Kirkeby, Nolbrant, et al., 2017; Kriks et al., 2011; Wakeman et al., 2017). Moreover, hESC-derived grafts have been shown to function to the same extent as primary human fetal VM tissue (Grealish et al., 2014). Studies using opto- and chemogenetics have further proven that the graft-induced functional effect in vivo is specifically mediated by DA neuron function (Chen et al., 2016; Steinbeck et al., 2015). With the aim to improve survival and standardization of the cell preparation, the cell product can be modified by different strategies prior to transplantation, including loading with biomaterials (Adil et al., 2017) or purification of DA progenitors by cell sorting (Bye, Jonsson, Bjorklund, Parish, & Thompson, 2015; Doi et al., 2014; Lehnen et al., 2017; Samata et al., 2016). Moreover, graft-derived innervation and synaptic integration may be further enhanced by modifying the cell-cell and cell-extracellular matrix interactions between the grafted neurons and the host (Battista, Ganat, El Maarouf, Studer, & Rutishauser, 2014; Kauhausen, Thompson, & Parish, 2015; Nishimura et al., 2016).
Figure 3. Alternative strategies for cell replacement therapy in Parkinson’s disease

Transplantable cell sources may originate from fetal tissue, pluripotent stem cells - including hESCs and iPSCs - or obtained via direct conversion of fibroblasts to neurons in vitro. Alternatively, cells in situ may be converted to neurons via viral-mediated delivery of reprogramming factors. In box are the strategies of interest for this thesis.
Direct conversion as a source of dopamine neurons

In 2010, Vierbuchen and colleagues showed that mouse fibroblasts could be directly converted into functional neurons, skipping an intermediate proliferative iPSC stage, by forced expression of three transcription factors: Ascl1, Brn2 and Myt1L (Vierbuchen et al., 2010). Since then, several reports have shown direct conversion of mouse and human fibroblasts, and astrocytes to TH-expressing neurons (Caiazzo et al., 2011; Dell’Anno et al., 2014; Pfisterer et al., 2011; Rivetti di Val Cervo et al., 2017; Torper et al., 2013). To date, human induced DA neurons survive poorly after transplantation, and their functionality in animal models of PD remains to be shown (Pereira et al., 2014; Torper et al., 2013). Nevertheless, direct conversion is an interesting method for generating patient-specific DA neurons, with great potential for disease modelling purposes (Drouin-Ouellet et al., 2017). With further improvements, they may become a viable source of cells for transplantation.

Building from the developments of in vitro direct conversion, another approach that has been developed in recent years, is transcription factor-mediated in vivo direct conversion. Residing glial cells in the rat brain, including astrocytes and NG2 glia, have been reprogrammed to neurons in situ, where endogenous cells were forced to express different reprogramming factors (Pereira et al., 2017; Rivetti di Val Cervo et al., 2017; Torper et al., 2015; Torper et al., 2013). This approach has the advantage of using endogenous cells as a reprogramming source, avoiding immunological rejection, and depleting the lesion site of glial-scar forming cells (Gascon, Masserdotti, Russo, & Gotz, 2017). However, the capacity to generate functional midbrain TH neurons has yet to be achieved.

Although at an early stage in the field, these studies show the potential of direct conversion in vitro and in vivo as potential sources of DA neurons for cell replacement therapy. However, the survival, subtype specification and functional maturation of converted neurons still needs to be improved.

Graft function and potential for circuitry repair

Several mechanisms have been proposed to be responsible for influencing animal behavior after transplantation of neuronal tissue in the brain. These mechanisms are context dependent and associated with the type of cells transplanted, the placement of the transplant and the pathophysiology of the targeted disease (Dunnett & Bjorklund, 2017). Such mechanisms include non-specific actions associated with surgery, neurotrophic support of degenerating neurons and axons, diffuse local delivery of neurotransmitters, glial support, promotion of host axonal outgrowth and full circuitry repair of the damaged host network (reviewed in Dunnett & Bjorklund, 2017). If circuitry repair is to be achieved, transplanted neurons must have the capacity to extend properly distal axonal projections towards correct anatomical targets. Moreover, grafted neurons must receive appropriate and functionally-relevant afferent innervation from the host and minimize disruptive forms of connectivity. To which extent grafted DA neurons have the capacity to repair the damaged circuitry, and to have their function regulated by the host, remains to be elucidated.

VM neurons from both primary and pluripotent origins have been shown to function in animal models of PD (Brundin et al., 1986; Dunnett et al., 1983; Kirkeby et al., 2012; Kriks et al., 2011). The depleted levels of DA on the lesioned side of the striatum can be replenished by up to 80% by fetal VM transplants (Strecker et al., 1987; Zetterstrom et al., 1986) and the therapeutic effect of PSC-derived transplants has been shown to depend on the activity of DA neurons present in the graft (Chen et al., 2016; Steinbeck et al., 2015). Hence, it is clear that the benefits elicited by VM-derived
grafts is depend on the physiological release of DA in the brain. However, to which extent DA release is modulated by the host and to which extent transplanted DA neurons integrate into host circuitry remain to be elucidated. Using anterograde tracers, early studies have shown that VM transplanted neurons receive afferent input from certain host anatomical structures such as striatum, frontal cortex and raphe nuclei, while lacking the full “repertoire” of endogenous nigral afferents (Doucet et al., 1989). However classical tract tracers cannot reveal new connections or if the host afferent terminal fields are able to establish functional synaptic machinery with grafted neurons. Electron microscopy studies combined with TH immunohistochemistry have shown that TH+ neurons in the graft can establish synaptic specialization with host striatal neurons (Clarke et al., 1988; Freund et al., 1985; Jager, 1985; Mahalik, Finger, Stromberg, & Olson, 1985), while electrophysiological studies reveal that grafted fetal DA neurons retain certain electrophysiological properties that characterize endogenous SNC neurons (Fisher, Young, Tepper, Groves, & Gage, 1991). Later, electrophysiological studies in slice preparations confirmed the presence of bi-directional functional synapses between host and transplant (Sorensen et al., 2005; Tonnesen et al., 2011). These studies indicate that transplanted VM neurons can elicit a certain level of circuitry integration and confirm the capacity of grafted neurons to establish bi-directional functional synaptic connectivity with host neurons.

Another important predictor of graft function and a key aspect for successful circuitry repair is the degree and specificity of axonal outgrowth derived from the transplanted neurons. Indeed, the extent of DAergic reinnervation of the host dorsolateral striatum closely correlates to the extent of functional recovery in animal models of PD (Bjorklund, Dunnett, et al., 1980; Bjorklund & Stenevi, 1979). Additionally, in human patients, an estimated 5 - 7 mm of axonal outgrowth from each graft deposit is required for full innervation of the putamen (Kordower et al., 1995). The first methodologies that allowed the study of graft derived axonal innervation of the host brain, relied on the immunohistochemical identification of graft derived fibers via neurotransmitter-specific (Bjorklund, Segal, & Stenevi, 1979; Bjorklund, Stenevi, & Svendgaard, 1976; Dunnett, Low, Iversen, Stenevi, & Bjorklund, 1982; Gage, Bjorklund, Stenevi, Dunnett, & Kelly, 1984) or species-specific antibody reactivity (Isacson & Deacon, 1996; Lund, Chang, Hankin, & Lagenaour, 1985; Stromberg, Bygdemain, & Almqvist, 1992; Wictorin, Brundin, Gustavii, Lindvall, & Bjorklund, 1990). Extensive axonal outgrowth via grey and white matter tracts was observed emerging from striatal neurons transplanted into the lesioned striatum (Isacson & Deacon, 1996; Wictorin, Clarke, Bolam, & Bjorklund, 1990; Wictorin, Lagenaour, Lund, & Bjorklund, 1991), cholinergic neurons transplanted into the lesioned septum (Leanza, Nikkhah, Nilsson, Wiley, & Bjorklund, 1996), and noradrenergic, serotonergic and cortical grafts in spinal cord (Foster et al., 1985; Y. Li & Raisman, 1993; Nornes, Bjorklund, & Stenevi, 1983). The axonal outgrowth pattern was even identified to be target-specific and reproduced the innervation patterns lost by the lesion (Isacson & Deacon, 1996; Nilsson, Clarke, Brundin, & Bjorklund, 1988). In xenograft models, human and porcine fetal VM neuroblasts grafted to the midbrain of 6-OHDA-lesioned rats were seen to efficiently innervate A9 and A10 forebrain targets via axonal extension through the medial forebrain bundle (MFB; Isacson & Deacon, 1996; Wictorin, Brundin, Sauer, Lindvall, & Bjorklund, 1992). The capacity of fetal neuroblasts to reconstruct the nigrostriatal and mesolimbocortical pathways was further confirmed in allograft mouse-to-mouse studies, where GFP-expressing fetal VM tissue harvested from transgenic mice was used for transplantation (Gaillard et al., 2009; L. H. Thompson, Grealish, Kikik, & Bjorklund, 2009). For a successful translational application of PSCs to the clinic, neurons derived from hESCs and iPSCs must display similar axonal outgrowth proper-
ties when placed in the adult lesioned brain. Indeed, neurons derived from human PSCs have been shown to extend long-distance axonal projections when placed in the adult and neonatal central nervous system (CNS; Denham et al., 2012; Lu et al., 2014; Steinbeck, Koch, Derouiche, & Brustle, 2012). Moreover, cortical neurons derived from human and mouse PSC have been shown to innervate host structures in a highly target-specific manner, with projection patterns being faithful to both the laminar identity and the regional phenotype of the transplanted cortical neurons (Espuny-Camacho et al., 2013; Espuny-Camacho et al., 2018; Gaspard et al., 2008; Ideguchi, Palmer, Recht, & Weimann, 2010; Michelsen et al., 2015). Similarly, hESC-derived VM neurons placed in the SN have the capacity to innervate, in a target-specific manner, the dopaminergic A9 and A10 forebrain targets structures and to the same extent as their fetal counterparts (Grealish et al., 2014). These transplantation studies serve as proof-of-concept that the adult CNS has the capacity to guide outgrowing axons over large distances and in a targeted manner, while also revealing that early post-mitotic neuroblasts maintain the capacity to recognize axon guidance cues when placed in the adult lesioned brain.

Dissecting graft function and synaptic integration

Early electron microscopy and electrophysiology studies have provided evidence that transplanted neurons can form synaptic specializations with host neurons, that are to some degree functional (see previous section). However, these methodologies do not allow for a whole-brain comprehensive overview of the extent and anatomical origin of host-to-graft synaptic inputs. While conventional tract-tracing studies do offer some insight into the anatomical origin of afferent innervation to grafted neurons (Doucet et al., 1989), these tracers are associated with technical limitations. First, these tracers do not allow specific targeting of a desired starter neuronal population, i.e., the tracing is initiated by any neuronal or glial population in the anatomical region where it is placed. Moreover, fibers of passage that are damaged during the injection procedure may also uptake the tracer, therefore providing false-positive labelling in regions that do not truly innervate neurons in the region of interest; Second, conventional tracers are progressively diluted as they label across higher order neurons; Third, conventional tracers do not enter neurons via a synapse-dependent mechanism, and as such they do not distinguish between innervation and synaptic coupling (Morecraft, Ugolini, Lanciego, Wouterlood, & Pandya, 2014).

Modern circuit tracing methods based on viral transneuronal tracers (i.e. alpha-herpesviruses and rabies virus) now offer the possibility for a more unambiguous interrogation of host-to-graft and graft-to-host synaptic integration, rather than only the close apposition of graft and host fibers. These techniques build upon the capacity of these neurotropic viruses to be transported along axonal tracts, to spread across synapses and to self-amplify in recipient neurons (Ugolini, 2010).

Monosynaptic tracing with modified rabies virus

Rabies virus is a single-strand negative RNA virus with a simple genome encoding only 5 proteins (Finke & Conzelmann, 2005). The external rabies glycoprotein (rGP) is the element responsible for binding, internalization and transsynaptic spread (Etessami et al., 2000). Rabies particles spread between synaptically-coupled neurons in a retrograde manner, involving loading into transport vesicles at synaptic terminals (Klingen, Conzelmann, & Finke, 2008) and hijacking of the p75 nerve growth factor receptor axonal transport machinery (Gluska et al., 2014).
Figure 4. Schematic representation of the monosynaptic tracing methodology.

Starter neurons (green and red) expressing the TVA receptor and rabies glycoprotein are selectively infectible by EnvA-pseudotyped ΔG-rabies. In starter neurons, rabies particles can transcomplement with the rabies glycoprotein, allowing the virus to spread transsynaptically, and in a retrograde manner, to traced neurons (red only). Traced neurons do not express rabies glycoprotein, therefore, ΔG-rabies cannot spread any further.

Recombinant rabies virus derived from the attenuated SAD-B19 strain, where the gene encoding rGP is deleted (SAD-ΔG), lacks the capacity to spread across synapses (Etessami et al., 2000; Mebatsion, Konig, & Conzelmann, 1996). However, the missing rGP can be transcomplemented by growing SAD-ΔG rabies in cells engineered to express rGP. This form of SAD-ΔG rabies has the capacity to infect neuronal terminals in vivo and to be transported to the cell body (Etessami et al., 2000). However, since the progeny rabies virus lacks rGP, the virus cannot spread across synapses...
Moreover, the rabies genome can be further modified to express fluorescent reporter proteins (Mebatsion, Schnell, Cox, Finke, & Conzelmann, 1996). Building upon these properties, Wickersham and colleagues developed a methodology to map first-order monosynaptic inputs to a defined starter neuron population, by including a fluorescent reporter gene (FRG) in the SAD-ΔG genome (SAD-ΔG-FRG; Wickersham et al., 2007). To tightly restrict the primary uptake of SAD-ΔG-FRG to defined starter cells, the virus was further pseudotyped with an avian retrovirus glycoprotein (EnvA). Hence, the resulting EnvA-SAD-ΔG-FRG virus can only infect starter cells engineered to express the appropriate avian receptor (TVA). Finally, by also forcing the expression of rGP in the starter cells, EnvA-SAD-ΔG-FRG can be transcomplemented with the rGP and spread retrogradely to first-order neurons that are synaptically coupled to the starter neuron. From there, EnvA-SAD-ΔG-FRG cannot spread any further as second-order neurons do not express the rGP (see Figure 4; Wickersham et al., 2007).

This method has been broadly used for circuit tracing in the CNS and peripheral nervous system (PNS). The complementation of this methodology with other technical developments, such as optogenetics (Xu et al., 2016), calcium imaging (Wertz et al., 2015) and tissue-clearing methods (Lerner et al., 2015; Menegas et al., 2015) has provided invaluable insight into neuronal networks connectome and functionality. In particular, the expression of rGP and TVA receptor can be targeted to specific Cre-expressing neurons to create genetically defined starter populations. Using Cre transgenic mice, this methodology has already yielded novel insight into the connectivity of the BG (Paget et al., 2016; Klug et al., 2018; J. B. Smith et al., 2016; Wall, De La Parra, Callaway, & Kreitzer, 2013; Watabe-Uchida et al., 2012) and other neuronal systems (Ogawa, Cohen, Hwang, Uchida, & Watabe-Uchida, 2014; Pollak Dorocic et al., 2014; Schwarz et al., 2015). Other applications of this methodology have allowed for retrograde mapping of inputs into newborn neurons in the dentate gyrus and olfactory bulb (Deshpande et al., 2013; Vivar et al., 2012). An important limitation of this methodology however, is that rabies virus infection can cause neurodegeneration after infection periods longer than seven days, and is therefore difficult to combine with functional manipulations or recordings. Recent developments have seen the establishment of “self-inactivating” or non-toxic forms of rabies tracing that can spread retrogradely and leave a permanent genetic “fingerprint” in the traced neurons (Chatterjee et al., 2018; Ciabatti, Gonzalez-Rueda, Mariotti, Morgese, & Tripodi, 2017). These “second generation” rabies viruses now present a promising tool-set for permanent genetic access to first-order neurons, and will enable more complex and long-term functional studies. Rabies virus can be further modified to carry the genes for a designer receptor exclusively activated by designer drugs (DREADDs) or channelrhodopsin, allowing for the specific manipulation of pre-synaptically labeled neurons (Ciabatti et al., 2017; Reardon et al., 2016).

Of particular interest for this thesis, is the possibility to apply the monosynaptic tracing methodology for assessing the synaptic integration of both transplanted and in vivo converted neurons (Grealish et al., 2015; Torper et al., 2015). We and others have used monosynaptic tracing to demonstrate that transplanted neurons can integrate into host circuitry, while also describing in detail the anatomical origin and extent of these afferent inputs in different disease paradigms (Adler, Lee-Kubli, Kumamaru, Kadoya, & Tuszyński, 2017; Doerr et al., 2017; Falkner et al., 2016; Grealish et al., 2015; Tornero et al., 2017). The application of monosynaptic tracing and the description of factors influencing synaptic integration in the grafted parkinsonian rat brain is the central theme of this thesis, and is discussed in detail in the results section.
Aims of the thesis

1. Assess the capacity of hESC-derived VM neurons to integrate host circuitry after transplantation to the parkinsonian rat brain using monosynaptic rabies tracing (Paper I).

2. Evaluate the capacity for \textit{in situ} converted neurons to integrate into host circuitry using monosynaptic rabies tracing (Paper II).

3. Evaluate the capacity for hESC-derived VM neurons to innervate correct forebrain structures and to receive appropriate synaptic inputs following transplantation into the substantia nigra of parkinsonian rats (Paper III).

4. Investigate the role of graft phenotype, graft placement and extent of host dopamine depletion on the connectivity between graft and host neurons (Paper IV).

5. Investigate the specific host-to-graft connectivity of A9- and A10-projecting neurons within the transplant, using a novel AAV and ΔG-rabies two-factor monosynaptic tracing approach (Manuscript under preparation)
Summary of results and discussion

Although 40 years have passed since the first transplantation studies in rodent models of PD were performed, the extent to which transplanted DA neurons integrate into the parkinsonian brain to elicit a more thorough reconstruction of the damaged host circuitry is still an open question. To address this question, a methodology to assess synaptic integration of transplanted and newly-generated neurons is presented herein (Paper I and II). In the subsequent studies, the extent and specificity of graft-derived innervation and synaptic integration, as well as the cell intrinsic and host variables influencing these outcomes, are evaluated (Paper III and Paper IV).

Establishing a rabies-based monosynaptic tracing approach to evaluate host-to-graft and graft-to-host synaptic integration (Paper I)

Protocols to generate bona-fide mesencephalic DA neurons in vitro (Kirkeby et al., 2012; Kriks et al., 2011; Nolbrant et al., 2017) now allow for a reproducible source of transplantable DA neurons that survive, innervate and function with equal potency as their fetal counterparts in vivo (Grealish et al., 2014).

Thus far, technical limitations to date have hindered the understanding of the full extent to which transplanted neurons can integrate into the pre-existing host circuitry. Traditional methods to analyze graft integration have relied on the combination of conventional tract tracing, electron microscopy and electrophysiology (Fisher et al., 1991; Freund et al., 1985; L. Thompson, Barraud, Andersson, Kirik, & Bjorklund, 2005; Tonnesen et al., 2011). However, these methodologies cannot produce a whole-brain “map” of the anatomical origin of host synaptic inputs, nor provide a comprehensive understanding of the extent of these inputs.

In this first study, we applied a rabies-based monosynaptic tracing method (Wickersham et al., 2007), widely used for mapping existing neuronal circuits in vivo (Miyamichi et al., 2011; Wall et al., 2013; Watabe-Uchida et al., 2012), to the transplantation context. Rabies virus has the characteristics to spread exclusively between synaptically coupled neurons in a retrograde manner. Here, a modified form of the rabies virus (ΔG-rabies), where the gene coding for rGP (necessary for transsynaptic spread) is replaced by a fluorescent protein (mCherry) and the envelope is pseudotyped (EnvA) to only infect TVA-expressing cells, was utilized as a transsynaptic retrograde tracer. By engineering cells to express TVA and rGP, we can specify starter neurons that are selectively infectible by EnvA-pseudotyped ΔG-rabies, and from where ΔG-rabies can subsequently spread across one synapse to first-order presynaptic neurons (traced neurons). Once there, ΔG-rabies cannot spread any further as traced neurons lack rGP (see Figure 4). We used this method to map the origin and extent of host synaptic inputs to grafted neurons (host-to-graft) and to assess the establishment of graft-to-host connectivity.
Figure 5. In vivo validation of the monosynaptic tracing methodology

(A, B) Schematic representation of the tracer and control lentiviral vectors used in this thesis. The tracing vector comprises of histone-tagged GFP, TVA receptor necessary for EnvA-pseudotyped ΔG-rabies infection and rabies rGP that allows ΔG-rabies to transmit across synapses. The control vector lacks rGP and thus ΔG-rabies cannot be transmitted transsynaptically.

(C) Injection of the tracing lentivirus to the rat striatum leads to the expression of nuclear GFP. After EnvA-ΔG-rabies injection, mCherry+ signal is observed co-localized with GFP+ nuclei. These GFP+/mCherry+ neurons are termed starter neurons. Starter neurons express rGP, hence ΔG-rabies can be transmitted retrogradely to label traced neurons.

(D-F) Traced mCherry+/GFP neurons were found in regions known to project to striatum, including the prefrontal cortex (D), thalamus (E) and substantia nigra pars compacta (F). From traced neurons, ΔG-rabies cannot transmit further due to the lack of GP.

(G-I) In animals injected with the control vector (lacking GP), no traced neurons (mCherry+/GFP) were detected in the brain, confirming that rGP is required for monosynaptic tracing events to occur.

PFC, prefrontal cortex; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STR, striatum; and Thal, thalamus. Scale bars represent 250 mm (C), 50 mm (D), 100 mm (E-I).
Figure 6. Host-to-graft synaptic integration of transplanted hESC-derived neurons

(A) Schematic illustration of generation of starter hESCs.
(B) Transplanted cells in the striatum expressed nuclear GFP.
(C) ΔG-rabies selectively infected the transplanted hESC-derived neurons.
(C') Close-up of the transplant core reveals that almost all GFP+ cells co-expressed rabies mCherry.
(C'') mCherry+/GFP− cells were detected in the host striatum at the edge of the graft, representing traced host neurons that connected to the transplant.
(D–F) Traced host neurons could also be detected in structures known to provide afferent inputs to the striatum and distal to the graft, including the prefrontal cortex (D), thalamus (E), and substantia nigra (F).

PFC, prefrontal cortex; SN, substantia nigra; STR, striatum; T, transplant; Thal, thalamus. Scale bars represent 400 mm (B and C), 25 mm (C'', D and E), and 50 mm (C' and F).
System validation *in vivo*

To validate the system *in vivo*, we injected a polycistronic lentiviral tracing vector (i.e., rabies helper) expressing histone-tagged GFP, TVA receptor and rGP (Figure 5A), driven by the synapsin promoter, into the striatum of naïve Sprague-Dawley rats. 4 weeks later, EnvA-ΔG-rabies was injected at the same site. When analyzed 1 week after EnvA-ΔG-rabies injection, a large number of GFP⁺/mCherry⁺ *starter* neurons were detected throughout the striatum (Figure 5C). Whole-brain analysis revealed the presence of GFP⁺/mCherry⁺ *traced* neurons in the cortex (Figure 5D), thalamus (Figure 5E), SNc (Figure 5F) and other brain structures. To control for unspecific labelling, we utilized a control vector (containing GFP and TVA but lacking rGP; Figure 5B), in a parallel experiment. Upon EnvA-ΔG-rabies infection, we also detected large numbers of GFP⁺/mCherry⁺ at the injection site of those animals. However, no tracing events (GFP⁻/mCherry⁺) were detected in these brains (Figure 5 G-I), confirming the dependence on rGP expression for transsynaptic spread of EnvA-ΔG-rabies.

Host-to-graft synaptic integration

After the system was validated *in vivo*, we applied it in a transplantation context in order to trace host-to-graft connectivity of transplanted hESC-derived neurons. For that purpose, hESCs lines that stably express the tracing construct were generated *in vitro* (Figure 6A). Then, these cells were differentiated to VM progenitors (Kirkeby et al., 2012) and transplanted into the striatum of 6-OHDA-lesioned Sprague-Dawley rats. 6 weeks post-transplantation, rats were injected with EnvA-ΔG-rabies at the site of transplantation and were perfused for histological analysis 7 days later. Grafted neurons were readily detectable by GFP immunostaining (Figure 6B) and a majority of GFP⁺ neurons were seen to be infected by EnvA-ΔG-rabies (Figure 6 C-C'). *Traced* neurons expressing mCherry but negative for GFP (GFP⁻/mCherry⁺) – hence representing host neurons that established monosynaptic contacts onto graft neurons – were found both in the vicinity of the transplant, corresponding to host striatal MSNs (Figure 6C’), and in regions distal to the graft, including the prefrontal cortex (Figure 6D), thalamus (Figure 6E), amygdala (not shown), dorsal raphe nucleus (not shown) and substantia nigra (Figure 6F).

Graft-to-host connectivity

We next sought to interrogate if the transplanted neurons themselves can equally establish local and distal afferent synaptic connectivity with host neurons, using a “reverse” approach of the mono-synaptic tracing methodology described above. For this purpose, 6-OHDA-lesioned athymic nude rats were first injected with the lentiviral tracing vector either into the striatum or prefrontal cortex (PFC). Four weeks later, wild-type hESC-derived VM progenitors were transplanted into the striatum and left to mature for 6 months *in vivo*. Next, EnvA-ΔG-rabies was injected at the same site of the original lentiviral injection to selectively infect host neurons (Figure 7A and F). Hence, host cortical or striatal neurons would be *starter* neurons, from where EnvA-ΔG-rabies could spread retrogradely to grafted neurons, and grafted neurons would be *traced* neurons if they established a direct presynaptic connection with a postsynaptic host *starter* neuron. Analysis of the animals that received lentiviral injection in the striatum (Figure 7 A-C), revealed the presence of several host GFP⁺/mCherry⁺ neurons surrounding the graft (HuNu⁺; Figure 7D). A closer look at the transplant revealed the presence of several mCherry⁺/HuNu⁺ neurons, indicating that grafted neurons can also establish post-synaptic connectivity with local host neurons (Figure 7E). In the PFC-injected animals (Figure 7 F-H), graft-
derived fibers (hNCAM+) could be seen reaching prefrontal cortex (Figure 7H) in close proximity with GFP+/mCherry+ host starter neurons (Figure 7I). Analysis of the graft core in striatum, also revealed the presence of mCherry+/HuNu+ neurons which is indicative of distal graft-to-host connectivity (Figure 7J).
To conclude, we established a tracing methodology that allows for a whole-brain, high-throughput analysis of both local and distal host-to-graft and graft-to-host connectivity, serving as a valuable complement to electrophysiology, conventional tracers and electron microscopy in the study of graft circuitry integration. Using this technique, we have shown that transplanted neurons integrate into the host circuitry to a larger extent than what was commonly thought possible (Tonnesen & Kokaia, 2012), and in a pattern consistent with the known anatomy of striatal afferents (Gerfen & Wilson, 1996) and with the striatal endogenous inputs mapped in this study (Figure 5). We also confirmed that transplanted neurons establish monosynaptic contacts onto host neurons, as has been previously suggested (Bolam, Freund, Bjorklund, Dunnett, & Smith, 1987; Freund et al., 1985; Sorensen et al., 2005; Tonnesen et al., 2011).

Synaptic integration of new neurons generated by in vivo reprogramming as assessed by monosynaptic tracing (Paper II)

Direct conversion of non-neuronal cells into neurons in vivo is a promising alternative approach to brain repair after injury or in neurodegenerative diseases. By viral-mediated expression of transcription factors, resident glial cells can be directly converted into neurons in vivo (Torper & Gotz, 2017). While offering a promising alternative to transplantation as a cell replacement therapy for PD, the identity and functionality of the newly generated neurons in vivo still needs to be resolved. In this study, we utilized Cre-dependent adeno-associated virus (AAV) to convert NG2-expressing glia into neurons in situ. Moreover, we used the monosynaptic tracing approach to investigate the capacity of in vivo converted neurons to integrate into the pre-existing host circuits.

AAV-mediated conversion of NG2 glia into neurons in vivo

To convert NG2-expressing glia to neurons in vivo, the reprogramming genes Ascl1, Lmx1a and Nurr1 (ALN), a combination previously described to convert mouse fibroblasts and glia to DA neurons in vitro (Addis et al., 2011; Caiazzo et al., 2011), was used in this study. For that purpose, the genes flanked by two pairs of antiparallel heterotypic loxP sites (Atasoy, Aponte, Su, & Sternson, 2008), were inserted in an antisense direction into Cre-dependent, flip-excision (FLEX) AAV vectors. The three conversion factors were placed under the control of the ubiquitous chicken b-actin (cbα) promoter (Figure 8A). To identify converted neurons, a GFP reporter was placed in a FLEXed AAV vector under the control of the neuron-specific synapsin promoter (Figure 8A). The reprogramming and reporter vectors were then injected into the striatum of NG2-Cre transgenic mice (Figure 8B). Hence, the conversion factors were exclusively expressed in NG2-expressing glia, while the GFP reporter is activated only in converted neurons originating from Cre-expressing cells. The animals were then sacrificed at different time points and analyzed for the presence of GFP+ converted neurons. By 4 and 12 weeks, several GFP+ converted neurons were identified in the striatum (Figure 8C), with quantifications revealing 6.912 ± 3.052 converted cells per animal at 4 weeks (Figure 8D). However, phenotypic characterization revealed that none of the converted neurons expressed TH (Figure 8E).
Monosynaptic tracing reveals local synaptic integration of reprogrammed neurons

Functional neuronal properties, using electrophysiological methods, have been described for in-vivo-reprogramed neurons (Heinrich et al., 2010; Niu et al., 2013). However, these methods cannot confirm the origin of the synaptic inputs to the newly generated neurons. We next implemented the monosynaptic tracing methodology in the in vivo conversion paradigm. For that purpose, we constructed a FLEXed AAV tracing vector containing the histone tagged GFP, TVA receptor and rGP under the synapsin promoter control (Figure 9A). This vector was injected in combination with the conversion factors into the striatum of NG2-cre mice. Hence, the tracing vector was selectively activated only in converted neurons originating from NG2 glia. After 12 weeks, EnvA-ΔG-rabies was injected into the same location in order to map the presynaptic inputs to the newly converted neurons, and animals were perfused one week later (Figure 9B). Histological analysis revealed the presence of converted neurons that were infected by EnvA-ΔG-rabies (GFP+/mCherry+) in the striatum (Figure 9C). Interestingly, several GFP+/mCherry+ neurons that express DARPP-32 were located in the striatum of these animals, representing local neurons that established a synaptic connection to the newly converted neurons (Figure 9 C-D”). However, no mCherry+ was detected outside the striatum, indicating that converted neurons only integrate into local circuitry (Figure 9 E-G). As a control, a FLEXed AAV containing the TVA receptor, but lacking the rGP, was injected together with the re-
programming factors in a parallel experiment. No tracing events (GFP+/mCherry+) were detected in the striatum of these animals.

In short, we have successfully applied the monosynaptic tracing methodology to a direct in-vivo conversion model, in order to investigate the capacity of newly generated neurons to integrate into pre-existing circuitry. We have shown the validity of utilizing the Cre recombinase system and FLEXed AAVs to deliver the rabies helper construct to genetically-defined neuronal populations, highlighting the versatility of this methodology.

Intranigral grafting in a rat model of Parkinson’s disease reveals target-specific axonal outgrowth and appropriate host synaptic input (Paper III)

An important predictor of successful DA graft function in PD patients, is the capacity for transplanted neurons to elicit long distance axonal outgrowth towards appropriate, and functionally relevant, host anatomical structures (W. Li et al., 2016). However, the majority of pre-clinical transplantation studies performed to date rely on transplanting DA neurons ectopically into the striatum, which lowers the distance requirement for graft-derived fibers to reach their target area, and limits long-distance axonal outgrowth investigations (Doi et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011). In this study, hESC-patterned VM neurons were transplanted homotopically into the midbrain.
of parkinsonian rats, allowing us to study the potential for these neurons to elicit target-directed, long-distance innervation of appropriate host structures. Moreover, taking advantage of the homotopic nature of this grafting paradigm, we utilized monosynaptic tracing to investigate the capacity for transplanted VM-patterned neurons to receive anatomically appropriate synaptic inputs from the host when placed in the midbrain.

Temporal assessment of graft-derived axonal outgrowth towards appropriate forebrain targets

To study the temporal profile of graft-derived axonal outgrowth, hESC-derived VM neurons were transplanted into the substantia nigra of 6-OHDA-lesioned rats, and animals were perfused at 6, 18 and 24 weeks post-grafting (Figure 10A). Histological analysis of the graft core at the three time points, revealed grafts rich in TH$^+$ neurons (Figure 10 B-B’’), that do not increase in volume over time (Figure 10C). Three-dimensional analysis of graft-derived fiber outgrowth, based on human-specific hNCAM immunostaining (see methods), revealed progressive axonal outgrowth along the MFB and the NSP, towards forebrain target structures, reaching as far as the prefrontal cortex (Figure 10 D-D’’). The forebrain target areas of A9 (dorsolateral striatum) and A10 (NAc and prefrontal cortex) mesencephalic DA neurons, were progressively innervated by graft-derived fibers over time (Figure 10 E-G’’), indicating that transplanted neurons elicited a specific, long-distance innervation of appropriate forebrain target regions.

Graft-derived dopaminergic innervation of dorsolateral striatum

Dopaminergic reinnervation of the dorsolateral striatum (DLS) is required for functional recovery in rat models of PD (Bjorklund, Dunnett, et al., 1980; Dunnett et al., 1983). While ectopic grafts of VM DA neurons in the striatum can strongly reverse amphetamine-induced motor asymmetries, the same has not been observed with homotopic grafting to the nigra (Winkler, Kirik, Bjorklund, & Dunnett, 2000). We next sought to assess the level of DA-specific innervation derived from the graft, by analyzing and quantifying the presence of hNCAM$^+$/TH$^+$ fibers in the DLS. Moreover, amphetamine induced rotations were performed at the three different timepoints to assess graft functionality. At 6 weeks, no double positive fibers were detected in DLS (Figure 11A and D) and no improvement in rotational bias was observed (Figure 11E). In contrast, at 24 weeks, several hNCAM$^+$/TH$^+$ fibers were present in the DLS (Figure 11B and D), which correlated with a reduction of amphetamine-induced rotational bias in two animals (grey and white circle in Figure 11E). Interestingly, in one grafted animal analyzed at 24 weeks, which showed absence of dopaminergic innervation in the DLS (Figure 11C), no recovery was seen in the amphetamine-induced rotation test (dark circle in Figure 11E). Taken together, these results reinforce the importance of proper dopaminergic innervation of the DLS for functional recovery, while demonstrating the capacity for hESC-derived DA neurons to properly reinnervate their forebrain target region long distances from the graft site.

Transplanted neurons in the midbrain receive a functionally-appropriate set of host synaptic inputs

We next used monosynaptic tracing to map the host inputs to hESC-derived VM-patterned cells transplanted into the substantia nigra. For this purpose, hESCs expressing the rabies helper con-
struct, as described in Paper I, were utilized for transplantation. EnvA-ΔG-rabies was then injected at the site of transplantation 5 weeks after grafting, and animals were sacrificed one week later (Figure 12A). As expected, histological analysis revealed that graft neurons were selectively infected by EnvA-ΔG-rabies, as revealed by the presence of GFP+ / mCherry+ neurons in the graft core (Figure 12B). Moreover, up to 37% of the starter neurons had matured into a dopaminergic phenotype
Figure 10. Temporal assessment of graft-derived innervation

(A) Schematic overview of the experimental plan for temporal assessment of graft-derived axonal outgrowth in intranigral transplants.
(B-B”) TH immunohistochemistry reveals location and size of the transplant at 6 weeks (B), 18 weeks (B’) and 24 weeks (B’’).
(C) Quantification of graft volume reveals no significant change in the three timepoints.
(D-D’’) 3D representation of graft-derived innervation based on hNCAM immunohistochemistry at 6 weeks (D), 18 weeks (D’) and 24 weeks (D’’).
(E) Quantification of the volume percentage innervated by the transplant over time demonstrates the progressive ramification of hNCAM+ fibers in host A9 and A10 target structures.
(F-G’’) Analysis of progressive graft-derived innervation, as revealed by hNCAM immunohistochemistry, reveals that 6 weeks post-transplantation no detectable fibers were seen in PFC (F) and only a few scattered axonal terminals were present in the dorsolateral striatum (G). At 18 weeks, hNCAM+ fibers were observed in the PFC (F’) and in the dorsolateral striatum (G’). At 24 weeks, abundant hNCAM+ innervation was readily detectable in the PFC (F’’) and dorsolateral striatum (G’’).

fmi = forceps minor; GP, globus pallidus; Hip, hippocampus; NAc, nucleus accumbens; PFC, prefrontal cortex; Sep, septum; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; Str, striatum; TH, tyrosine hydroxylase; Tha, thalamus; Tx, transplant. Scale bars represent 200 µm (B, B’ and B’’), and 100 µm (F-G’’).

(GFP+/mCherry+/TH+; Figure 12 C-C’’). Three-dimensional analysis of tracing events throughout the brain (see methods), revealed extensive synaptic integration of the transplanted neurons into the host circuitry (Figure 12D). Specifically, mCherry+ neurons were detected in host structures including the somatosensory cortex (Figure 12E), striatum (Figure 12F), hypothalamus (Figure 12G) and dorsal raphe nucleus (Figure 12H). In a parallel experiment, we utilized lentiviral tracing vectors and EnvAΔG-rabies, as described in Paper I, to map the presynaptic inputs to endogenous VM neurons (Figure 12A and I). Interestingly, the pattern of synaptic input to transplanted neurons and to endogenous neurons were similar (compare Figure 12D and I), and in accordance with known anatomical afferents to the region (Gerfen & Bolam, 2010; Watabe-Uchida et al., 2012). These results indicate that VM-patterned graft neurons are able to extensively integrate into host circuitry and receive the correct afferent synaptic inputs from the host when placed in their proper anatomical region.

In paper III, we confirmed previous observations that transplanted hESC-derived VM neurons can innervate across long-distances (up to 10 mm) to innervate appropriate target structures in the forebrain (Grealish et al., 2014), a distance observed in post-mortem tissue to be sufficient to provide full reinnervation of the putamen of PD patients transplanted with multiple deposits of fetal tissue (W. Li et al., 2016). Moreover, we have shown that grafted DA neurons can project along the NSP towards the DLS, to elicit functional recovery as assessed by amphetamine-induced rotations. Finally, we have shown that grafted neurons have the capacity to receive the correct set of host presynaptic inputs when in their homotopic location. Taken together, these results support the capacity of graft-derived DA neurons to elicit a more extensive repair of the damaged circuitry in PD.
Figure 11. Graft-derived dopaminergic innervation of dorsolateral striatum and related functional outcome
(A–C) Analysis of double positive hNCAM+/TH+ fibers in the dorsolateral striatum at 6 weeks (A) revealed the absence of graft-derived dopaminergic innervation at this stage. After 24 weeks, significant hNCAM+/TH+ innervation was seen in the dorsolateral striatum (B) which correlated with behavioral improvement (white and grey circle in E). Analysis of graft-derived dopaminergic innervation in one animal in the 24-week group with no behavioral recovery (dark circle in E) showed sparse hNCAM+/TH+ fibers reaching the dorsolateral striatum (C).
(D) Quantification of hNCAM+/TH+ positive fibers in the dorsolateral striatum revealed progressive increase in the density of graft-derived dopaminergic fibers in this structure over time (Kruskal–Wallis, $\chi^2_{2}\text{-df} = 7.658$, $p < .01$, Dunn’s multiple comparisons test revealed a significant difference between the 6 and 24 week groups, $p < .05$).
(E) Assessment of amphetamine-induced rotations before and after transplantation revealed progressive graft mediated functional recovery over time.
Data presented as mean ± SEM, * = $p < .05$; DL, dorsolateral; Scale bars represent 100 µm (A, B and C).
Figure 12. hESC-derived neurons placed in the midbrain receive appropriate synaptic input from the host

(A) Experimental design for assessment of endogenous and host-to-graft connectivity using monosynaptic tracing. 

(B-C'') Histological analysis of transplants in the midbrain revealed that grafted neurons expressed GFP and were selectively infected by rabies (B). A significant proportion of starter neurons (GFP/mCherry+) also expressed TH (C-C'').

(D) 3D representation of whole brain monosynaptic inputs to grafted neurons at 6 weeks. Each dot represents the location of an identified traced neuron in one representative animal.

(E-H) Analysis of traced neurons (GFP/mCherry+) in the host brain revealed extensive synaptic inputs to grafted neurons originating from regions known to project to the midbrain, including the somatosensory cortex (E), striatum (F), hypothalamus (G), and dorsal raphe nucleus (H).

(I) 3D representation of endogenous synaptic inputs to the midbrain of un-lesioned rats, revealed a pattern of connectivity similar to that observed in transplanted animals (compare with D).

Ctx, cortex; GP, globus pallidus; Hyp, hypothalamus; PFC, prefrontal cortex; PVH, paraventricular hypothalamic nucleus; RN, raphe nucleus; SN, substantia nigra; Som ctx, somatosensory cortex; Str, striatum; Tha, thalamus; VM str, ventromedial striatum. Scale bars represent 100 µm (B), 40 µm (C, C' and C''), and 200 µm (E-H).
Cell intrinsic factors and host environment affect graft-derived innervation and host synaptic input (Paper IV)

In the previous work, we described the capacity for transplanted neurons to project long distances in a targeted manner, while also revealing the extent to which they can integrate into host circuitry. In paper IV, we further explore the effect of graft phenotype, placement and extent of host lesion on graft-derived axonal outgrowth and synaptic integration. By understanding the dominant factors controlling host-graft connectivity, we aim to collect information that may allow further refinement of hESC-derived VM neurons cell therapy for the treatment of PD.

Target-specific innervation by transplanted neurons is determined by cell phenotype

When placed in the midbrain of 6-OHDA-lesioned rodents, transplanted human DAergic neuroblasts can innervate appropriate forebrain targets (Cardoso, Adler, et al., 2018; Grealish et al., 2014; Wictorin et al., 1992). However, these studies do not reveal whether grafted cells intrinsically dictate axonal outgrowth patterns, or if this outgrowth is mandated by host guidance cues regardless of graft cell phenotype. In this study, specifically, we wanted to elucidate if the specificity of axonal outgrowth is dependent on the correct phenotype of the grafted neurons, or if any neuronal cell type placed in the DA depleted host “milieu” would naturally outgrow axonal projections via the lesioned MFB towards the forebrain. For that purpose, hESC-derived progenitors were patterned either to a VM fate, as performed in the previous studies of this thesis, or to a forebrain (FB) fate as described in Kirkeby et al, 2012, and grafted into the midbrain of 6-OHDA lesioned athymic nude rats (Figure 13A). 24 weeks later, the brains were analyzed and revealed that both groups contained grafts of similar size and were discretely placed in the midbrain (Figure 13B and D). As expected, only VM-patterned grafts contained TH+ neurons (Figure 13C and E), showing that VM- and FB-patterned grafts generate distinct neuronal phenotypes in vivo. Analysis of graft-derived innervation, as visualized by hNCAM immunostaining, revealed a distinct pattern of innervation (Figure 13F and G). While VM-patterned neurons innervated A9 and A10 target structures as described in the previous study, FB-patterned neurons scarcely innervated the striatum and showed extensive axonal outgrowth towards cortical structures instead (Figure 13 H-K). Hence, we show that the profile of graft-derived innervation of host, is dependent on the type of cell transplanted, and is therefore determined by cell intrinsic factors.

Host-to-graft integration determined by graft placement

We next sought to assess the effect of graft placement and phenotype on host-to-graft synaptic integration, using rabies-based tracing. VM- and FB-patterned cells expressing the rabies helper construct were grafted into the midbrain, or to the striatum of parkinsonian rats. 24 weeks later, EnvA-DG-rabies was injected into the site of transplantation to trace host presynaptic inputs to grafted neurons (Figure 13A). Whole-brain analysis of tracing events (GFP/mCherry+ neurons), revealed that graft neurons received a distinct set of synaptic inputs from the host, when placed in a different location (Compare Figure 14 A to C and 14B to D). Curiously, graft phenotype had no detectable effect on host-to-graft synaptic integration at this level of analysis, as the origin of presynaptic inputs to VM- and FB-patterned neurons when in the same location originated from the same anatomical re-
Figure 13. Graft-derived innervation is determined by graft cell phenotype

(A) Schematic overview of the experimental setup to assess graft-derived fiber outgrowth and synaptic integration
(B and C) hESC-derived ventral midbrain neurons placed into the substantia nigra (B) generate grafts rich in dopamine TH+ neurons (C).
(D and E) FB-patterned grafts (D) do not generate TH+ neurons in vivo (E), showing that VM- and FB-patterned graft have a different neuronal phenotype.
(F and G) Schematic representation of graft-derived innervation reveals VM-patterned and FB-patterned neurons innervate different host structures in the forebrain.
(H-K) hNCAM staining reveals that VM-patterned neurons extensively innervate the dorsolateral striatum (H) and only scarcely the host cortex (I). The inverse was seen for FB-patterned neurons (J and K).

ac, anterior commissure; dlStr, dorsolateral striatum; FB, forebrain; fmi, forceps minor; hNCAM, human neural cell adhesion molecule; HuNu, human nucleus; INS, insular cortex; NAc, nucleus accumbens; PFC, pre frontal cortex; Sep, septum; Str, striatum; TH, tyrosine hydroxylase; VM, ventral midbrain. Scale bars represent 100 µm (B and D), 50 µm (C and E) and 20 µm (H-K).
Figure 14. Effect of graft placement and phenotype on synaptic integration

(A-D) Schematic representation of host synaptic inputs to grafted neurons reveals that graft placement determines the anatomical origin of host inputs (compare A to C and B to D). Graft phenotype had no detectable effect on synaptic integration (compare A to B and C to D). Each dot represents a traced neuron (mCherry+) in a 1:8 series from of mCherry stained sections.

(E and F) Quantification of the percentage of traced neurons in each anatomical structure over the total number of traced neurons per brain, confirms observations above. Data presented as mean + SEM.

Amyg, amygdala; FB, forebrain; GPe, globus pallidus; Hyp, hypothalamus; SN, substantia nigra; Thal, thalamus; VM, ventral midbrain.
Figure 15. Phenotypic description of synaptic inputs to ectopic and homotopic VM-patterned grafts

(A-F) Fluorescent immunohistochemistry reveals that CTIP2+ pyramidal neurons in the motor cortex (A and B), MOR+ medium spiny neurons in the striatum (C and D) and PV+ neurons in the globus pallidus connect to both ectopic (intrastriatal) and homotopic (intranigral) grafts containing VM-patterned neurons.

(G) Schematic summary of the origin of afferent inputs to VM neurons grafted in the striatum (left) and substantia nigra (right). Solid lines represent anatomical regions with considerable synaptic input to transplanted neurons, while dashed lines represent structures with sparse inputs.

Hyp, hypothalamus; MOR, µ-opioid receptor; SN, substantia nigra; Str, striatum; thal, thalamus. Scale bars represent 20 µm (A-F).
regions (Compare Figure 14 A to B and 14C to D). Quantification of traced events per anatomical region confirmed this observation (Figure 14E and F). Taken together, these results indicate that host-to-graft synaptic integration seem to be largely determined by placement within the host and that graft phenotype had no influence in the origin of afferent input. However, considering that hESC-derived neurons express TVA and rGP under a pan-neuronal synapsin promoter, any subtype of neuron in the heterogenous transplant will be a potential starter neuron.

Figure 16. Synaptic integration of hESC-derived neurons transplanted to intact vs 6-OHDA lesioned rats

(A-B) hESC-derived VM-patterned cells grafted in the striatum of intact (A) or 6-OHDA-lesioned rats (B) generate grafts of comparable size that are selectively infected by EnvA-ΔG-rabies in an equal manner.

(C) Quantification of the total number of traced neurons per brain reveals no significant difference in the overall extent of synaptic integration between both groups (not significant in unpaired two-tailed test).

(D-E) Histological analysis of rabies-labelled cells in the globus pallidus reveals more tracing events in this region in lesioned rats (E) compared to intact rats (D).

(F) Quantification of the percentage of synaptic inputs per host structure confirms the difference in synaptic inputs from the globus pallidus to intact vs lesioned rats (two-way ANOVA with Bonferroni-corrected post-hoc testing, p < .05)

Data presented as mean + SEM. * = p < .05; ctx, cortex; GPe, globus pallidus; PF, parafascicular thalamic nucleus; Str, striatum.

Scale bars represent 500 µm (A and B), and 200 µm (D and E).
Phenotypic description of synaptic inputs to ectopic hESC-derived VM-patterned grafts

In clinical trials performed to date, DA neurons have been transplanted in the striatum of PD patients, thereby maximizing the reinnervation of this structure to a level required for functional recovery (Barker et al., 2013). However, the ectopic placement of these cells could seem to imply that the full repertoire of “natural” presynaptic inputs – that would interact with the transplant if placed in its homotopic location – may be lacking in this setting. Indeed, we showed above that VM-patterned cells received a distinct set of synaptic inputs from the host when placed in striatum or substantia nigra (Figure 14). Nonetheless, a certain overlap in presynaptic inputs to ectopic and homotopic grafts was observed in functionally relevant anatomical structures for motor control, including those from the striatum, motor cortex and GPe (Figure 14). To assess if the phenotype of the traced neurons in these three structures are the same in both transplantation paradigms, we performed immunohistochemical staining for relevant molecular markers that identify neurons known to innervate both the striatum, and the substantia nigra via collateral projections (Molyneaux, Arlotta, Menezes, & Macklis, 2007; Saunders, Huang, & Sabatini, 2016; J. B. Smith et al., 2016). Indeed, traced neurons expressing CTIP-2 (marker for corticofugal neurons) in cortex, MOR (marker for direct pathway MSNs) in the striatum and parvalbumin (marking a subpopulation with projections to the striatum, STN and SNr/c) in the GPe were detected in both groups (Figure 15 A-F), which points to the possibility that ectopically placed neurons are nevertheless regulated by anatomically appropriate host neurons involved in BG motor control (Figure 15G).

Effect of dopamine lesion on the synaptic integration of graft neurons

Previous studies have shown that lesion of the host DA system may improve fiber outgrowth of transplanted neurons (Doucet, Brundin, Descarries, & Bjorklund, 1990; L. Thompson et al., 2005). Here, we used monosynaptic tracing to investigate the effect of DA depletion on synaptic integration, by transplanting hESC-derived neurons into the striatum of intact vs 6-OHDA-lesioned rats. At 6 weeks post-transplantation, histological analysis revealed that graft survival, placement and EnvA-ΔG-rabies infection was comparable in both groups (Figure 16A and B). Analysis of tracing events (GFP/mCherry⁺), revealed that transplanted neurons receive synaptic inputs from a similar number of host neurons in both groups (Figure 16C), with inputs originating from the same anatomical regions (Figure 16F). Interestingly, a quantification of the percentage of input per region revealed that grafted neurons receive more inputs from the GPe when placed in lesioned rats (Figure 16 D-F). This region has been described to change its firing rate and pattern following lesion of the DA system (Mastro et al., 2017), while another study using monosynaptic tracing demonstrated that rabies labeling in the GPe increased with higher firing activity (Beier et al., 2017). Taken together, these results suggest that this structure may play an important role in modulating the graft neurons in lesioned rats, and may have future implications for the stratification of patient populations based on the state of PD progression.

We have shown in Paper IV that variables relevant for clinical translation, including the type of cell transplanted, the location of the graft, and the lesion environment, influence graft-derived innervation and host-to-graft synaptic integration, providing valuable information for better understanding and optimizing graft function in the clinical setting.
Circuit integration of hESC-derived neurons based on their target projections as assessed by a novel two factor monosynaptic tracing (manuscript under preparation)

Despite the continuous efforts to establish more stringent differentiation protocols (Kirkeby, Nolbrant, et al., 2017), hESC-derived VM neurons still generate highly heterogeneous transplants when placed in vivo. We have shown in Paper III that transplanted neurons innervate both forebrain targets of A9 and A10 DA neurons, suggesting the presence of both populations in the graft. Moreover, in Paper IV we show that VM-patterned grafts also contain considerable amounts of non-DAergic neurons. One important limitation associated with the host-to-graft monosynaptic tracing studies described in this thesis, was the non-restricted expression of the synapsin-driven tracing construct (hisGFP-TVA-rGP) to all neuronal populations in the transplant. Therefore, any grafted neuron is a potential starter neuron. Indeed, when we quantify the number of graft-derived TH+ neurons infected by EnvA-DG-rabies, we observed that up to 37% of the starter neurons were of the dopaminergic phenotype. Hence, with this setup, we cannot identify which of the host neurons are specifically connected to the dopaminergic neurons in the transplant. In light of this, we have designed an experiment aimed at restricting the expression of the tracing construct to a defined neuronal population on the graft, based on their innervation profile (Schwarz et al., 2015), using novel retrogradely transported AAV vectors (Davidsson et al., 2018).

**Dissecting host-to-graft connectivity based on graft-derived innervation profile**

Cre-expressing hESC-derived VM neurons were transplanted into the substantia nigra of 6-OH-DA-lesioned athymic nude rats. 24 weeks post-grafting, we utilized a novel AAV with enhanced retrograde transport properties (AAV-MNM008; Davidsson et al., 2018), and expressing Cre-inducible FLP, into either the PFC (target of A10 DA neurons), or into the striatum (target of A9 DA neurons; Figure 17A-D). At the same time, AAV8 expressing the FLP-inducible TVA receptor (required for EnvA-DG-rabies infection) and rGP (here termed oG; required for DG-rabies spread), was injected at the site of the transplant (Figure 17A-D). Hence, FLP is only expressed in Cre-expressing hESC-derived neurons that project to either PFC or striatum, while rGP and TVA are selectively expressed in FLP-expressing neurons. After 4 weeks, EnvA-DG-rabies was injected at the site of transplantation (Figure 17A-D). Hence, this system allowed to selectively trace presynaptic inputs to a defined neuronal population in the graft, based on their projection profile. The experiment was also done in non-transplanted, non-lesioned, transgenic TH-Cre rats, in order to map endogenous connectivity to PFC- and striatum-projecting DA neurons in the midbrain (Figure 17A-D).

In both experimental paradigms, traced neurons (mCherry+) were detected in all the host regions expected and as previously described (see Paper III; Watabe-Uchida et al., 2012). These structures include the striatum, GPe, hypothalamus and the rostral half of the cortical sheet (Figure 18A and B). Interestingly, when the number of inputs per anatomical structure was quantified, we noted that striatum-projecting neurons (A9-like) in the transplant receive more number of inputs from motor BG structures, including the striatum, GPe and subthalamic nucleus, in comparison to PFC-projecting (A10-like) neurons (Figure 18C). Moreover, the distribution of synaptic inputs in the striatum differed between both groups. While the inputs to transplanted A9-like neurons originated from the
A lateral portion of the striatum, A10-like neurons connected preferentially to the ventral portion of the striatum. Differences in connectivity were also observed in some cortical regions, with motor and somatosensory cortex preferentially connected to A9-like neurons and orbitofrontal cortex to A10-like neurons (Figure 18C).

This novel approach to determine host-to-graft connectivity based on the innervation pattern of the graft, thus allowed us to refine the understanding of inputs to the graft. We show that striatum-projecting and PFC-projecting neurons within the graft, receive a distinct set of synaptic inputs from the transplant, that is in accordance with the mapped endogenous connectivity and with literature (Watabe-Uchida et al., 2012). These results reveal the capacity for different subtypes of neurons within the transplant to receive distinct set of afferent input from the host and suggest that transplanted DA neurons can evoke an even more refined and specific circuitry repair (Cardoso, Aldrin-Kirk, et al., 2018).

Figure 17. Experimental design for two-factor monosynaptic tracing

(A-D) AAV MNM008-DIO/FLP was injected either into the rat striatum (A) or prefrontal cortex (C). AAV8-FLP/TVA and AAV8-FLP/αG was injected in the substantia nigra of TH-Cre and transplanted rats. Four weeks post AAV injections rats received an injection of EnvA-ΔG Rabies to map host-to-graft connectivity (B, D).
Figure 18. Synaptic input to PFC- vs striatum-projecting neurons in the transplant

(A-B) 3D representation of host afferent inputs to striatum- (A) or PFC-projecting (B) neurons in the transplant, reveals distinct pattern of synaptic integration of A10- and A9-like neurons within the graft.

(C) Quantification of traced neurons per brain region in PFC- vs striatum-projecting neurons, and in intact and transplanted animals, confirms the different pattern of synaptic integration between both types of neurons in the brain and in the transplant.
Conclusion and Perspectives

Pluripotent stem cells are steadily moving towards clinical testing as an alternative to fetal tissue in cell replacement therapy for PD (Kirkeby, Parmar, & Barker, 2017; Studer, 2017; J. Takahashi, 2017). While the safety and efficacy of these cells in vivo has been widely demonstrated in preclinical testing (Doi et al., 2014; Grealish et al., 2014; Kikuchi et al., 2017; Kirkeby et al., 2012; Kriks et al., 2011), the mechanism of graft function and the potential for bona fide circuitry repair remains not entirely clear. Elucidating the factors controlling circuit reconstruction by grafts, and identifying host connections that may most potently modulate graft neurons, will be key for further refinement of graft function in the context of cell replacement therapy for PD. Emergent technologies such as optogenetics (Deisseroth et al., 2006), chemogenetics (Armbruster, Li, Pausch, Herlitze, & Roth, 2007) and rabies-based tracing (Wickersham et al., 2007), have opened new avenues of investigation (Chen et al., 2016; Doerr et al., 2017; Grealish et al., 2015; Steinbeck et al., 2015).

In Paper I and Paper II, we have applied the monosynaptic tracing methods – which allows for the precise identification of presynaptic inputs to a genetically defined neuronal population using rabies virus as a retrograde transsynaptic tracer – to a cell replacement context in order to map novel connectivity established between host and hESC-derived graft neurons, and between pre-existing host circuitry and in situ converted neurons. This is the first time this method has been used to assess synaptic integration of transplanted neurons, allowing for a high-throughput detection and analysis of direct presynaptic inputs to transplanted neurons.

In Paper III we utilize monosynaptic tracing to reveal that hESC-derived neurons placed in their homotopic location, the substantia nigra, receive a full complement of “correct” afferent synaptic input from the host neurons. We also describe the capacity of grafted neurons in the substantia nigra to extend axons across long distances, and specifically towards A9 and A10 forebrain targets regions. These findings point to the capacity of graft neurons to elicit a more complete circuitry repair to a greater extent than what was previously thought possible (Tonnesen & Kokaia, 2012).

In Paper IV, we assessed the effect of graft phenotype, placement and lesion in host-to-graft synaptic integration and graft-derived innervation. We conclude that graft-derived innervation is determined by cell intrinsic factors, as only the phenotypically correct VM-patterned neurons innervated the appropriate forebrain target regions, while graft placement largely determined synaptic integration. Interestingly, and relevant for clinical translation purposes, we identify that ectopic VM-patterned grafts placed in the striatum receive synaptic input from anatomically relevant populations involved in motor control and that equally connect to homotopic VM-patterned grafts. These connections possibly play an important role in modulating the function and release of DA from transplanted neurons (Piccini et al., 1999; Piccini et al., 2000), and support the validity of ectopic transplantation in cell replacement therapy for PD.
Finally, we show that specific subtypes of neurons within the hESC-derived grafts, as determined by their projection pattern towards A9 and A10 forebrain targets, receive a distinct set of synaptic inputs from the host that closely match the described presynaptic endogenous connectivity of these two populations in intact animals (Watabe-Uchida et al., 2012). These observations serve a proof-of-principle that subtypes of neurons within the graft connect with the host differently, and hint at the capacity for transplanted DA neurons to elicit an even more refined and appropriate integration into the damaged circuitry.

Taken together, the studies performed in this thesis provide new and unprecedented insight into the functionality of transplanted hESC-derived neurons, and identify important variables that influence graft-derived axonal outgrowth and host-to-graft synaptic integration. The findings herein will serve as an important basis for optimizing the functionality of graft neurons, prompting further functional studies in pre-clinical models and may eventually impact clinical use: First, by reinforcing the importance of having a defined neuronal product for transplantation and supporting the optimization of differentiation protocols aimed at enriching for the therapeutically beneficial A9 population (Gre- alish et al., 2010); Second, by supporting the validity of transplanting hESC-derived VM-patterned progenitors ectopically into the putamen of PD patients, as some of the major midbrain input nuclei may also send collaterals to the transplant in this context; Finally, these results prompt further pre-clinical investigations, by identifying major presynaptic neuronal populations in the host cortex, striatum and GPe that may play an important role in the modulation of graft function, and describing the presence of refined host-to-graft connectivity to specific subtypes of neurons within the graft.
Material and Methods

In this section I will provide a general overview of the materials and methods used in the different studies included in this thesis. I will focus on transplantation and animal related studies, including hESC transgenesis and differentiation, surgery details (lesion, grafting, virus injections), behavior, histology and 3D mapping analysis. For additional information on cloning, virus production and procedures used in in-vivo conversion studies, I kindly refer the reader to the respective papers (see appendix).

![Figure 19. General overview of experimental design to study host-to-graft integration and endogenous connectivity.](image)

All lesions of the mesencephalic dopaminergic system in this study were performed by injection of 6-OHDA into the medial forebrain bundle of Athymic Nude or Sprague-Dawley rats. In order to assess host-to-graft synaptic integration, hESCs expressing the rabies-helper construct were used for transplantation. To investigate endogenous connectivity, a lentiviral vector expressing the rabies-helper construct was injected into intact animals. EnvA-ΔG-rabies was injected at the site of transplantation or lentiviral infection, to reveal pre-synaptic inputs to transplanted or endogenous neurons respectively. All animals were sacrificed for histological analysis 7 days after EnvA-ΔG-rabies infection.
hESC transgenesis, culture and differentiation

Two different hESC cell lines, H9 (WA09, passage 31-45; Paper I and III) and RC17 (Roslin, passage 28; Paper III and IV) were utilized in the transplantation studies included in this thesis. For the production of control and tracer cell lines to be used in host-to-graft monosynaptic tracing experiments, the cells were transduced with lentiviral vectors containing the (Syn-HisGFP-TVA) and (Syn-HisGFP-TVA-rGP) constructs respectively, at a multiplicity of infection (MOI) of 30. In paper III and IV, in order to “boost” the expression of the tracer construct and increase the retrograde tracing efficiency in vivo, cells were re-infected repeatedly with lentivirus at MOI of 1.5, at day 4 and 11-15 of differentiation.

Differentiation of hESCs to authentic mesencephalic DA neural progenitors was achieved via floor-plate induction. Activation of the canonical WNT pathway with the appropriate concentration of GSK3 inhibitor (CHIR99021) leads to the appropriate caudalization to midbrain fate. While potent SHH signaling (SHH-C24II) induces strong ventralization of the culture. With efforts to generate more reliable, consistent and GMP-compliant cell products for transplantation, this differentiation protocol was subject to improvements during the time course of this work (Kirkeby et al., 2012; Kirkeby, Nolbrant, et al., 2017). Here I will provide an overview of the latest protocol in use (for a detailed description see Nolbrant et al., 2017). Culture and differentiation details for each individual study can be found in the method section of the respective paper.

Undifferentiated hESCs cells were maintained on plates coated with Lam-521 (0.5 µg/cm²) in iPS-Brew medium and passaged with EDTA (0.5 mM). Differentiation was started by plating colonies onto Lam-111 coated culture plates, in differentiation medium containing DMEM/F12:Neurobasal (1:1), N2 supplement (1:100), SB431542 (10 µM), rhNoggin (100 ng/ml), SHH-C24II (200 ng/ml) and CHIR99021 (0.7 µM) from day 0-9. On day 9 of differentiation, FGF8b (100 ng/ml) was added to the culture medium to enhance caudal patterning. On day 11, cells were dissociated to single cells with accutase and replated onto Lam-111 coated plates in Neurobasal, B27 supplement without vitamin A (1:50), brain derived neurotrophic factor (BDNF; 20 ng/ml) and ascorbic acid (200 mM). On day 16 of differentiation, patterning was complete, and cells were ready for transplantation.

For paper IV, hESCs were patterned to a forebrain fate by removing CHIR99021 and FGF8b from the differentiation medium, therefore generating a neuronal population with rostral identity.

Animal studies

Throughout all the studies included in this thesis, adult female Sprague-Dawley (SD) rats were used as recipients for transplantation experiments lasting 18 weeks or less. To avoid immune rejection, SD rats received a daily intraperitoneal (i.p.) injection of ciclosporin A (10mg/kg) for the entire duration of the experiment. For transplantation experiments lasting above 18 weeks, immunocompromised female adult athymic nude rats were utilized as recipients. Animals were kept under a 12-hr light/dark cycle with free access to water and food. All the animal procedures used in this work were conducted with permission and in accordance of local ethical committee and Swedish law.
General surgery considerations

All surgical procedures were performed under general anesthesia by i.p injection of 6ml/kg of a 20:1 solution of the m-opioid agonist, fentanyl citrate (Fentanyl) [300mg/kg], and the α2-agonist, medetomidine hydrochloride (Domitor) [0.3mg/kg]. After surgery, the anesthesia was reversed by subcutaneous (s.c) injection of 0.5ml of the α2-antagonist, atipamazole (Antisedan) [0.3mg/kg] diluted in sterile saline, while post-operative analgesia was achieved by s.c. administration of 0.5ml of the μ-opioid agonist, buprenorphine (Temgesic) [45μg/kg] diluted in sterile water. All surgical procedures were performed in Kopf and Stoelting stereotaxic frames. A Hamilton syringe with a 22 gauge needle fitted with a glass capillary was used for all surgical procedures in order to minimize unspecific tissue damage. Stereotaxic coordinates for MFB lesions, transplantations, and virus injections were optimized from study to study, and strain to strain, with the most up-to-date coordinates found in Table 2. For each individual study, detailed coordinates can be found in the methods section of the paper.

6-hydroxydopamine lesions

The injection of 6-OHDA into the MFB causes the rapid and selective degeneration of the A9 and A10 DA neurons (Ungerstedt, 1968). 6-OHDA is an hydroxylated form of DA with high affinity for the dopamine transporter (DAT). When injected into the brain parenchyma, the toxin enters DA neurons via the DAT transporter and easily forms free radicals in the cytoplasm, which causes oxidative stress damage. 6-OHDA also acts as a potent inhibitor of mitochondrial respiratory chain complexes I and IV (Glinka, Gassen, & Youdim, 1997). These properties indicate this toxin for standard use to model PD in rodents.

Here, 4μl of 6-OHDA at a freebase concentration of 3.5μg/μl was injected into the right MFB (coordinates described in Table 1) to render rats parkinsonian. To avoid the rapid oxidation of the molecule, 6-OHDA is diluted in a solution of 0.02% ascorbate in sterile saline and kept in the dark on ice. Moreover, the aliquots are kept only for 2 hours after dilution, to ensure toxicity. Coordinates used for MFB injections can be found in Table 2.

Transplantation of hESC-derived neurons

On day 16 of hESC neuronal patterning, the cells were dissociated to single-cell suspension, washed, counted and resuspended in the adequate volume of Hank’s Balanced Salt Solution (HBSS) + DNase (1:10 in HBSS) for achieving the desired concentration for transplantation (see Table 2). The cells were kept on ice during the entire duration of the surgical procedures and transplanted in a 4-5 hour window from the time the cells were prepared. In order to ensure an homogenous density of the cell preparation, cells were gently mixed with a sterile pipette prior to loading into the glass capillary. After intracerebral injection, the glass capillary was left in place for 2 minutes and removed at a slow pace, in order to avoid reflux of the cells along the injection tract. Coordinates used for transplantation can be found in Table 2.

Lentivirus and EnvA-DG-rabies injections

The detailed information for the production of lentivirus and rabies virus can be found in the method sections of the respective papers. For intracerebral injection, frozen aliquots were thawed,
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utilized and discarded after the end of the surgical procedures. A diffusion time of 2 minutes was used to minimize reflux via the injection tract after injection. Details on speed, volume and coordinates for injection are found in Table 2.

Lentivirus injections of the control vector (Syn-HisGFP-TVA) and the tracing vector (Syn-HisGFP-TVA-rGP) were performed to validate the monosynaptic tracing system in vivo and to map endogenous connectivity of the striatum and substantia nigra (Paper I and II). The virus titers and dilutions used for intracerebral injection are described in the methods section of each paper. EnvA-∆G-rabies, expressing a mCherry reporter, was used to map the retrograde connectivity to endogenous starter neurons and to transplanted hESC-derived neurons. EnvA-∆G-rabies titers were 20-30 x 10^6 TU/ml and a dilution of 5% was used for intracerebral injections. The injections were performed in order to maximize the infection of the starter endogenous neurons and transplanted neurons.

Behavior

The behavioral tests performed in this thesis are based on the imbalance of DA in the right vs left rat brain hemispheres caused by the unilateral 6-OHDA lesion to the right MFB. Since the left side of the brain controls movements in the right side of body and vice-versa, this DA imbalance causes motor deficits specifically in the contralateral side of the lesion, leaving the ipsilateral side intact. This causes the animal to have the tendency to rotate towards the lesion side, i.e., clockwise. Therefore, these hemispheric asymmetries can be exploited in behavioral tests to evaluate both the extent of the lesion (Ungerstedt & Arbuthnott, 1970) but also the therapeutic benefit of DA neuronal grafts (Torres & Dunnett, 2007). Amongst the most popular behavioral tests associated to the 6-OHDA model are amphetamine induced rotations. Amphetamine causes the release of DA from stored vesicles and also stops DA reuptake by blocking the activity of the DAT. This leads to a massive sustained increase of DA levels at the synapse (Sulzer et al., 1995). In the unilateral 6-OHDA lesion model, amphetamine leads to the release of DA only in the intact hemisphere, hence, exacerbating the ipsilateral rotational bias. These rotational biases can be quantified with the appropriate equipment and apparatus (Ungerstedt & Arbuthnott, 1970). In short, the rat is placed in a bowl, injected with amphetamine i.p. and attached to a harness that is directly connected to an automated counter. In turn, this automated counter is connected to a computer that measures the clockwise and counter-clockwise rotations over a defined period of time, and then calculates the net clockwise rotations. Simplistically, this test is a measure of the absence of DA release in the lesioned hemisphere. Hence, it provides an estimation of the extent of lesion but also the capacity of transplanted neurons to normalize DA levels in the lesioned side.

Here, d-amphetamine induced rotations were used to assess extent of lesion and graft-derived recovery. The rats were placed in the bowls and left for 5 minutes for environment habituation prior to amphetamine challenge. Then, rats were injected i.p. with 2.5mg/kg of d-amphetamine diluted in sterile saline and the rotational response was measured over a period of 90 minutes. As a rule of thumb, rats that elicited a net rotation response of 5 or above clockwise turns per minute were considered good lesions and used for assessing graft-induced recovery.
Perfusion and Histology

Rats were given a terminal anesthetic dose of sodium pentobarbital i.p. and then transcardially perfused with room-temperature 0.9% saline followed by ice-cold 4% paraformaldehyde (PFA) for protein fixation. After perfusion, the brains were removed from the skull and post-fixed in 4% PFA for 2 hours. Next, the brains were switched to 25% sucrose solution in 0.1M PBS and stored for 48 hours in 4°C, or until the brains had sunk to the bottom of the vials, indicating complete sucrose penetration. PFA solution was prepared fresh, filtered for particles, and kept at physiological pH (7.4±0.2) at 4°C.

Immunohistochemistry was performed on free floating rat brain sections for the analysis of graft survival, differentiation and axonal outgrowth, as well as whole-brain rabies labeling. For that purpose, fixed rat brains were sectioned on the coronal plane using a freezing microtome, at a thickness
of 35mm and collected in a 1 in 8 series. Sections were then subjected to an antigen retrieval protocol by incubation with TRIS-EDTA at 80°C for 30 minutes. Next, sections were incubated with primary antibodies overnight in 5% serum for the species specific to the secondary antibody, in a 0.1 M KPBS solution containing 0.25% Triton-X. The detergent Triton-X permeabilizes the cell membrane facilitating the antibody access to intracellular antigens. In certain cases, the removal of the detergent facilitated the antibody-antigen binding (see Table 3). Following primary antibody incubation, sections were blocked for 1 hour at room temperature with same serum solution as above. Then, sections were incubated for 1 hour with secondary antibodies coupled with either a fluorophore to allow for fluorescent detection (1:500), or conjugated with streptavidin for di-aminobenzidine (DAB) detection (1:200). For DAB detection, sections were incubated with peroxidase conjugated avidin-biotin (ABC) kit solution for 1 hour, washed thoroughly and then revealed by peroxide-catalyzed DAB precipitation. After washing, sections were mounted on gelatin-coated slides. DAB-revealed sections were dehydrated in an ascending series of alcohols, cleared with xylene and coverslipped with DPX mountant. Immunofluorescent sections were coverslipped with PVA-DABCO. For certain antibodies, a biotinylated tyramide signal amplification (TSA) protocol (Adams, 1992) was used to boost detection of fluorescently-labeled sections (see Table 3). After labeling with primary antibodies, sections were incubated in biotinylated secondary antibodies for 1 h at room temperature, washed, and then

Figure 20. 3D representation of whole-brain graft-derived innervation and traced neurons
To generate a comprehensive and tridimensional map of graft-derived axonal outgrowth and rabies tracing (A), entire 1:8 series of 35 mm-thick coronal brain sections were immunostained for hNCAM and mCherry respectively. The sections were then imaged and placed in the proper anatomical plane of a 3D rendered model of the rat brain, based on the Paxinos and Watson rat brain atlas proportions (A). The spatial location of mCherry+ neurons (B; represented as a red dot) and the area covered by hNCAM+ fibers (C), were then imprinted into the 3D rat brain template (A).
incubated with ABC solution for 30 min. Sections were washed, then incubated with biotinyl tyramide (1:2,500 in KPBS containing 0.009% H2O2) for 30 minutes. Sections were then fluorescently labeled by 2 h incubation with fluorophore-conjugated streptavidin (1:500). Finally, sections were washed and mounted as described above. For a detailed information of antibodies and dilutions used in this thesis, see Table 3.

3D mapping analysis

A 3D template of the rat brain, built with the Cinema 4D software and with structural proportions based on the Paxinos and Watson rat brain atlas (Paxinos & Watson, 2005), was utilized in Paper III and Paper IV for a whole-brain comprehensive analysis of graft-derived innervation and host-to-graft synaptic input. For representation of graft-derived innervation, an entire 1:8 series was immunohistochemically stained for hNCAM (specific for neuronal bodies and fibers of human origin) and developed by DAB. Sections were then imaged and placed in the appropriate anatomical plane in the 3D brain template, to which the area covered by hNCAM+ processes was mapped. For the spatial representation of rabies labelled neurons in the host brain, an entire 1:8 series immunohistochemically stained for mCherry were used instead. Similarly, the sections were imaged and placed in the correct anatomical plane in the 3D brain template, with each dot representing the location of an mCherry+ neuron.
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Gerfen, C. R. (1992). The neostriatal mosaic: multiple levels of compartmental organization. Trends Neurosci, 15(4), 133-139.


Now comes the most difficult, but also the most gratifying chapter to write in this entire thesis. I have met and interacted, both at the professional and personal level, with the most truly inspiring, talented, smart, kind and fun people during these almost 5 years of PhD and life in Sweden. The following words are, therefore, an expression of gratitude to everyone that, in one way or another, enlightened me and helped reach this milestone.

First and foremost, I would like to thank my supervisor Malin for providing me the opportunity to have conducted my doctoral studies in such a fantastic and high-quality scientific, professional and co-operative environment. Despite your busy and frantic schedule, and your deserved professional reputation and status, your approachability, openness and scientific acuity are truly inspiring. I am immensely grateful for what I have grown as a researcher and scientist under your supervision. Thank you very much! I would also like to thank my co-supervisor, Cilla for the scientific inputs and discussions.

I would like to express my immense gratitude to my lab “chaperone” during my first year in Lund, Shane, who also happens to be one of the most intelligent and talented persons I have ever met. What I have learned from you, including technical and theoretical knowledge, but above all, a highly rigorous scientific standard, is invaluable. I could not have asked for a better tutor. I also want to give a special thanks to Andi, your teachings and help were extremely important to get me going on my feet during my lab beginnings.

Next it’s you Andrew! The new perspectives, insights and inputs you brought to the projects herein included were crucial. It wouldn’t have been the same without you! Also, thanks for making those surgery sessions more fun with those misheard lyrics and for the great scientific and non-scientific discussions. Thank you very much brother! I would also like to acknowledge Anders for the vital scientific input given to the studies herein included. Your passion for science and encyclopedic knowledge is truly remarkable and inspiring. Also, a very special thank you to Bengt for giving all these papers and figures a keen artistic touch, but also for the important scientific input stemming from your original analytic perspectives. It was a pleasure to work with you.

A special thanks to the fantastic technicians without whom none of this would be possible: Ulla, for the infinite expertise and passion for histology! And for always knowing how to solve our staining problems. Micke and Susanne for assisting with in vivo work and for keeping the animals studies running smoothly like clockwork. Paulina, for fixing all of our travel, grants, and administration related problems…and so much more!

I want to express my special gratitude to all the co-authors and collaborators that have contributed to the scientific content of this thesis, but also to other studies not herein included. First, I would like to acknowledge Agnete, Sara and Jenny W. for the meticulous in vitro work. You are true stem
cell magicians. Deirdre for the excellent animal work and scientific input. Olof, Daniella, Maria P. and Shong for the truly elegant in vivo reprogramming studies. Marie J., Jenny J. and Johan for the crucial contribution to establishing the monosynaptic tracing methodology. Patrick, Marcus D. and Tomas for the creativity and originality of the AAV studies and the fruitful scientific discussions. Daniela L. for the MACS sorting project.

A great thank you to everyone I have met in conferences and courses throughout the world for the great scientific inputs and conversations around posters and seminars, and for the good times outside the conference halls. Carlos, Jonathan, Wala’a, Brett, Michiya and many others...

Also, I want to acknowledge all the past and current members of the Parmar, Jakobsson, Bjorklund and Lundberg labs for always providing a great and friendly environment in the offices, labs and around the coffee table, and for enriching scientific exchanges: Agnete, Pedro, Janelle, Maria P., Martin, Shong, Ulrich, Alessandro, Andrew, Daniella, Deirdre (thanks the proof-reading!), Fredrik, Jenny W., Jessica, Marcella, Olof, Sara (thanks for the Swedish summary!), Shelby, Sol, Yogita, Yu, Josephine, Liana, Malin A., Monika, Rebecca, Daniela, Diahnann, Jenny J., Johan, Karolina, Kritika, Marie J., Marie P., Per, Pia, Raquel, Ludivine, Poonam, Marcus L. and Luis. But also other great colleagues and friends that I have had the pleasure to meet and work with during these five years at BMC: Alex S., Antonio, Carla, Christine, Cristina, Daniel, Frank, Isaac, Isak, Itzia, Jonas, Margarita, Maria J., Michaela, Michael, Miriana, Thomas, Valentina and many others.

I would like to use the next few paragraphs to express my gratitude to all the great people that I have met during these years, and that made my “extra-lab” time in Lund so pleasant and extraordinary. Though much of you have now moved on and started new lives in other lands, you have undoubtedly left your mark on me. Maria P., for your friendship, kindness, helpfulness in and outside the lab, and for introducing me to the Lundian-Iberian circle. João for being such a nice and fun chap, for our Benfica, football and politics discussions, our FIFA battles (that I always win) and for your witty sense of humour. And the king Afonsito for the laughs! Marina for your invaluable friendship (it’s an honour to have you as my toastmaster). Markos and Nikoletta for being such great friends. I will never forget that fantastic, fun and idyllic Greek wedding and that amazing tour of Athens! The “fofinhos” Diogo and Patricia, thank you very much for your friendship and great times in Lund and Odense. Miguel M., for the after work beers, fun discussions and laid-back attitude. Alex (what a “killer” PhD party man...) and Marina A. for being the nicest, fun and coolest people. Big thanks for Diana, Victor, Javier and Elisa for the great dinners, brunches and parties! Also, Sofia, Rita and Raquel for bringing a little bit of Portugal to Sweden.

A special thanks to Per (my social media nemesis 😊) and Matilde F., for the friendship and all the good times. And to Karolina and Peti for being the best neighbours one could ask for!

Thanks to all the past and current “creatives”, “igornants” and “festives” for the beers, wine, cheese, parties, hikes, trips but most of all laughs and good times. The list is long but I hope I don’t forget anyone: Alba, Alex S., Ana, Bianca, Carletto, David, Eliska, Fabio R., Fabio D., Henrique, Isak, Jessica, Katarina, Kritika, Laura D., Laura TG, Marcus L., Maria R., Marine, Marta RV., Marta S., Martino, Matilde N., Mert, Nic, Quentin, Roberta, Tiago, Tommaso and Oscar.
I would also like to give a special thanks to all the past and current football crew and teammates for those immensely fun, gratifying and competitive matches at Victoria! And for almost making me feel like my professional football career dreams actually came true! Robert (inga problem!), Andreas, Pablo, Diogo, Fabio D., Mariano, Jonas, Robin, Per, Edin, Ricard, Martino, Mohammad, Filip, Albert, Stefano, Paolo it was a lot of fun! Also thanks to Luis for those great martial art trainings and teachings, I truly enjoyed it!

Finally, I will reserve the last words of this chapter – which will be written in my mother tongue – to express my infinite appreciation and gratitude to my family, for all the love and support provided to me throughout these years abroad and my entire life.

Por fim, quero deixar aqui expresso o meu apreço e gratidão aos meus pais, Arcidres e Susana, pelo apoio emocional e financeiro, e pelo amor e carinho com que sempre me presentearam. Nada disto seria possível sem vocês. Foram 5 anos de altos e baixos, mas mesmo à distância, vocês estiveram sempre presentes. Espero que este trabalho seja para vocês um motivo de orgulho. Muito obrigado! Uma palavra de apreço á minha irmã Patricia, pelo apoio durante todos estes anos e por estar presente na minha defesa. Obrigado! E também á Emmita por ser tão fofo. Finalmente um muito obrigado á minha avó Clara, á minha madrinha Lurdes, ao meu tio Dinis e aos meus primos Bruno e Miguel R. por estarem presentes na minha defesa, uma tão importante façanha da minha vida.
Monosynaptic Tracing using Modified Rabies Virus Reveals Early and Extensive Circuit Integration of Human Embryonic Stem Cell-Derived Neurons

Shane Grealish,1,2,4 Andreas Heuer,1,2,4 Tiago Cardoso,1,2 Agnete Kirkeby,1,2 Marie Jönsson,1,2 Jenny Johansson,3 Anders Björklund,1 Johan Jakobsson,2,3 and Malin Parmar1,2,*

1Department of Experimental Medical Science, Developmental and Regenerative Neurobiology, Wallenberg Neuroscience Center
2Lund Stem Cell Center
Lund University, 22184 Lund, Sweden
3Department of Experimental Medical Science, Molecular Neurogenetics, Wallenberg Neuroscience Center, Lund University, 22184 Lund, Sweden
4Co-first author
*Correspondence: malin.parmar@med.lu.se
http://dx.doi.org/10.1016/j.stemcr.2015.04.011
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SUMMARY

Human embryonic stem cell (hESC)-derived dopamine neurons are currently moving toward clinical use for Parkinson’s disease (PD). However, the timing and extent at which stem cell-derived neurons functionally integrate into existing host neural circuitry after transplantation remain largely unknown. In this study, we use modified rabies virus to trace afferent and efferent connectivity of transplanted hESC-derived neurons in a rat model of PD and report that grafted human neurons integrate into the host neural circuitry in an unexpectedly rapid and extensive manner. The pattern of connectivity resembled that of local endogenous neurons, while ectopic connections were not detected. Revealing circuit integration of human dopamine neurons substantiates their potential use in clinical trials. Additionally, our data present rabies-based tracing as a valuable and widely applicable tool for analyzing graft connectivity that can easily be adapted to analyze connectivity of a variety of different neuronal sources and subtypes in different disease models.

INTRODUCTION

Dopamine (DA) cell replacement for Parkinson’s disease (PD), using transplantation of human fetal ventral mesencephalic tissue to the striata of PD patients, has provided proof of principle that DA-neuron-rich grafts can survive long term and restore striatal D\(_{\text{A}}\)-ergic function (Barker et al., 2013). The lack of available fetal tissue, as well as the logistic and ethical issues associated with the use of obtaining such tissue, has spurred the development of protocols for the generation of authentic and functional midbrain DA neurons from human embryonic stem cells (hESCs) (Kirkeby et al., 2012; Kriks et al., 2011). Pre-clinical validation of such hESC-derived neurons in a rat model of PD shows that they function with equal potency and efficacy to fetal DA neurons after transplantation (Grealish et al., 2014). With the overall goal of mapping how transplants of stem cell-derived human neurons integrate in the host brain in a preclinical rat model of PD, we used a method based on modified rabies virus (\(\Delta G\)-rabies) that allows for the tracing of afferent and efferent connections of transplanted human neurons. \(\Delta G\)-rabies is a deletion mutant virus in which the gene coding for glycoprotein (GP; necessary for transsynaptic spread) is replaced by the gene coding for a fluorescent protein, and the envelope is pseudotyped to only infect cells expressing the TVA receptor (Wickersham et al., 2007). Therefore, the initial infection can be targeted to any cell engineered to express the TVA receptor. If that cell is modified to also express GP, the \(\Delta G\)-rabies can assemble into infectious particles in this cell and spread retrograde across one synapse. This system has previously been used to trace connectivity of endogenous neural circuitry (Miyamichi et al., 2011; Watabe-Uchida et al., 2012), as well as newly born neurons in the olfactory bulb and hippocampus (Deshpande et al., 2013; Vivar et al., 2012). Here, we use the system to trace connectivity of transplanted hESC-derived neurons in a rat model of PD.

RESULTS

We used a polycistronic lentiviral tracing vector (Miyamichi et al., 2011) expressing a histone-tagged GFP; the TVA receptor, which allows for selective infection of \(\Delta G\)-rabies; and rabies GP to allow for transsynaptic spread (Figure 1A). To control for unspecific labeling, we used a non-synaptic spreading of \(\Delta G\)-rabies, a control vector containing GFP and TVA, but lacking GP (Figure 1A) in parallel. The targeted cells, here termed starter neurons, can be visualized by nuclear GFP expression (Figure 1B). Upon infection with the \(\Delta G\)-rabies vector encoding mCherry, the targeted starter neurons expressing the TVA receptor can be infected by \(\Delta G\)-rabies and easily identified by expression of GFP and mCherry (Figure 1B). These neurons are engineered to contain GP, and hence, \(\Delta G\)-rabies can assemble into infectious particles in the starter cells. Any neuron that forms presynaptic contacts with the starter...
Figure 1. Overview of Monosynaptic Tracing Methodology
(A) Schematic representation of the lentivector constructs used in the study. The tracing vector labels cells with a histone-tagged GFP, the TVA receptor necessary for ΔG-rabies infection as well as the rabies GP that allows ΔG-rabies to transmit across synapses. The control vector lacks the GP and thus cannot transmit.
(B) Neurons that have been infected with the tracing construct are termed starter neurons. After ΔG-rabies infection, these cells turn mCherry+, and due to the presence of GP in the starter neuron, ΔG-rabies is transmitted retrogradely to label the traced neuron and cannot transmit further due to the lack of GP.
(C) Upon injection of the tracing lentivector to the rat striatum (n = 6), strong nuclear GFP+ expression is observed after 4 weeks. (D and E) At 7 days after ΔG-rabies injection, a clear mCherry+ signal is observed co-localized with GFP+ nuclei. (F) The main starter neuron population in this paradigm was DARPP32-expressing MSNs of the striatum. (G–H′) Traced mCherry+/GFP+ neurons could also be observed in rostral structures, such as the prefrontal and lateral orbital cortex and close to the injection site within the striatum.

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neuron, here called traced neuron, will be transduced with mCherry due to the selective transmission of rabies virus retrogradely across active synapses (Ugolini, 1995). The traced neurons can be easily identified from the starter neurons, as they express mCherry, but not GFP (Figure 1B). Further propagation of the ΔG-rabies vector does not occur as expression of the GP is restricted to the starter neuron and therefore only first-order synapses are traced (Etessami et al., 2000).

First, we traced endogenous striatal connections by injecting adult rats with either the tracing or control lentiviral vector into the striatum, and after 4 weeks, we injected the ΔG-rabies vector into the same site. When analyzed 1 week after ΔG-rabies injection, we found a large number of GFP+/mCherry+ starter neurons throughout the striatum (Figures 1C–1E). The vast majority of the GFP+ starter neurons co-expressed the medium spiny neuron (MSN) marker DARPP-32 (Figure 1F). Selective infection via the TVA receptor by ΔG-rabies vector was further confirmed by injecting ΔG-rabies into naive animals, where mCherry expression was never observed (not shown). mCherry+/GFP+ neurons could also be found in the prefrontal, lateral orbital, and sensorimotor cortices (Figures 1G–1H), as well as some scattered cells in thalamus, amygdala, and midbrain (Figures 1I–1J). In animals that received the control lentiviral vector prior to injection of ΔG-rabies (i.e., where the targeted cells expressed TVA receptor but lacked GP), a large number of starter cells were observed expressing GFP and mCherry (Figure S1B). In these animals, however, we did not observe any tracing events (Figures S1A–S1H), confirming previous reports documenting that spread depends on expression of GP and occurs only via synaptically connected neurons (Etessami et al., 2000).

The tracing observed is consistent with the known anatomy of striatal afferents (Gerfen and Wilson, 1996) and confirms the usefulness of the rabies-based tracing method for the visualization of striatal afferents throughout the brain.

To trace host-to-graft connectivity of transplanted hESC-derived neurons, we next generated hESC lines that stably expressed the tracing and control constructs (Figures 2A and S2A). The engineered hESCs were differentiated using a protocol that results in a high yield of authentic and functional ventral midbrain patterned DA neurons (Kirkeby et al., 2012). The midbrain-patterned cells (Figures S2C–S2F) were transplanted into a 6-hydroxydopamine (6-OHDA)-lesion model of PD, with athymic, nude rats as hosts (n = 8 per group). At 6 weeks post-transplantation, two rats per group were injected with ΔG-rabies within and adjacent to the graft core and perfused 7 days later. Remaining animals were left for their grafts to mature for 6 months, as detailed in the experimental timeline (Figure S2B). At this time point, the grafts were rich in DA neurons (Figures S2G and S2H). Based on quantifications of mCherry, GFP and tyrosine hydroxylase (TH) expression (Figure S2H), we could determine that 44.7% ± 15.7% of the starter neurons expressed TH. We also stained for serotonergic neurons and found only a small number (2.1% ± 0.4%) of the starter neurons expressed 5-HT (Figure S2J).

Already 6 weeks after transplantation, grafts were readily detectable using GFP immunostaining (Figure 2B), and we observed that the majority of GFP+ grafted neurons were infected with ΔG-rabies (Figures 2C–2D). Interestingly, mCherry+ neurons that did not co-express GFP (thus representing host cells that had made synaptic contacts with the transplanted neurons) could be observed adjacent to the graft core (Figure 2D†). In areas distal to the transplant, we observed mCherry+/GFP+ host neurons within the prefrontal and sensorimotor cortices (Figures 2E and 2I), the thalamus (Figures 2F and 2I), and a few as far caudal as dorsal raphe nucleus and the substantia nigra, which is ≥ 5 mm away from graft core (Figures 2G and 2I).

At 6 months post-transplantation we confirmed that all starter cells (as detected by GFP expression) co-expressed human nuclei (HuNu; Figure 2H). At this time point, traced mCherry+/GFP+ host neurons could be found in the striatum, close to the graft core, as well as in distal structures matching those observed at 6 weeks (Figure 2I). Quantifications showed that the majority of neurons connecting to the graft at this time point were located in the cortex, but connected neurons were also detected in thalamus, striatum, substantia nigra, and amygdala (Figure 2K; n = 5). The traced neurons in the prefrontal cortex were located mainly in layers III/V, displaying a classical pyramidal (Figure 3A) or basket cell (Figure 3B) morphology, and co-expressed the cortical marker TBR1 (Figure 3C). In the thalamus, the traced neurons were located in all anterior intralaminar nuclei (central medial, paracentral, and central lateral) (Figures 3D and 3F), which are the thalamic regions that normally project to the striatum (Van der Werf et al., 2002). These thalamic cells expressed Calbindin (CALB), but not Parvalbumin (PV) (Figures 3G and 3H). The traced host striatal cells connecting the grafted cells were distributed around the periphery of the graft and displayed (I–J†) In more caudal sections, traced neurons were found in regions known to project to the striatum, such as thalamus, amygdala, and substantia nigra pars compacta.

AC, anterior commissure; Amyg, amygdala; FMI, forceps minor; LOC, lateral orbital cortex; PFC, prefrontal cortex; RF, rhinal fissure; SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STR, striatum; and Thal, thalamus. Scale bars represent 250 μm (C–E), 30 μm (F), 1 mm (G–J), 100 μm (G–J), 50 μm (G and H†) and 100 μm (I and J†). See also Figure S1.
Monosynaptic Tracing using Modified Rabies Virus Reveals Early and Extensive Circuit Integration of Human Embryonic Stem Cell-Derived Neurons

A: Schematic diagram showing the process from H9 cells to Starter-hESC.

B-C: Fluorescence images showing GFP, mCherry, and GFP/mCherry expression in different brain regions.

D: Magnified view of a brain region with GFP/mCherry expression.

E-H: Images of PFC, Thal, and SN regions 6 weeks and 6 months post-transplantation.

I-J:脑片显示不同区域的mCherry表达。

K: Graph showing the number of traced cells in different brain regions 6 months post-transplantation.

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a typical MSN morphology (Figures 2D’ and 3I) and expressed DARPP32 (Figure 3I). Thus, the connectivity observed after 6 weeks remained stable for up to 6 months post-transplantation, and the host brain regions that provide input to the grafted human neurons matched those that connected to endogenous striatal neurons.

In the next set of experiments, we altered our experimental design to trace graft-to-host connectivity. Again, we used 6-OHDA lesioned nude rats, but this time the rats had been pre-injected with the tracing or control lentiviral vectors 4 weeks prior to transplantation. These virus injections were placed into two regions known to be densely innervated by transplanted human DA neurons: striatum (n = 6 for tracing and n = 4 for control) and prefrontal cortex (n = 5 for tracing and n = 4 for control). Thus, on the day of transplantation, the host brain contained GFP+ TVA-GP-expressing starter neurons and served as the recipient environment for the transplanted neurons.

Figure 2. Host-to-Graft Connectivity of Intrastriatal Grafts of hESC-Derived Neurons
(A) Schematic illustration of generation of starter hESCs.
(B) Transplanted cells were detected based on GFP expression (n = 2 rats).
(C and D) ΔG-rabies selectively infected the transplanted tracer hESC-derived neurons.
(D’–G) Within the core of the transplant almost all GFP+ nuclei co-expressed mCherry.
(D”–G) mCherry+/GFP+–traced neurons were found in the host striatum at the graft edge.
(E–G) Traced host neurons could be detected in distal structures known to provide afferent inputs to striatum, such as the prefrontal cortex (E), thalamus (F), and substantia nigra (G).
(H) All GFP+ co-expressed the human-specific marker HuNu.
(I and J) Schematic illustration of the locations of the graft core, and distribution of mCherry+/GFP+–traced neurons in a brain 6 weeks (n = 2) and 6 months (n = 6) post-grafting.
(K) Quantifications of traced neurons in different host regions 6 months after transplantation (n = 5 rats, mean ±SEM).

Amyg, amygdala; DRN, dorsal raphe nucleus; PFC, prefrontal cortex; SNr, substantia nigra pars reticulata; SMC, sensory motor cortex; STR, striatum; T, transplant; Thal, thalamus. Scale bars represent 400 μm (B–D), 25 μm (D”, E, F, and H), and 50 μm (D’, J, G, and K). See also Figure S2.
midbrain patterned WT hESCs (Figures S3A and S3B). Six months after transplantation, cells in the grafts had matured into functional DA neurons as confirmed by recovery in amphetamine-induced rotation (Figure S3C). At this time point, ΔG-rabies was injected at the same host brain regions that were initially injected with lentiviral vectors, resulting in selective infection of the modified host neurons via the TVA receptor. Subsequently, retrograde labeling of transplanted hESC-derived neurons that connected to host neurons in these regions should occur in animals pre-injected with tracing, but not control, vectors.

Local connectivity was assessed in animals pre-injected with lentiviral vectors into the striatum prior to grafting (Figure 4A). In these animals, a widespread mCherry expression in host striatum was observed (Figures 4B and 4C), and a distinct mCherry+ graft core could be easily delineated (Figures 4B and 4C, dotted line demarcates graft-host border in B). In higher magnification, we observed traced mCherry+ neurons within the graft core of the animals pre-injected with the tracing vector (Figure 4D), but not in animals with the control vector (Figure 4E), indicating retrograde synaptic transfer of ΔG-rabies from starter neurons in the host striatum only in the presence of GP. In both groups, the grafted cells could be detected by the expression of the human-specific marker HuNu (blue cells in Figures 4F and S3D). The ΔG-rabies selective infection of the host starter neurons was confirmed by co-expression of GFP and mCherry (Figures 4G and S3E), whereas no HuNu+ human cells were found to co-express GFP (Figures 4G and S3E).

Long distance connectivity was assessed in the animals pre-injected with lentiviral vectors into the prefrontal cortex prior to grafting (Figure 4H). The hESC-derived neurons had extended axons rostral innervating the prefrontal cortex (Figures 4I and 4J), where human neural cell adhesion molecule (hNCAM)-expressing axons could be found closely intermingled with mCherry+/GFP+ host starter neurons (Figure 4K). Analysis of the graft core, located in the striatum, 5 mm away from the site of the starter host neurons, revealed mCherry+/GFP−/HuNu+ neurons scattered within the graft core (Figure 4M). Phentypic assessment of the traced mCherry+/GFP− cells showed that they co-expressed TH (Figure 4M), but not serotonin (5-HT) (Figure 4L), confirming that the grafted hESC-derived DA neurons had established synaptic contacts with the host neurons.

**DISCUSSION**

We have used monosynaptic tracing based on modified rabies virus to analyze circuit integration of transplanted hESC-derived DA neurons in a rat model of PD. The technique allowed us to confirm the presence of graft-to-host and host-to-graft connectivity of transplanted human neurons, which is in line with previous studies of transplanted primary rodent DA neurons using electrophysiology, electron microscopy, or conventional tract-tracing methods (Fisher et al., 1991; Sørensen et al., 2005; Thompson et al., 2005; Tønnesen et al., 2011).

Our results revealed that the grafted hESC-derived DA neurons have a propensity for integrating in the host brain at a level exceeding what is commonly thought possible to achieve (Tønnesen and Kokaia, 2012). Additionally, it allowed for the precise identification of the source of host afferent monosynaptic inputs to the grafted cells, which has not been feasible until now due to limitations in existing technology. Our dataset shows that besides adjacent striatal cells, cortico-striatal input represents the most significant population of host afferents connecting with the grafted human neurons, but that also host neurons in the thalamus, dorsal raphe nucleus, amygdala, and substantia nigra innervate the grafted cells. This largely matches the endogenous striatal afferents mapped in this study and is in line with what has been previously described for transplanted striatal cells (Dunnett et al., 2000; Gerfen and Wilson, 1996). Interestingly, midbrain DA neurons normally receive afferent inputs from these cortical regions, as well as from striatal projection neurons (Watabe-Uchida et al., 2012), suggesting that the host brain may provide a regulatory control of the grafted DA neurons similar to that exerted by the intrinsic cortical and striatal afferents. One noteworthy and unexpected observation in this study is that the host afferents are formed as soon as 6 weeks after transplantation, at a time point much earlier than functional effects of transplants of human DA neurons normally can be detected.

Monosynaptic tracing using modified rabies virus has previously been successfully used to trace existing neuronal circuits in vivo (Wall et al., 2013; Watabe-Uchida et al., 2012) and also to map the integration of newborn neurons in the olfactory bulb and hippocampus (Deshpande et al., 2013; Vivar et al., 2012). Adapting it for tracing the connectivity of transplanted hESC-derived neurons, as done here, overcomes some limitations of existing tracing technology and opens up many new possibilities to analyze circuit integration after transplantation. First, it enables mapping in 3D of pre-synaptic inputs from host cells to grafted neurons in whole brains, which has not previously been possible because of a lack of adequate technology. Second, experiments in vitro and in vivo have confirmed the selective spread of ΔG-rabies only to synaptically coupled neurons (Wickersham et al., 2007), making it possible to distinguish between innervation and synaptic coupling. Third, the use of genetic targeting allows for analysis of integration of very defined cell populations. This makes...
Figure 4. Graft-to-Host Connectivity of Intrastriatal Grafts of hESC-Derived Neurons

(A and H) Sites of injection of the ΔG-rabies vectors used to visualize graft-to-host connectivity. (B and C) mCherry expressed in the host starter neurons was confined to the host striatum, while the transplant (T) was seemingly void of mCherry expression.

(D and E) In high-power magnification, mCherry+ neurons could be detected within the graft in the tracing group (n = 6; D), but never in the control group (n = 4; E).

(F and G) The HuNu+ graft was surrounded by GFP+ host nuclei, but none of the HuNu+ cells co-expressed GFP.

(I–K) Immunostaining for hNCAM showed that the grafts had established a dense innervation with the surrounding striatum (I), as well as the prefrontal cortex (J), including the site of prefrontal cortex that was targeted with the tracing lentivirus (K) (n = 5).

(L and M) The mCherry+ traced cells within the transplant core did not express 5HT (L), but were found to co-express HuNu (M) and TH (N), confirming that grafted human DA neurons had established synaptic contacts with neurons in the surrounding host striatum/prefrontal cortex.

PFC, prefrontal cortex; STR, striatum; T, transplant. Scale bars represent 1 mm (B, C, I, and J), 500 μm (D and E), 10 μm (G), 100 μm (K and F), 50 μm (L), and 25 μm (M and N). See also Figure S3.
the technology more refined than the use of conventional synaptic tracers, and it has already been used to re-define knowledge of neural circuits in vivo (Watabe-Uchida et al., 2012). One should keep in mind, though, that the technology is qualitative rather than quantitative and that it underrepresents the actual number of connected cells (Marshel et al., 2010).

Nevertheless, the technology offers new possibilities to qualitatively study functional integration of grafted neurons and serves as a valuable complement to electrophysiology, conventional tracers, and electron microscopy, as it allows for higher throughput analysis, mapping of both afferent and efferent connectivity, as well as visualization of connectivity from both local and distal brain structures.

**EXPERIMENTAL PROCEDURES**

All data are presented as mean ± SEM.

**Research Animals and Ethical Permissions**

Adult (≤180 g) athymic, nude female rats (n = 40) were purchased from Harlan Laboratories (Hsd:RH-Foxn1nfgnu). They were housed in individual ventilated cages, under a 12-hr light/dark cycle with ad libitum access to sterilized food and water. Female adult (225–250 g) Sprague Dawley rats (n = 12) were purchased from Charles River and were used to validate all viral vectors for these experiments; they were housed as described above but in standard caging.

All procedures were conducted in accordance with the European Union Directive (2010/63/EU) and were approved by the ethical committee for the use of laboratory animals at Lund University and the Swedish Department of Agriculture (Jordbruksverket).

Details of transplantation procedures are provided in detail in Supplemental Experimental Procedures.

**Lentiviral Production**

The constructs for the tracing and control vectors (as detailed in Figure 1A) were purchased from AddGene (IDs: 30195 and 30456, respectively). High-titer preparations of lentiviral particles were produced as previously described (Zufferey et al., 1997). Titers for the control vector were 3.1 × 10^8 U/ml and were used in vivo at a concentration of 20%. Titers for the tracing vector were 3.9 × 10^8 U/ml and were used in vivo at a concentration of 10%.

**ΔG-Rabies Production**

We produced our own pseudotyped rabies vectors according to Osakada and Callaway (2013) with minor adjustments. The protocol was stopped after step 60 as the virus was concentrated via ultracentrifugation only once and no sucrose cushion was used. Titering was performed using TVA-expressing HEK293T cells as defined in the protocol. Titers were 20–30 × 10^6 TU/ml, and for in vivo experiments were used at a dilution of 5%, as determined by testing different dilutions for a concentration that gave specific infection and tracing, in the absence of toxicity.

**Differentiation of hESCs**

Human ESCs H9 (WA09, passage 31–45) were differentiated to a ventral midbrain fate, using the protocol as described in detail in Supplemental Experimental Procedures, and generation of transgenic lines is also provided.

**Microscopy and Quantifications**

All bright-field images were captured using a Leica DMi6000B microscope, while all fluorescent images we acquired using a TCS SP8 laser-scanning confocal microscope. A full description of quantifications performed is provided in Supplemental Experimental Procedures.

**Immunohistochemistry**

All samples, cultured cells and brain tissue, were fixed in fresh 4% paraformaldehyde. A complete list of suppliers and concentrations of primary and secondary antibodies used is detailed in Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.04.011.

**AUTHOR CONTRIBUTIONS**

S.G., A.H., T.C., and M.J. performed and designed experiments. A.B., J.J., and M.P. designed experiments, guided preparation of the data and figures, and led data interpretation and analysis. S.G. and M.P. wrote the manuscript. All authors gave input for the manuscript.

**ACKNOWLEDGMENTS**

We would like to thank Dr. Ed Callaway for help with establishing the technique and virus production and Bengt Mattsson, Michael Sparrénius, Ingar Nilsson, and Ulla Jarl for excellent technical assistance. This study was supported by grants from the European Community’s 7th Framework Programme through NeuroStemcellRepair (number 602278), the Strategic Research Area at Lund University Multipark (Multidisciplinary research in Parkinson’s disease), the Swedish Research Council (70862601/Bagadillico, K2012-99X-22324-01-5 and K2014-61X-20391-08-4), and the Swedish Parkinson Foundation (Parkinsonfonden). S.G. was supported by a postdoctoral stipend from the Swedish Brain Foundation (Hjärnfonden). The research leading to these results has received funding from the European Research Council under the European Union’s 7th Framework Programme (FP/2007-2013)/ERC Grant Agreement number 309712.

Received: December 19, 2014
Revised: April 22, 2015
Accepted: April 22, 2015
Published: May 21, 2015

**REFERENCES**


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**Figure S1. In vivo testing of control lentiviral constructs.**

**Related to Figure 1**

**(A-B)** DAB immunohistochemistry for mCherry revealed that host neurons of the infected striatum (STR) had mCherry⁺ fibres extending rostrally to the prefrontal cortex (PFC) (n=6). **(C, D)** At sites distal to the injection, only mCherry⁺ fibres and no cell bodies, could be observed. The fibres observed included a bundle of mCherry⁺ fibres extended along the nigrostriatal pathway (NSP), giving rise to a terminal network in the substantia nigra pars reticulata (SNr). **(E-H)** At no anatomical level throughout the brain was a single traced neuron observed in any animal, confirming the specificity of the ∆G-rabies and also that the presence of rabies glycoprotein is necessary for monosynaptic tracing events to occur.

Scale bars: (A-D) = 1 mm; (E-H) = 100 µm
Grealish et al. Figure S2.
Figure S2. *In vitro* and *in vivo* validation of transgenic tracer- and control-hESCs.

Related to Figure 2

(A) Schematic illustration of generation of control hESCs, and (B) time-line of the host-to-graft experiment. (C-F) Both tracer and control hESCs could efficiently be patterned into midbrain identity as detected by co-expression of FOXA2, LMX1a and OTX2. (G) Grafts of tracer (n=6) and control (n=6) hESCs gave rise to a transplant (T) rich in TH⁺ dopaminergic neurons. (I) Many of the GFP/mCherry expressing starter neurons expressed TH (J) but very few expressed 5HT.

Scale bars: (B-E) = 100 µm; (F-I) = 500 µm.
Figure S3. *In vitro* midbrain patterning of wildtype hESCs used in graft to host experiments.

Related to Figure 4

(A, B) Wild type hESCs were efficiently patterned towards midbrain identity as detected by co-expression of FOXA2, LMX1A and OTX2. (C) Amphetamine-induced rotation showed that the transplants analyzed in this study were functional, when assessed 6 months after transplantation (n= 5 rats; mean ± SEM; p < 0.01 compared to post-lesion; two-tailed paired t-test). (D) In animals receiving the control lentiviral vector in the striatum (n=4), no tracing events within the transplant could be observed. (E) Any mCherry+ neurons present were at the perimeter of the HuNu+ transplant, and none of the HuNu+ cells co-expressed GFP or mCherry.

Scale bars: (A-B) = 1 mm; (D) = 100 μm; (E) = 10 μm.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**hESC culture, transgenesis and differentiation**

Human ESCs H9 (WA09, passage 31-40 from WiCell) were maintained on γ-irradiated mouse embryonic fibroblasts in DMEM/F12, 20% KSR, 0.05 mM 2-mercaptoethanol, 0.5% Pen/Strep and 10 ng/mL FGF-2 (R&D Systems), and passaged using EDTA. For production of tracer and control cell lines, cells were transduced at a multiplicity of infection (MOI) of 30 with lentiviral particles of the Syn_HisGFP_TVA and the Syn_HisGFP_TVA_Gp constructs, respectively. We enriched transgenic cells in the population based on weak leakage of GFP from the synapsin promoter in undifferentiated cells. Cells with the strongest fluorescence in the GFP channel (gating 30-40% of total population) were sorted out and used for continued experiments. Cells were differentiated towards a VM phenotype using a previously described protocol (Kirkeby et al., 2012). Briefly, for patterning towards a VM fate 10 μM SB431542, 100 ng/ml rhNoggin, 200 ng/ml Shh-C24II and 0.7 μM CHIR99021 was added to the culture medium from day 0-9 of differentiation. From day 11 of differentiation, BDNF (20 ng/ml), GDNF (10 ng/ml) and ascorbic acid (200 μM) was added to the medium, and on day 16, cells were dissociated with accutase and resuspended in HBSS + 0.05% DNAse to a concentration of 75,000 cells/μl for transplantation.
**Surgical Procedures**

All surgical procedures were done under general anaesthesia using a fentanyl and medetomidine (20:1) solution injected intraperitoneally. All rats received a unilateral 6-hydroxydopamine lesion of the right medial forebrain bundle with a volume of 4 µl, at a freebase concentration of 3.5µg/µl, to the following co-ordinates relative to bregma: A/P -4.4; M/L -1.2; D/V (from dura) -7.8; tooth bar -2.4. Two weeks later animals were screened for lesion success using amphetamine-induced rotations. Briefly, the rats were injected with 2.5 mg/kg, i.p. (Apoteksbolaget, Sweden) and recorded using an automated rotometer system (Omnitech Electronic Inc., USA). The animals were recorded for 90 minutes, and only full body turns were counted and then expressed as net turns per minute, with rotations towards the side of the lesion given a positive value. All rats were stratified across the experimental groups.

For the host to graft experiment the host rats were grafted with a total of 300 000 modified hESCs at day 16 of differentiation towards a ventral midbrain fate of either control or tracing lines to the striatum in a volume of 2 µl, at a concentration of 75 000 cells/µl, to each of the following co-ordinates relative to bregma: (1) A/P +1.2; M/L -2.6; and (2) A/P +0.5; M/L -3.0; D/V (from dura) -4.5; tooth bar -2.4. One week prior to termination, the rats underwent injection of ΔG-rabies at a dilution of 5% of the stock at two sites (one within and one adjacent to each graft site) with 2 deposits at each site. Each deposit consisted of a volume of 1µl injected at a rate of 0.5 µl per minute and a diffusion time of 2 minutes, delivered at the following co-ordinates: (1) A/P +1.2; M/L -2.6 and -3.0; and (2) A/P +0.5; M/L -3.0 and -2.6; D/V (from dura) -4.0 and -5.0; tooth bar -2.4.
For the graft to host experiment the rats were allocated to receive either control or tracing lentivector into the striatum or PFC 4 weeks prior to transplantation. To target the striatum 2 µl per site of lentivector was injected into the following co-ordinates: (1) A/P +1.2; M/L -2.6; and (2) A/P +0.5; M/L -3.0; D/V (from dura) -4.5; tooth bar -2.4. To target the PFC 1 µl per deposit was injected at: (1) A/P +3.5; M/L -0.8; D/V -2.5; and (2) A/P +2.5; M/L -0.8; D/V -3.7; tooth bar -4. After 4 weeks, all hosts received and intra-striatal transplants of wild-type hESCs at day 16 of differentiation patterned towards a midbrain fate. 300 000 cells were grafted in a volume of 2 µl, at a concentration of 75 000 cells/µl, to each of the following co-ordinates relative to bregma: (1) A/P +1.2; M/L -2.6; and (2) A/P +0.5; M/L -3.0; D/V (from dura) -4.5; tooth bar -2.4. One week prior to perfusion the rats underwent injection of ΔG-rabies at a dilution of 5% of the stock at sites flanking the original deposits of lentivector. For the striatum, each deposit consisted of a volume of 0.75 µl injected at a rate of 0.2 µl per minute and a diffusion time of 2 minutes, delivered at the following co-ordinates: (1) A/P +1.5; M/L -2.6; (2 and 3) A/P +1.2; M/L -2.2 and -3.0; (4 and 5) A/P +0.5; M/L -3.3 and -2.6; (6) A/P 0.0; M/L -2.6; (for all sites) D/V (from dura) -4.0 and -5.0; tooth bar -2.4. For the PFC each deposit consisted of a volume of 0.5 µl injected at a rate of 0.2 µl per minute and a diffusion time of 2 minutes at the following co-ordinates: (1) A/P +3.5; M/L -0.8; D/V -3.7 and -2.5; (2) A/P +3.0; M/L -0.8; D/V -3.7 and -2.5; (3) A/P +2.5; M/L -0.8; D/V -3.7 and -2.5; tooth bar -4.
**Immunohistochemistry**

All procedures and protocols were performed as detailed in (Grealish et al., 2014). All animals were transcardially exsanguinated with physiological saline solution before perfusion with fresh, ice-cold 4% paraformaldehyde. The brains were extracted and post-fixed for 2 hours, before cryopreservation in 25% sucrose solution prepared using PBS. The brains were sectioned on a freezing microtome in a 1:8 series at a thickness of 35 µm.

For this study all primary antibodies were used as follows: rabbit anti-5-HT (1:10 000; Incastar 20080); rabbit anti-DARPP32 (1:4 000; Santa Cruz SC11365); chicken anti-GFP (1:1 000; Abcam ab13970); goat anti-FOXA2 (1:500; Santa Cruz M-20); mouse anti-human NCAM (1:1 000; Santa Cruz Eric-1); mouse anti-HuNu (1:200; Millipore MAB1281); rabbit anti-LMX1A (1:5 000; Millipore ab10533); mouse anti-mCherry (1:1 000; Abcam ab65856); goat anti-OTX2 (1:1 000; R&D AF1979); chicken anti-TBR-1 (1:1 000; Millipore AB2261); mouse anti-tyrosine hydroxylase (1:5 000; Immunostar); rabbit anti-tyrosine hydroxylase (1:1 000; Millipore ab152).
**Quantifications**

To assess the relative proportion of dopaminergic and serotonergic starter neurons one anterior section and one section of the graft core was stained for mCherry, GFP and TH or mCherry, respectively. For each rat (n=7) these two sections were imaged at x20 magnification using a Leica DMI6000B microscope and all single mCherry, all single TH, as well as all co-expressing mCherry/TH expressing cells were counted off-line using Adobe Photoshop. To quantify the number of traced neurons in different host structures, all coronal sections (1:8 series) stained for mCherry were imaged at x20 magnification using a Leica DMI6000B microscope. Using Adobe Photoshop all sections were scanned visually for tracing events, which were recorded and registered. Coronal sections with tracing events were overlaid with the appropriate coronal image from the Paxinos and Watson rat brain atlas and the location of the traced cells was registered in the respective structure.

**REFERENCES**

Graphical Abstract

Highlights
- Ascl1, Lmx1a, and Nurr1 convert striatal NG2 glia into functional neurons
- Cre-inducible AAV conversion vectors result in efficient neural conversion in vivo
- A neuron-specific reporter allows for long-term phenotypic and functional analysis
- Rabies-based tracing show that converted neurons integrate into local circuitry

Authors
Olof Torper, Daniella Rylander Ottosson, Maria Pereira, ..., Tiago Cardoso, Shane Grealish, Malin Parmar

Correspondence
malin.parmar@med.lu.se

In Brief
Torper et al. have developed an AAV-based system for in vivo neural conversion with neuron-specific reporters allowing for long-term histological and functional characterization. The in-vivo converted neurons are shown to be functional and integrate into host neural circuits, as assessed with rabies-virus-based tracing.

Torper et al., 2015, Cell Reports 12, 1–8
July 21, 2015
© 2015 The Authors
http://dx.doi.org/10.1016/j.celrep.2015.06.040
In Vivo Reprogramming of Striatal NG2 Glia into Functional Neurons that Integrate into Local Host Circuitry

Olof Torper,1,2 Daniella Rylander Ottosson,1,2 Maria Pereira,1,2 Shong Lau,1,2 Tiago Cardoso,1,2 Shane Grealish,1,2 and Malin Parmar1,2,*

1Department of Experimental Medical Science, Wallenberg Neuroscience Center
2Lund Stem Cell Center
Lund University, 221 84 Lund, Sweden
*Correspondence: malin.parmar@med.lu.se

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SUMMARY

The possibility of directly converting non-neuronal cells into neurons in situ in the brain would open therapeutic avenues aimed at repairing the brain after injury or degenerative disease. We have developed an adeno-associated virus (AAV)-based reporter system that allows selective GFP labeling of reprogrammed neurons. In this system, GFP is turned on only in reprogrammed neurons where it is stable and maintained for long time periods, allowing for histological and functional characterization of mature neurons. When combined with a modified rabies virus-based trans-synaptic tracing methodology, the system allows mapping of 3D circuitry integration into local and distal brain regions and shows that the newly reprogrammed neurons are integrated into host brain.

INTRODUCTION

Lineage conversion, where one terminally differentiated cell is converted into another terminally differentiated cell type via viral-mediated expression of transcription factors, has recently been achieved for many cell types (Vierbuchen and Wernig, 2011), including cells of the CNS such as neurons, astrocytes, oligodendrocytes, and neural progenitor cells (Caiazzo et al., 2015; Thier et al., 2012; Vierbuchen et al., 2010; Yang et al., 2013). In the brain, resident glia and transiently appearing proliferating progenitors have been successfully converted into neurons or neural progenitor cells in situ using defined combinations of transcription factors (Guo et al., 2014; Torper et al., 2013), and in some cases, functional properties such as action potentials and/or postsynaptic currents have been observed (Heinrich et al., 2014; Niu et al., 2013). However, to date, it is not clear if the new neurons can integrate into the host neuronal circuitry, and studies of this are partly hampered by lack of good experimental systems.

In this study, we present an adeno-associated virus (AAV)-based conversion system that can be used to specifically target and convert resident glia at high efficiency. When combined with a neuron-specific reporter system, it allows for long-term monitoring and functional analysis of the reprogrammed neurons. Using this system, we show that the striatal NG2 glia can be converted to GABAergic and glutamatergic neurons that remain stable over a long period of time and display electrophysiological properties of functional neurons. By combining the reporter system with virus-based tracing methodology, we show that the new neurons integrate into local circuitry in an efficient manner.

RESULTS

To specifically target NG2-expressing cells, we developed Cre-recombinase-dependent AAV vectors. In these flip-excision (FLEX) vectors, the conversion factors and reporter genes are inserted in an antisense direction, flanked by two pairs of antiparallel, heterotypic loxP sites (Atasoy et al., 2008). Each of the three conversion factors, Ascl1, Lmx1a, and Nurr1 (ALN), are placed under the control of the ubiquitous chicken β-actin (cba) promoter on individual vectors (Figure S1A). To construct a reporter system that restricts GFP expression to reprogrammed neurons, we placed GFP under the control of the neuron-specific synapsin promoter in a FLEX vector (Figure S1A) so that GFP would be expressed only in neurons originating from a Cre-expressing cell.

To validate the vectors, we first transfected T293 cells and transduced the cultures with a lentiviral vector expressing Cre. In the absence of Cre, there is no transgene expression. In the presence of Cre, the transgene is excised and recombined stably in a correctly reinserted orientation, allowing the transgene to be transcribed and protein to be expressed (Figure S1B). Next, we injected our vectors into the striatum of transgenic mice aged 12–16 weeks (Figure 1A) that express Cre under the control of the mouse NG2 (Cspg4) promoter/enhancer (Zhu et al., 2008). When injecting a ubiquitous reporter vector (cba-GFP, n = 5; Figure S1A) that expresses GFP in all Cre-positive cells, we found that GFP was exclusively expressed in NG2 glia and co-localized with NG2 (Figure 1C), SOX10, and PDGFRα (Figures S1F and S1G). When we injected our new neuron-specific reporter (syn-GFP) into the striatum of NG2Cre-expressing mice aged 12–16 weeks, we did not detect any GFP-expressing...
cells (Figure 1B), with the exception of 1 animal out of 11 analyzed, where we observed a total of 18 GFP+ cells. We also injected the cba-GFP and syn-GFP vectors into wild-type mice and never detected any GFP expression (Figure S1 C; n = 10 for each reporter). Taken together, these data confirm a specific Cre-dependent control of gene expression only in NG2-expressing cells in vivo and establish that the neuron-specific reporter is not active in these cells. All subsequent experiments were performed in mice aged 12–16 weeks; we included control animals injected with syn-GFP only in each experiment and never observed any conversion in these animals (Figures 1G and 1I).
To analyze the potential for neural conversion of striatal NG2 glia using ALN as conversion factors, we injected a mix of ALN and syn-GFP (1:1:1:1) into the striatum of NG2-Cre mice and also injected syn-GFP alone as control (n = 4 for each time point and vector combination). Animals were sacrificed 2 days and 1, 4, and 12 weeks after vector injections. In the ALN-injected group, no GFP-positive cells could be detected when analyzed after 2 days of injection (n = 4, not shown), and only a few GFP-positive cells could be detected when analyzed 1 week after injection (n = 6; Figures 1D and 1D’). The number of GFP-positive cells increased by 4 and 12 weeks (n = 4 and 7 for each time point, respectively; Figures 1E, 1E’, 1F, and 1F’). No GFP-expressing cells could be detected in the control-injected animals (Figures 1G, S1D, and S1E). Quantifications showed an increasing number of converted GFP+ cells over time, reaching 6,912 ± 3,052 per animal at 4 weeks (n = 3). As Ascl1 alone can convert somatic cells into neurons whereas Lmx1a and Nurr1 cannot (Chanda et al., 2014, Pfister et al., 2011), we next performed injections with Ascl1 only and compared these mice with ALN-injected and control animals. We found some converted neurons in the Ascl1-only group (Figure 1H), albeit in much lower numbers compared to ALN-injected animals (Figure 1I). The control animals injected with syn-GFP only did not contain any reprogrammed neurons (Figure 1I).

To establish a neuronal phenotype of the ALN-converted cells, we stained for the neuronal marker NeuN. The GFP+ cells co-expressed NeuN (Figures 2A–2A’), and the proportion of converted cells co-expressing GFP and NeuN increased from 20.8% ± 5.9% (n = 3) at 4 weeks to 46.8% ± 2.9% (n = 3) after 12 weeks (Figure 2B). The reprogrammed cells also co-expressed MAP2, and confocal analysis confirmed the presence of cells positive for syn-GFP, NeuN, and MAP2, further confirming a mature neuronal phenotype of the converted cells (Figures 2C–2C’). Staining for NG2 and GFP confirmed that no cells co-expressed GFP and NG2, suggesting a rapid loss of glial properties in the converted cells (Figures 2D–2D’).

Next, we co-stained GFP with a panel of subtype-specific markers: tyrosine hydroxylase (TH) for dopaminergic neurons, GAD65/67 for GABAergic neurons, vGlut1 for glutamatergic neurons, DARPP32 for striatal projection neurons, and paravalbumin (PA), calbindin (CALB), and choline acetyltransferase (Chat) for striatal interneurons. The gene combination used in this study (ALN) has been shown to convert mouse fibroblasts into functional neurons, including dopamine neurons, in vitro (Caiazzo et al., 2011). However, we did not detect any converted neurons expressing TH, even though ALN was used to drive neural conversion in vivo (Figure S2F). Given that all factors were delivered on separate vectors, the lack of TH could potentially be explained by lack of co-expression of the dopamine fate determinants in the reprogrammed neurons. However, when analyzing this, we found a large overlap in expression when staining for GFP and NURR1 as well as for GFP and LMX1A.
Figure 3. Reprogrammed Neurons Show Electrophysiological Properties of Functional Neurons

(A) Converted neurons acquire a mature neuronal morphology and exhibit dendrite spine-like protrusions. (B) Example of a cell selected for patch-clamp recordings 12 weeks after conversion. (C and D) Reprogrammed cells evoke action potentials (AP) upon depolarizing current injection (C) and inward sodium (Na+) and outward potassium (K+) current upon depolarization in artificial cerebrospinal fluid (ACSF) (D). (E) Action potentials and inward outward current could be blocked by adding the neurotoxin TTX to the buffer. (F) Examples of spontaneous post-synaptic currents.

See also Table S1.

(Figures S1H–S1I”). Quantifications showed that 79% ± 8% and 83.7% ± 6% of the GFP+ neurons co-expressed LMX1A and NURR1, respectively. To exclude that the lack of a dopamine-like phenotype is due to the cell of origin, we repeated the experiment using astrocytes instead of NG2 glia as a starter population for neural conversion. In these studies, we used a GFAP-Cre mouse to specifically target the resident astrocyte population. We injected syn-ALN and syn-GFP into the striatum of GFAP-Cre mice as well as syn-GFP only (n = 5 for each group). 6 weeks after vector injections, we could detect a high level of neural conversion as assessed by GFP expression (Figure S2F) in the animals injected with ALN, but not in animals injected with syn-GFP alone, matching what we have reported from reprogramming GFAP-Cre glia using Ascl1, Brn2a, and Myt1 (Torper et al., 2013). Co-staining with TH showed that the ALN-converted neurons originating from GFAP+ astrocytes, like the ALN-converted neurons originating from NG2+ glia, do not express TH (Figure S2F). 16% of the reprogrammed neurons expressed vGlut1 (Figure 2E), and 68% expressed GAD65/67 (Figures 2F and S2A), while none were found to co-express DARPP32, PA, CALB, or CHAT (Figures S2B–S2E).

Twelve weeks after conversion, the reprogrammed neurons had acquired a mature neuronal morphology, and dendritic spine-like protrusions could be observed (Figure 3A). At this time point, we used patch-clamp electrophysiology to investigate the functional properties of the neurons obtained (Figure 3B). All the GFP-expressing neurons recorded showed functional electrophysiological properties of neurons, with a resting membrane potential (Vm) of –61.4 ± 9.7 mV (n = 5) and the ability to evoke repetitive action potentials after current injections, with a mean amplitude of 33.5 ± 2.29 mV and an action potential threshold of 25 ± 7.19 pA (Figure 3C; Table S1; n = 6). Furthermore, all the converted cells recorded from showed fast-inactivated inward and outward currents characteristic of sodium and delayed-rectifier potassium currents, with a peak amplitude of \( I_{Na} \) at 625 ± 131 pA and \( I_K \) at 2,020 ± 290 pA (Figure 3D; n = 6). Both action potentials and inward currents could be blocked with the neurotoxin tetrodotoxin (TTX) (Figure 3E). We could also detect post-synaptic events with a mean frequency of 3.67 ± 1.93 Hz and a peak amplitude of 5.03 ± 0.64 pA, indicative of formation of a functional postsynaptic compartment and possible establishment of synaptic networks (Figure 3F; Table S1).

Functional neuronal properties, including post-synaptic events, have previously been described for in-vivo-reprogrammed striatal astrocytes and for reprogrammed cortical NG2 glia (Heinrich et al., 2014; Niu et al., 2013), but these types of recordings cannot distinguish between functional integration with host neurons and communication among reprogrammed neurons. To confirm circuitry integration, and to map what neurons form synaptic inputs to the newly reprogrammed cells, we used modified rabies virus (ΔG-rabies) to trace connectivity of the reprogrammed neurons. ΔG-rabies is a deletion mutant virus where the gene coding for glycoprotein (GP; necessary for trans-synaptic spread) is replaced by the gene coding for mCherry (Wickersham et al., 2007a, 2007b) and pseudotyped to infect cells only via the avian tva receptor (Barnard et al., 2006). To selectively trace connectivity of our in-vivo-reprogrammed neurons, we constructed a FLEX tracing vector with a tricistronic construct encoding for nuclear GFP (ncGFP), tva receptor, and rabies GP under the synapsin promoter (Figure S3A). Expression of this vector is exclusive to the reprogrammed neurons and renders them infectable with Arabies via the tva receptor. Since the reprogrammed neurons also express GP from the tracing construct, the rabies can assemble into infectious particles in the reprogrammed neurons and transmit mCherry retrogradely across one synapse to host neurons that provide efferent input. Thus, targeted reprogrammed neurons (starter neurons) will appear red and green, while traced host neurons that synapse...
onto the starter neurons appear red only (schematic in Figure 4A). As a positive control, we used injection of unflexed tracing vectors containing the same synapsin-driven tricistronic construct with ncGFP, tva, and GP (Figure S3B). Because it is not FLEXed, this vector infects host neurons and thus traces the connectivity of endogenous striatal cells. We also injected animals with a vector containing ncGFP and tva but lacking GP (Figure S3B). This control vector allows for Δrabies infection via the tva receptor but no transsynaptic spreading, as the cell lacks GP (Wickersham et al., 2007a).

Three groups of animals were injected with (1) unflexed tracing vector as a positive control (tracer-control), (2) flexed tracing vector together with ALN to trace connectivity of reprogrammed cells (FLEX tracer + ALN), and (3) control vector lacking GP. 12 weeks after injection of these tracing and control vectors, Δrabies was injected in the same area, and 1 week later, the animals were perfused and used for histology (Figure 4B). In animals injected with tracer control (that traces from endogenous neurons), we could detect targeted host starter cells expressing GFP and mCherry at the injection site (Figure 4C), as well as traced-red-only cells (i.e., striatal neurons that have made a synaptic contact with the targeted cells) (Figure 4C). Outside the striatum, we could also detect traced mCherry*/GFP− neurons in structures that normally innervate the striatum, such as the motor cortex (Figure 4D), thalamus (Figure 4E), and substantia nigra (Figure 4F). In the animals injected with control vector lacking GP, no tracing was observed (Figure S3C), confirming the transsynaptic spread of mCherry.

To trace connectivity of the reprogrammed neurons, animals were injected with the FLEX tracer vector in combination with ALN (Figure 4B). In these animals, we detected targeted reprogrammed starter cells expressing GFP and mCherry at the injection site (Figure 4G). Also in this group, red-only-traced cells representing endogenous host neurons that have made a synaptic contact with the reprogrammed neurons could be detected (Figure 4G). Co-staining of mCherry with DARPP32 showed that host cells that connect with the reprogrammed neurons are of the medium spiny neuron subtype (Figures 4K–4K′). Interestingly, no mCherry-positive neurons could be detected outside the striatum in this group, indicating that the new neurons preferentially integrate into local circuitry (Figures 4H–4I).

The starter neurons and the traced neurons were evenly distributed within the striatum (Figure 4L). Quantifications of the reprogrammed starter neurons and the traced host neurons showed that each animal contained more traced host neurons than reprogrammed starter neurons (Figure 4M) and that, on average, each reprogrammed neuron was contacted by three or four host neurons (Figure 4N).

**DISCUSSION**

Recent studies have shown that it is possible to convert endogenous glia into functional neurons in vivo, which offers new possibilities for brain repair (Amamoto and Arlotta, 2014). With few exceptions (Heinrich et al., 2014; Niu et al., 2013), the neuronal properties of the in-vivo-reprogrammed neurons have mostly been assessed based on marker expression (Guo et al., 2014; Magnusson et al., 2014; Torper et al., 2013) or in vitro (Guo et al., 2014).

In this study, we have developed an AAV-based vector system for in vivo neural conversion that results in efficient targeting and conversion of glia in situ. Importantly, the reporters used in this system are designed to be exclusive to the newly reprogrammed neurons and remain stable over time, allowing for long-term analysis of phenotype and function of the new neurons. The reporter can be combined with virus-based tracing methodology, which permits mapping of circuitry integration and allows for the precise identification of the source of host afferent inputs to the reprogrammed neurons.

The vector combination used in this study (ALN) has previously been used to reprogram mouse fibroblasts and glia into dopamine neurons in vitro (Addis et al., 2011; Caiazzo et al., 2011; Torper et al., 2013), yet no TH-expressing neurons could be detected when astrocytes or NG2 glia were converted in vivo. This mimics previous findings on astrocyte conversion, where several factors have been shown to convert astrocytes into neurons in vitro (Benniger et al., 2007; Heinrich et al., 2010) yet fail to do so in vivo (Su et al., 2014).

Nevertheless, the use of ALN results in functionally mature neurons in larger proportions than previously reported for conversion of resident glia using Sox2 alone or in combination with Mash1 (Heinrich et al., 2014), without the need for treatment with neurotrophic factors or histone deacetylase inhibitor as previously reported as a requirement for neuronal maturation (Niu et al., 2013). We made use of a monosynaptic tracing methodology based on modified rabies virus to analyze if and how the newly reprogrammed neurons integrate with existing host neurons. Rabies-based tracing has previously been used to trace connectivity of endogenous neural circuitry (Miya et al., 2011; Watabe-Uchida et al., 2012), as well as newly born neurons in the olfactory bulb and hippocampus (Deshpande et al., 2013; Vivar et al., 2012). Here, we used it to map efferent connectivity to the newly reprogrammed neurons and revealed that they are extensively innervated by host striatal projection neurons.

**EXPERIMENTAL PROCEDURES**

**Animals and Surgery**

All experimental procedures were carried under the European Union Directive (2010/63/EU) and approved by the ethical committee for the use of laboratory animals at Lund University and the Swedish Department of Agriculture (Jordbruksverket). Surgery was performed under general anesthesia using 2% isoflurane in a mix of air and N2O at a 4:1 ratio. For conversion, 1 μl vector was injected in the striatum of each animal at a rate of 0.2 μl/min and a diffusion time of 2 min at the coordinates A/p = +0.5, M/L = −2.0, and D/V = −2.7, toothbar = flat. For tracing experiments, tracing vectors plus ALN were injected at the same dilutions and volume as above at the same coordinates, and tracing vector alone was injected contra-laterally (M/L −2.0). Animals were kept for 11 weeks, and 1 week prior to perfusion, animals were injected with Δrabies diluted to 5% of stock (20–30 x 105 T.U/ml) in three consecutive injections of 0.5 μl each (0.5 μl deposit) flanking the original injection at coordinates (1) A/P +1.4; M/L −2.0; (2) A/P +0.4; M/L −2.0; or (3) A/P +0.8; M/L −1.5. A depth of D/V = −3 was used for all sites.

Patch-clamp electrophysiology was performed on coronal brain slices at 12 weeks post-conversion. Mice were killed by decapitation, and brains were rapidly taken out and cut on a vibratome at 350 μm. Slices were transferred to a recording chamber and submerged in a continuously flowing Krebs solution gassed with 95% O2 and 5% CO2 at 28 °C. The composition of the
Please cite this article in press as: Torper et al., In Vivo Reprogramming of Striatal NG2 Glia into Functional Neurons that Integrate into Local Host Circuitry, Cell Reports (2015), http://dx.doi.org/10.1016/j.celrep.2015.06.040

(legend on next page)
standard solution was 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4 \cdot H2O, 1.3 mM MgCl2 \cdot 6H2O, and 2.4 mM CaCl2 \cdot 2H2O. Voltage-gated sodium channels were blocked with 1 \mu M TTX (Tocris). Recordings were made using Multi-clamp 700B (Molecular Devices), and signals were acquired at 10 kHz using pClamp10 software and a data acquisition unit (Digidata 1440A, Molecular Devices). Input resistances and injected currents were monitored throughout the experiments.

Converted cells were identified with their GFP fluorescence and patched with borosilicate glass pipettes (3–7 MΩ) filled with the following intracellular solution: 122.5 mM potassium glutamate, 12.5 mM KCl, 0.2 mM EGTA, 10 mM HEPES, 2 mM MgATP, 0.3 mM NaGTP, and 8 mM NaCl adjusted to pH 7.3 with KOH. Resting membrane potentials were monitored immediately after breaking-in and clamping mode. Thereafter, currents were injected from -20 pA to +90 pA with 10-pA increments to induce action potentials. For sodium and potassium current measurements, cells were clamped at -70 mV and voltage-depolarizing steps were delivered for 100 ms at 10-mV increments. Transient activity was measured in voltage-clamp mode using the same internal solution at resting membrane potentials. Group mean values were calculated using GraphPad Prism.

For details on cloning, virus production, immunohistochemistry, and quantifications, please see the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at Supplemental Information.

ACKNOWLEDGMENTS

We would like to thank Ed Callaway for valuable help establishing monosynaptic tracing and producing modified rabies virus; Johan Jakobsen and Anders Bjerkland for scientific discussions and proof reading of manuscript; and Ulla Jarl, Bengt Mattsson, Michael Sparrenius, Jenny Johansson, and Ingar Nilsson for excellent technical assistance. This study was supported by grants from the European Community’s 7th Framework Programme through NeuroStemcellRepair (nr. 602278), The Strategic Research Area at Lund University (multidisciplinary research in Parkinson’s disease), the Swedish Research Council (20136201/Bagadilico and 2014-61X-20397-08-4), and the Swedish Parkinson Foundation (Parkinsonfonden). S.G. was supported by a postdoctoral stipend from the Swedish Brain Foundation (Hjärnfonden). The research leading to these results has received funding from the European Research Council under the European Union’s 7th Framework Programme (FP7/2007-2013)/ERC grant agreement 309712.

REFERENCES


Figure 4. Reprogrammed Neurons Are Innervated by the Pre-existing Local Neural Circuitry

A) Schematic of modified-rabies-mediated monosynaptic tracing. Reprogrammed cells infected by modified rabies appear with a green nucleus and a red cytoplasm, whereas any neuron providing synaptic input to the reprogrammed neurons appears as red only.

B) Experimental timeline.

(C) Injection of unflexed tracer serves as positive methodological control. Targeted host neurons (green and red) receive synaptic input from local striatal neurons (red only).

(D–F) Traced neurons (DAB and red) are also detected in the motor cortex (PFC), thalamus (Thal), and substantia nigra (SN).

(G) Tracing of reprogrammed neurons using FLEX tracer + ALN (red and green) shows traced host neurons (red).

(H–J) The absence of traced neurons in PFC, Thal, and SN suggest that the reprogrammed cells integrate only locally.

(K–K’) Co-labeling of mCherry and DARRP32 (blue) shows that the traced neurons are of a medium spiny neuron subtype.

(L) Distribution of the reprogrammed neurons (yellow) and traced neurons (red) in a representative animal.

(M and N) Quantifications of reprogrammed (gray bars) and traced (white bars) neurons (n = 7).

Scale bars represent 50 \mu m in (C) and (D–H) and 10 \mu m in (I–J). Error bars represent SEM. See also Figure S3.


In Vivo Reprogramming of Striatal NG2 Glia into Functional Neurons that Integrate into Local Host Circuitry

Olof Torper, Daniella Rylander Ottosson, Maria Pereira, Shong Lau, Tiago Cardoso, Shane Grealish, and Malin Parmar
Figure S1 - related to Figure 1

(A) Map of AAV constructs used to target NG2 cells in NG2-Cre expressing mice. (B) Validation of the expression of the constructs by transfection of T293 cells in the presence or absence of cre. (C) Wild type littermates injected with the same vectors never recombine to express GFP or the factors. (D-E) Transgenic NG2-Cre mice injected with flexed Syn-GFP never express GFP 4 or 12 weeks after injection confirming the reporting of GFP to reprogrammed cells. (F-G) Transgenic NG2-Cre mice injected with a flexed cba-GFP reporter construct confirms the exclusive recombination of the vectors in NG2 cells by the co expression of Sox10 (blue) and PDGFR-α (red). (H-H’’) The majority of the reprogrammed cells co-express the reprogramming factors Nurr1 (red) or (I-I’’) Lmx1a (red). Scale bars in (B) and (H-I’’) = 100 µm, (C-E) = 500 µm and 25 µm for (F-G).
Figure S2 - related to Figure 2

(A) Most reprogrammed cells express GAD65/67 (red). (B-E) Reprogrammed cells are negative for striatal markers DARPP32 (blue), parvalbumin (PA, blue), calbindin (calb, blue) and choline acetyltransferase (Chat, blue). (F) GFAP-Cre transgenic animals injected with cre inducible AAV vectors for ALN fail to convert into dopamine neurons. Scale bars (A-F) = 50 µm.
Figure S3 - related to Figure 4

(A) Map of constructs used for the flexed-tracing + ALN injections. (B) Map of constructs used for control tracing. (C-F) Micrographs using control vector lacking GP to show that the tracing is synaptic. Scale bar in (C) = 50 µm and in (D-F) = 100 µm. Conversion = 1305 +/-143 (n=4).
Table S1

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Table S1 - related to Figure 3

Electrophysiological properties of converted cells recorded from.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cloning

Cre-inducible AAV5 vectors were created by inserting cDNA for GFP, Ascl1, Lmx1a or NR4A2 (Nurr1) in a reverse orientation flanked by two pairs of heterotypical, antiparallel LoxP (Flex) sequences. Expression of GFP was under the control of either a ubiquitous expressed chicken beta actin (cBA) promoter or a synapsin promoter, and Ascl1, Lmx1a and Nurr1 were all regulated by the cBA promoter. All constructs were sequenced prior to use. The constructs for the tracing and control vectors (as detailed in Figure S4A-B) were purchased from Addgene (IDs:30456, control vector and 30195, tracing vector) and cloned into the flexed vectors as described above.

Viral Vector production

High-titer preparations of lentiviral particles were produced as previously described and similar cre inducible AAV5 vectors as described above were produced for the flex-tracer (Zolotukhin et al., 1999; Zufferey et al., 1997). Pseudotyped rabies vectors were produced in house from starter cells provided by Edward Callaway at the Salk Institute, USA, according to (Osakada and Callaway, 2013) with minor adjustments.

Immunohistochemistry

At experimental endpoints all animals were trans-cardially perfused in ice cold 4% PFA, brains were removed and post fixed 12 h in 4% PFA. The following day brains were put in 25% sucrose for 12 h, frozen and then cut on a microtome in 35 µm sections in a series of 8.

Sections were stained by standard protocols using the following antibodies: PDGFR-α (rabbit, Santa Cruz, 1:200), SOX10 (goat, Santa Cruz, 1:2500), Nurr1 (rabbit, Santa
Cruz, 1:2000), Chat (goat, Milipore, 1:500), DARRP32 (rabbit, Milipore, 1:1000), NG2 (rabbit, Milipore, 1:200), NeuN (mouse, Milipore, 1:800), Lmx1a (rabbit, Milipore, 1:1000), GFP (rabbit, Milipore, 1:5000; chicken, Abcam, 1:1000), Mash1 (rabbit, Abcam, 1:500), Calbindin (mouse, Sigma, 1:1000) Parvalbumin (mouse, Sigma 1:2000) and MAP2 (mouse, Sigma, 1:500). For fluorescent stainings, Alexa 488 (goat, Life Technologies, 1:500) and Cy3/Cy5 (donkey, Jackson, 1:200) or biotinylated (Goat, Jackson, 1:200) with a Cy5 streptavidine (Jackson, 1:200) antibody were used as secondary antibody. For stainings in DAB, horseradish peroxidase conjugated secondary antibody (horse, Vector laboratories, 1:400) was used. All brightfield images were taken using an inverted Leica microscope equipped with a DFC450C camera and fluorescent images were taken using either a DFC360 FX camera mounted on the same microscope or a Leica TCS SP8 confocal laser-scanning microscope.

Quantifications and statistical analysis

The total number of GFP positive cells with a neuronal morphology was counted in 3 sequential sections in a series of every eight coronal section/animal. To correct for the sampling bias we utilized the Abercrombie correction N = Counts Raw x (section thickness µ) / (section thickness µ + average cell diameter µ). Cells were counted offline in tiled pictures taken using a microscope with a 20x objective. The same strategy was applied for NeuN quantifications and conversion efficiencies where the total number of NeuN or Nurr1 positive cells was divided by the total number of GFP positive cells. The number of traced neurons (all red only) was divided by the number of GFP/RFP (reprogrammed cells) double positive cells. All data is presented as Mean ± SEM. All quantifications were performed in blind.
SUPPLEMENTAL REFERENCES


Target-specific forebrain projections and appropriate synaptic inputs of hESC-derived dopamine neurons grafted to the midbrain of parkinsonian rats

Tiago Cardoso1,2 | Andrew F. Adler1,2 | Bengt Mattsson1,2 | Deirdre B. Hoban1,2 | Sara Nolbrant1,2 | Jenny Nelander Wahlestedt1,2 | Agnete Kirkeby1,3 | Shane Grealish1,2 | Anders Björklund1 | Malin Parmar1,2

1Developmental and Regenerative Neurobiology, Department of Experimental Medical Science, Lund University, Lund, Sweden
2Lund Stem Cell Center, Lund University, Lund, Sweden
3Danish Stem Cell Center (DanStem), University of Copenhagen, Copenhagen, Denmark

Correspondence
Malin Parmar, Department of Experimental Medical Science, Lund Stem Cell Center, Lund University, Lund 22184, Sweden.
Email: malin.parmar@med.lu.se

Funding information
Hjärnfonden; Parkinsonfonden; Seventh Framework Programme, Grant/Award Number: ERC Grant Agreement no. 30971 NeuroStemcellRepair (no. 602278); Vetenskapsrådet, Grant/Award Number: 2016-0087370862601/ Bagadilico; New York Stem Cell Foundation; Strategic Research Area at Lund University Multipark

Abstract
Dopamine (DA) neurons derived from human embryonic stem cells (hESCs) are a promising unlimited source of cells for cell replacement therapy in Parkinson’s disease (PD). A number of studies have demonstrated functionality of DA neurons originating from hESCs when grafted to the striatum of rodent and non-human primate models of PD. However, several questions remain in regard to their axonal outgrowth potential and capacity to integrate into host circuitry. Here, ventral midbrain (VM) patterned hESC-derived progenitors were grafted into the midbrain of 6-hydroxydopamine-lesioned rats, and analyzed at 6, 18, and 24 weeks for a time-course evaluation of specificity and extent of graft-derived fiber outgrowth as well as potential for functional recovery. To investigate synaptic integration of the transplanted cells, we used rabies-based monosynaptic tracing to reveal the origin and extent of host presynaptic inputs to grafts at 6 weeks. The results reveal the capacity of grafted neurons to extend axonal projections toward appropriate forebrain target structures progressively over 24 weeks. The timing and extent of graft-derived dopaminergic fibers innervating the dorsolateral striatum matched reduction in amphetamine-induced rotational asymmetry in the animals where recovery could be observed. Monosynaptic tracing demonstrated that grafted cells integrate with host circuitry 6 weeks after transplantation, in a manner that is comparable with endogenous midbrain connectivity. Thus, we demonstrate that VM patterned hESC-derived progenitors grafted to midbrain have the capacity to extensively innervate appropriate forebrain targets, integrate into the host circuitry and that functional recovery can be achieved when grafting fetal or hESC-derived DA neurons to the midbrain.

KEYWORDS

1 INTRODUCTION

Stem cell therapy for Parkinson’s disease (PD) is rapidly moving towards clinical trial (Barker, Parmar, Studer, & Takahashi, 2017). Several recently published studies have demonstrated maturation and function of dopamine (DA) neurons derived from human pluripotent stem cells (hPSCs) after transplantation (Chen et al., 2016; Grealish et al., 2014; Kikuchi et al., 2017; Kirkeby et al., 2012; Kriks et al., 2011; Steinbeck et al., 2015). With few exceptions, these studies have been conducted after ectopic, intrastratal graft placement in rodents.
and primates—mimicking the graft placement in clinical trials conducted with fetal ventral midbrain (VM) tissue (Barker, Drouin-Quellet, & Parmar, 2015). These studies clearly demonstrate the ability of stem cell-derived DA neurons to release DA and restore motor functions in animal models of PD with an efficacy and timing comparable to primary human fetal cells.

An important predictor of successful graft function in patients is the ability of the grafted DA neurons to reinnervate the denervated striatum in the host brain (Li et al., 2015). A critical step in developing effective stem cell-based transplantation therapies is to better understand the innervation capacity of the transplanted cells, as well as the extent of afferent inputs from the host, with the aim of re-establishing connectivity that is similar to healthy endogenous circuitry. The ability of hPSC-derived neurons to extend axons across large distances sufficient to reinnervate the human brain has been modeled experimentally using intrastratial transplantation in non-human primates (Kikuchi et al., 2017; Morizane et al., 2017; Wakeman et al., 2017). Transplantation of VM patterned progenitors into the ventral midbrain of rat models of PD—that is, the site where these cells normally reside—offers an interesting alternative model to explore outgrowth capacity and specificity in greater detail. We have previously shown that intranigral grafts of human embryonic stem cell (hESC)-derived VM progenitors, like their fetal counterparts, can extend axons over long distances to reinnervate distant forebrain targets (Grealish et al., 2014; Wictorin, Brundin, Sauer, Lindvall, & Bjorklund, 1992).

In this study, we further develop this approach to transplant VM patterned hESC-derived neural progenitors to the midbrain of a rat model of PD to assess temporal aspects of long-distance fiber outgrowth and graft integration. Using three-dimensional (3D) brain reconstruction of the distribution of graft-derived fibers, we assessed target-specific innervation along the nigrostriatal pathway (NSP) and the medial forebrain bundle (MFB) toward target forebrain structures. These target structures include the dorsal striatum (the prime target of A9 nigral neurons), as well as targets of the A10 neurons of the ventral tegmental area (VTA), that is, nucleus accumbens (NAC), septum and medial prefrontal cortex (PFC). Rabies-based monosynaptic tracing has already provided a more comprehensive identification of normal inputs to DA neurons in the VTA and substantia nigra (SN) in the intact brain (Beier et al., 2015; Faget et al., 2016; Lerner et al., 2015; Menegas et al., 2015; Watabe-Uchida, Zhu, Ogawa, Yamanara, & Uchida, 2012), and we now use this technique to map the origins and extent of synaptic inputs to graft-derived neurons and determine how hESC-derived neurons integrate into the host brain when grafted into their normal anatomical location, the midbrain.

The results demonstrate that while the transplanted neurons progressively innervate their normal target structures over 6 months, they have been contacted by appropriate presynaptic host neurons already by 6 weeks after grafting. Analysis of behavioral recovery suggests that the timing of graft-mediated functional recovery after intranigral grafting corresponds to the time of arrival of graft-derived axonal innervation in the correct target structures, rather than that of the establishment of presynaptic partners from the host, which also matches the timing of behavioral recovery reported in other studies where the cells are transplanted to the striatum (Brundin et al., 1986; Lelos et al., 2016).

2 | METHODS

2.1 | Research animals

Adult (<180 g) female, athymic “nude” rats were purchased from Harlan Laboratories (Hsd:RH-Foxn1nu™) and housed in individual ventilated cages with ad libitum access to food and water, under a 12-hr light/dark cycle. Adult (>1 year) male, athymic “nude” rats (Crl:Nih-Foxn1nu™) were purchased from Charles River as hosts for the human fetal midbrain tissue. Female adult (225–250 g) Sprague–Dawley rats were purchased from Charles River and housed as described above but in standard caging. All procedures were conducted in accordance with the European Union Directive (2010/63/EU), follow 3R principles, and were approved by the local ethical committee for the use of laboratory animals and the Swedish Department of Agriculture (Jordbruksverket).

2.2 | Experimental overview

All rats received a unilateral injection of 6-hydroxydopamine to the MFB, and the efficacy of lesion was confirmed by amphetamine-induced rotations 4 weeks later.

Sprague–Dawley rats were used as recipients for transplantation experiments lasting 18 weeks or less. These animals were immunosuppressed with daily injections of cyclosporine (10 mg/kg/day, intraperitoneally; Apoteksbolaget, Sweden), starting 2 days prior to transplantation until the end of the experiment to prevent graft rejection. Athymic nude rats were used as recipients for transplantation experiments lasting >18 weeks.

2.2.1 | Temporal profiling of graft-derived fiber outgrowth

For temporal assessment of graft-derived fiber outgrowth, all animals (n = 30) received a transplant of hESC-derived neurons into the midbrain and were allocated to three different groups pertaining to three different graft survival time points: 6 weeks (n = 10), 18 weeks (n = 10), and 24 weeks (n = 10).

Animals that died before the experimental endpoint were removed from further analysis. Following perfusion and histological analysis, only the animals with surviving grafts that were discretely placed within the midbrain were included in the final analysis: 6 weeks (n = 6), 18 weeks (n = 6), and 24 weeks (n = 5).

2.2.2 | Endogenous connectivity and host-to-graft tracing experiments

To study endogenous connectivity in the midbrain, un-lesioned age- and sex-matched Sprague–Dawley rats (n = 5) were injected in the midbrain with lentivirus expressing the rabies helper construct. Four weeks later, these animals received ΔG-rabies injection at the same site and were perfused 7 days later. To assess host-to-graft connectivity at 6 weeks, animals (n = 10) were transplanted with hESC-derived neurons expressing the rabies helper construct into the midbrain. ΔG-rabies was injected at the site of transplantation after 5 weeks and the animals were perfused 7 days later for histological analysis.
2.3 | ΔG-rabies and lentivirus production

EnvA-pseudotyped ΔG-rabies was produced as described in Grealish et al. (2015). Titers were 20–30 × 10^6 TU/ml and a working dilution of 5% was used for injection in vivo. The construct for the tracing vector was purchased from Addgene (ID: 30195). High-titer preparations of lentiviral particles were produced as previously described (Zufferey, Nagy, Mandel, Naldini, & Trono, 1997). Titers were 3–9 × 10^5 U/ml and a dilution of 20% was used for in vivo experiments.

2.4 | Differentiation of hESCs and transgene expression

Human ESCs H9 (WA09, passage 31–45) and RC-17 (Roslin, passage 28) were patterned toward a ventral midbrain fate as described previously (Nolbrant, Heuer, Parmar, & Kirkeby, 2017) and used for assessing graft outcome. For the tracing studies, hESCs were repeatedly transduced with the tracing construct (Grealish et al., 2015), and the cells were transduced again at day 4 and 11–15 of differentiation using lentivirus vectors at a Multiplicity of Infection (MOI) of 1.5.

2.5 | Human fetal tissue

Human fetal tissue was obtained from legally terminated embryos with approval of the Swedish National Board of Health and Welfare in accordance with existing guidelines including informed consent from women seeking elective abortions. The gestational age of each embryo was determined by measuring the crown-to-rump length and/or estimated by ultrasound measurements. Embryos were then staged according to weeks post-conception (p.c.). Fetal tissue was prepared and transplanted into the midbrain of 6-OHDA lesioned athymic nude rats that were perfused after 24 weeks (Nag, Mandel, Naldini, & Trono, 1997). Titers were 3–9 × 10^5 U/ml and a dilution of 20% was used for in vivo experiments.

2.6 | Amphetamine-induced rotations

Lesion-induced rotational bias was assessed by amphetamine challenge (intrapertoneal injection of 2.5 mg/kg of amphetamine; Apoteksbolaget, Sweden) and recorded over 90 min using an automated system (Omnitech Electronics). Full body turns toward the side of the lesion side were given positive values and turns to the opposite side given negative values—and data expressed as net turns per minute. Animals with a net score of five turns per minute or greater were considered to have successful lesions and were included in the behavioral analysis in Figure 4k.

2.7 | Surgical procedures

All surgical procedures were performed under general anesthesia using a solution of fentanyl and medetomidine (20:1) injected intraperitoneally (1 ml/kg; Apoteksbolaget, Sweden). Rats were rendered parkinsonian by unilateral injection of 6-hydroxydopamine into the right MFB, with a volume of 4 μl at 20.6 mM to the following coordinates relative to bregma: A/P -4; M/L -1.2; D/V (from dura) -7.5; tooth bar -4.

Four weeks after 6-OHDA lesion, host rats received a total dose of 75,000 hESCs at day 16 of differentiation into the midbrain in a volume of 2 μl, at a concentration 37,500 cells/μl at a rate of 1 μl per minute and diffusion time of 2 min, to the following coordinates relative to bregma: A/P -5.2; M/L -2.3; D/V (from dura) -7; adjusted to flat head.

For fetal VM tissue transplantation, individual fetuses aged 5.5 or 8 weeks p.c. were dissociated in 20 μl and 1.5 μl per rat was transplanted to the following coordinates relative to bregma: A/P -4.6; M/L -2.2; D/V (from dura) -7; tooth bar -2.4.

For host-to-graft monosynaptic tracing experiments, ΔG-rabies at a dilution of 5% of the stock was injected in a volume of 2 μl, at a rate of 0.5 μl per minute and diffusion time of 2 min, into the transplantation site: A/P -5.2; M/L -2.3; D/V (from dura) -7; adjusted to flat head.

For the mapping of endogenous midbrain connectivity, the tracing lentiviral vector was injected into the midbrain in a volume of 2 μl, at the following coordinates relative to bregma: A/P -5.2; M/L -2.3; D/V (from dura) -7; adjusted to flat head. Four weeks later ΔG-rabies was injected in volume of 2 μl at the same site.

2.8 | Immunohistochemistry

Prior to perfusion, rats were given terminal anesthesia with a lethal dose of 60 mg/kg sodium pentobarbitone injected intraperitoneally (Apoteksbolaget, Sweden). The animals were transcardially perfused with physiological saline solution followed by ice-cold 4% paraformaldehyde. Brains were post-fixed for 2 h in 4% paraformaldehyde, transferred to 25% sucrose for 48 h and sectioned at a 35 μm thickness (1.8 series) using a freezing microtome. Immunohistochemistry was performed on free floating sections that were incubated with primary antibodies overnight in 0.1 M PBPS solution containing 0.25% Triton-X and 5% serum for the species specific to the secondary antibody. Sections were incubated with secondary antibodies for 1 hr in the same solution. Secondary antibodies coupled with biotin or conjugated with a fluorophore were used for dianaminobenzidine (DAB) or fluorescent detection, respectively. All stained sections were mounted on gelatin-coated microscope slides. DAB-developed sections were dehydrated in an ascending series of alcohols, cleared with xylene and coverslipped using DPX mountant. Fluorescent immunostainings were coverslipped using polyvinyl alcohol mounting medium with DABCO (PVA-DABCO, Sigma-Aldrich, St. Louis, Missouri, USA) and left to dry overnight.

A biotinylated tyramide signal amplification (TSA) protocol (Adams, 1992) was used to boost detection of fluorescently labeled human neural cell adhesion molecule (hNCAM) positive axons and μ-opioid receptor (MOR) positive cell bodies. After labeling with primary antibodies, sections were incubated in biotinylated secondary antibodies for 1 hr at room temperature, washed, and then incubated with ABC solution for 30 min. Sections were washed, then incubated with biotinyl tyramide (1:2,500 in KPBS containing 0.009% H2O2) for 30 min. hNCAM-stained sections were further amplified with a second round of ABC and biotinyl tyramide incubation. Sections were then fluorescently labeled by 2 hr incubation with fluorophore-conjugated streptavidin (1:500). Finally, sections were washed and mounted as described above.
2.9 Antibody characterization

The following primary antibodies were used: mouse monoclonal anti-human NCAM (1:1,000 for DAB reactions, and 1:5,000 for TSA reactions; Santa Cruz Biotechnology, Dallas, Texas, USA Cat# sc-106; RRID: AB_627128), immunogen raised against CD56 positive cells of human origin and recognizes band corresponding to hNCAM in western blot of transfected 293T whole lystate (manufacturer information); rabbit polyclonal anti-TH (1:1,000; Millipore, Burlington, Massachusetts, USA Cat# AB152, RRID: AB_390204), raised against denatured tyrosine hydroxylase from rat pheochromocytoma, selectively labels a single band at ~62 kDa in western blot of PC12 lysates (manufacturer information); rabbit polyclonal anti-NeuN (1:1,000; Millipore, Burlington, Massachusetts, USA Cat# ABN78, RRID: AB_10807945), raised against Glutathione S-transferase-tagged recombinant mouse NeuN N-terminal fragment, specificity evaluated by western blotting in mouse brain nuclear extract (manufacturer information); mouse monoclonal anti-red fluorescent protein (RFP) (1:1,000; Abcam, Cambridge, UK Cat# ab65856, RRID: AB_1141717), raised against recombinant RFP protein expressed in Escherichia coli, positive control: recombinant RFP protein (manufacturer information); goat polyclonal anti-mCherry (1:1,000; SIGKEN Cantanhege, Portugal Cat# AB0040-200, RRID: AB_2333092), raised against purified recombinant peptide produced in E. coli, detects 29 kDa band in western blot of 293HEK cell transfected with cDNA plasmid (manufacturer information); chicken anti-green fluorescent protein (GFP) (1:1,000; Abcam, Cambridge, UK Cat# ab13970, RRID: AB_300798), raised against recombinant full length protein corresponding to GFP, positive control: ICC/IF of GFP-transfected NIH3T3 cells (manufacturer information); guinea pig polyclonal anti-MOR (1:1,000; Millipore, Burlington, Massachusetts, USA Cat# ab5509, RRID: AB_177511), recognizes C-terminus of rat MOR corresponding to residues 384–398 (manufacturer information); rabbit polyclonal anti-Barhl1 (1:500; NovusBio, Littleton, Colorado, USA Cat# NB1-86513, RRID: AB_11034569), developed against recombinant Barhl1 protein, specificity verified on a protein array containing target protein plus 383 other nonspecific proteins (manufacturer information); rabbit polyclonal anti-5-HT (1:10,000; ImmunoStar, Hudson, Wisconsin, USA Cat# 20080; RRID: AB_572263), raised against serotonin coupled to BSA with paraformaldehyde, the antiserum demonstrated significant labeling of rat hypothalamus, raphe nuclei, and spinal cord using indirect immunofluorescent and biotin/avidin-horseradish peroxidase techniques (manufacturer information).

2.10 Microscopy and quantifications

All brightfield images were captured using a Leica DMi6000B microscope, while fluorescent images were acquired using a TCS SP8 laser scanning confocal microscope. For hNCAM fiber density quantifications, an ImageJ (FIJI) macro was used to automatically apply a Gaussian blur, set a local intensity threshold of a defined radius around each pixel, and quantify the pixels within the threshold and regions of interest defined with the aid of an atlas of the rat brain.

To assess the number of graft-derived dopaminergic fibers innervating the dorsolateral striatum, one section per animal depicting the same anatomical area (corresponding to +0.72 A/P relative to bregma from the Paxinos and Watson atlas of the rat brain) was immunostained with hNCAM (amplified with two rounds of TSA) and tyrosine hydroxylase (TH). Identical anatomical regions were imaged at 20× using the confocal microscope, and double positive (hNCAM+/TH+) fibers per field were manually quantified in a blinded fashion.

2.11 3D brain reconstruction

The 3D template of the rat brain used in this study for mapping of fiber outgrowth and ΔG-rabies traced neurons was built using a 3D software (Cinema 4D), with structural proportions based on the Paxinos and Watson rat brain atlas (Paxinos & Watson, 2005). For representation of graft-derived fiber outgrowth an entire 1:8 series of hNCAM DAB stained sections were imaged and placed in the appropriate anatomical plane in the 3D brain template. The area covered by hNCAM+ outgrowth was then mapped onto each anatomical plane with outgrowth in between sections being extrapolated. Quantifications of graft volume and fiber outgrowth were performed based on this 3D map using the same Cinema 4D software. For the representation of host synaptic inputs to the graft, 1:8 series of mCherry DAB stained sections were used, with each dot representing the location of an mCherry+ neuron.

2.12 Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed with GraphPad Prism 6. Statistical tests and biological replicates, representing individual animals are stated in the figures legends or results. A significance level of α = 0.05 was set for all comparisons.

In Figure 4j, Kruskal–Wallis test was performed followed by Dunn's multiple comparisons test to compare double hNCAM+/TH+ fibers at 6, 18, and 24 weeks. In Figure 4k, Student t-test was used to assess graft-mediated recovery in amphetamine-induced rotation asymmetry, 24 weeks after transplantation with fetal tissue.

3 RESULTS

3.1 hESC-derived neurons grafted to the midbrain progressively extend axons to innervate appropriate forebrain targets over the course of six months

To study the dynamics of graft-derived axonal outgrowth, hESCs were patterned toward a VM fate in vitro as previously described (Nolbrant et al., 2017) and transplanted into the midbrain of 6-OHDA lesioned adult immunosuppressed or athymic (nude) rats. After 6, 18, or 24 weeks of graft maturation, the animals were sacrificed for histological analysis (Figure 1a). The results show that transplanted VM patterned neural progenitors can generate grafts rich in TH+ neurons that survive long term after implantation into the midbrain (Figure 1b–b′), and that the graft core does not increase in volume between 6 and 24 weeks (Figure 1c). For analysis of graft survival, size, and pattern of fiber outgrowth, we performed a 3D virtual reconstruction of the host brain using histological sections immunostained with antibodies specific for hNCAM, that labels both human neuronal cell bodies and fibers (Figure 1d–d′).
Using the 3D reconstructed images, we estimated the area covered by hNCAM+ fibers at 12 evenly spaced levels along the antero-posterior (A-P) axis (Figure 2a), comparing the different groups to assess the temporal effects of fiber outgrowth along the MFB and the NSP, as well as the progressive innervation of distant A9- and A10-related forebrain target structures over the course of 24 weeks (Figure 2b,c).

At 6 weeks, the majority of hNCAM+ fibers had entered the MFB and NSP, reaching the posterior hypothalamus (Figures 1d and 2b,c—corresponding to levels 4 and 5 in Figure 2a,c). At this timepoint, scarce axonal fibers were detected in the caudal striatum (corresponding to levels 6 and 7 in Figure 2a,c). Counterstaining of striatal gray matter for NeuN revealed that hNCAM+ fibers were almost exclusively confined to areas devoid of neuronal cell bodies (NeuN−) (white arrow in Figure 2d). Indicating that the earliest graft-derived axons to reach the striatum extended rostrally along white matter tracts. Analysis of more rostral structures, including dorsolateral striatum, NAc, and PFC, demonstrated an absence of significant graft-derived innervation of more distant target structures (Figure 2a–d).

By 18 weeks, prominent hNCAM+ projections were seen to extend along the entire caudo-rostral extent of the MFB, into NAc, ventral striatum and septum—with some fibers reaching as far rostral as the medial PFC and the olfactory bulb (Figure 1d1 and corresponding to levels 7–11 in Figure 2a,c). At this stage dense hNCAM+ terminal networks had developed in NAc and septum, as well as in part of the medial PFC (Figures 2b and 3e–g), but we could detect only few hNCAM+ axon terminals in dorsolateral striatum (Figures 2b and 3h). Over the subsequent 6 weeks, we observed a marked expansion of the hNCAM+ terminal network in the main A9 target, striatum, as well as in the A10 targets NAc, ventral striatum, and septum (Figures 2b, 3i–l). At this timepoint (24 weeks), we could observe an extensive ramification of hNCAM+ fibers into striatal parenchyma (Figure 3i) that extend to striatal gray matter, as revealed by NeuN counterstaining, (white arrowhead in Figure 2e). These findings were confirmed by quantifying the density of fibers using automated image analysis in two of the principal A9 and A10 target structures, striatum and NAC (Figure 2f).
In summary, hESC-derived neurons placed in the midbrain progressively extend axons along the MFB and NSP, directed toward anatomically appropriate forebrain targets. Over the course of 24 weeks, we observed a progressively increased coverage of the forebrain areas normally innervated by midbrain DA neurons, with extensive axonal branching into the dorsal striatum (the prime target of the A9 nigral neurons), as well as into NAc, ventral striatum, septum, and medial PFC (targets of the A10 neurons in the VTA).

### 3.2 Graft-derived dopaminergic innervation of dorsolateral striatum is associated with reduction in rotational bias

Specific dopaminergic (TH+) innervation targeted to dorsolateral striatum is required for graft-mediated recovery of motor asymmetry in the 6-OHDA rat model (Bjorklund, Dunnett, Stenevi, Lewis, & Iversen, 1980; Dunnett, Bjorklund, Schmidt, Stenevi, & Iversen, 1983). Therefore, we proceeded to analyse the pattern of graft-derived dopaminergic outgrowth at 6 and 24 weeks after transplantation by co-staining for TH and hNCAM (green) and NeuN (blue) in the striatum demonstrates that graft-derived fibers are confined to white matter tracts at 6 weeks (white arrow in (d)), expanding into a fiber network in the striatal gray matter at 24 weeks (white arrowhead in (e)).

**FIGURE 2** Progressive reinnervation of host forebrain target structures by graft-derived fibers. (a) 3D template of the host brain showing the position of the 12 anterior/posterior levels (white circles labeled 1–12) used to measure graft-derived axonal outgrowth at 6, 18, and 24 weeks in (c). (b) Quantification of the percentage of host target structure volume innervated by hNCAM+ fibers at 6 weeks (n = 6), 18 weeks (n = 6), and 24 weeks (n = 5) post-grafting, demonstrates the progressive ramification of hNCAM+ fibers over time. (c) Area innervated by hNCAM+ fibers at 12 selected anterior/posterior levels (as shown in (a)) at 6 weeks (n = 6), 18 weeks (n = 6), and 24 weeks (n = 5) shows continuous outgrowth and progressive reinnervation of forebrain target structures over time. Immunostaining for hNCAM (green) and NeuN (blue) in the striatum demonstrates that graft-derived fibers are confined to white matter tracts at 6 weeks (white arrow in (d)), expanding into a fiber network in the striatal gray matter at 24 weeks (white arrowhead in (e)).
FIGURE 3  Graft-derived reinnervation of A9 and A10 target structures. (a–d) Analysis of graft-derived innervation as revealed by hNCAM immunohistochemistry 6 weeks post-transplantation, demonstrates no detectable graft fibers in PFC (b) and only few scattered axonal terminals in NAc (c) and dorsolateral striatum (d). (e–h) At 18 weeks, hNCAM+ fibers were observed in the PFC (f), with extensive graft-derived innervation of the NAc (g). Individual hNCAM+ fibers can be detected in dorsolateral striatum (h) at this timepoint. (i–l) At 24 weeks, abundant hNCAM+ innervation was readily detectable in PFC (i) and NAc (k). At this timepoint, an extensive network of graft-derived axonal projections were observed also in dorsolateral striatum (l). Black arrow in (b) and (j) denotes the lateral ventricle. Scale bars: (a, e, i) = 1 mm. (b–d, f–h, j–l) = 100 μm. Insets in (b–d, f–h, j–l) = 100 μm. ac = anterior commissure; DL = dorsolateral; fmi = forceps minor; LV = lateral ventricle; NAc = nucleus accumbens; PFC = prefrontal cortex; Sep = septum; Str = striatum.
no behavioral recovery was observed at this stage (squares, Figure 4k). At 18 weeks post-grafting, the appearance of graft-derived hNCAM\(^+/\)TH\(^+\) axonal projections in the dorsolateral striatum (Figure 4j) coincided with a trend of reduction in the amphetamine-induced rotation scores (triangles in Figure 4k). At 24 weeks, more hNCAM\(^+/\)TH\(^+\) fibers were present in the caudal and dorsolateral striatum (Figure 4e,f,g), and at this timepoint two out of three animals showed recovery in rotation scores (circles, Figure 4k). At this stage, double hNCAM\(^+/\)TH\(^+\) fibers could also be detected in other dopaminergic target structures, such as NAc and cortex as previously described (Grealish et al., 2014). However, the overall distribution of hNCAM\(^+/\)TH\(^+\) fibers was more extensive than the hNCAM\(^+/\)TH\(^+\) fibers, reflecting the heterogenous composition of the transplants.

A closer look at individual post-transplantation rotation scores from the 24-week survival group revealed two distinct behavioral responses elicited by the amphetamine challenge. One rat (black circle in Figure 4k) showed no behavioral recovery (net score pre-transplantation = 13 turns/min; post-transplantation = 13 turns/min) and two rats (gray and white circles in Figure 4k) exhibited a pronounced rotational bias attenuation (gray circle: net score pre-transplantation = 7 turns/min; post-transplantation = 1 turns/min; white circle: net score pre-transplantation = 6 turns/min; post-transplantation = 1 turns/min). Despite the similarities between graft volume and placement, the rat that did not recover (black circle in Figure 4k) revealed more limited hNCAM\(^+/\)TH\(^+\) outgrowth directed toward the striatum suggesting that sufficient innervation is necessary for behavioral recovery (Figure 4g–i).

This is supported by previous experiments using intrastratal transplantation of fetal and hESC-derived cells (Kirkeby et al., 2012; Rath et al., 2013). Here, we confirmed these observations by assessing rotational recovery in animals transplanted with human fetal VM tissue. We have previously shown that fetal VM cells provide significant innervation of the A9 target structures in a more extensive manner than hESC-derived progenitors when analyzed at the same timepoint (Grealish et al., 2014). Here, we analyzed graft-mediated functional recovery data in the amphetamine-induced rotation test performed on the animals transplanted with fetal VM tissue. This revealed that grafts of human fetal VM tissue placed in the midbrain more consistently normalized amphetamine-induced rotations by 24 weeks (diamonds, Figure 4k) compared to grafts of hESC-derived progenitors (circles, Figure 4k), associated with a dense hNCAM\(^+/\)TH\(^+\) innervation of dorsolateral striatum (Figure 4f).

In summary, this data highlights that long-distance, target-specific dopaminergic innervation to the dorsolateral striatum from human VM progenitors of hESC or fetal origin coincides temporally with graft-mediated normalization of rotational bias at 24 weeks.

### 3.3 | Monosynaptic tracing reveals early host-to-graft connectivity

Monosynaptic tracing allows for unambiguous identification of first-order inputs onto a defined starter population of neurons, by taking advantage of the selective retrograde transsynaptic spread of rabies virus (Wickersham et al., 2007). First, we used the system to create a reference map of the endogenous connectivity of the neurons located in the rat midbrain. For this purpose, a lentivirus expressing the rabies helper construct under control of the human synapsin promoter that includes histone-tagged GFP (for unambiguous identification of starter cells); TVA receptor (required for selective primary infection with EnvA-pseudotyped rabies); and rabies glycoprotein (required for transsynaptic spread of glycoprotein-deleted \(\Delta G\) rabies), was injected into the midbrain of naïve Sprague–Dawley rats at the same coordinates used for transplantation. Four weeks later, EnvA-pseudotyped \(\Delta G\)-rabies was injected at the same site to label synaptic inputs to starter neurons located in the area of the midbrain corresponding to the graft site (Figure 4a). As evident from the 3D reconstruction (Figure 5b), the input neurons labeled are in agreement with current knowledge of basal ganglia anatomy (Gerfen & Bolam, 2010) and the pattern offferent connectivity to the dopaminergic neurons of the midbrain reported previously (Watabe-Uchida et al., 2012).

Next, we mapped the host presynaptic input to the grafted cells 6 weeks after transplantation. For that purpose, hESC-derived neurons expressing the rabies helper construct were transplanted into the midbrain. Five weeks post-transplantation EnvA-pseudotyped \(\Delta G\)-rabies encoding mCherry was injected at the transplant site to label presynaptic inputs to the graft (Figure 4a). Hence, grafted starter neurons expressed both GFP (green) and mCherry (red), while host neurons making synaptic contact onto grafted neurons expressed mCherry only. One week after \(\Delta G\)-rabies injection the animals were sacrificed for histological analysis.

![FIGURE 4](image-url) Outgrowth of graft-derived dopaminergic fibers and related functional outcome. (a–c) Analysis of double positive hNCAM\(^+/\)TH\(^+\) fibers at different levels in the host brain at 6 weeks post-grafting revealed the presence of some graft-derived dopaminergic fibers coursing through the cerebral peduncle/nigrostriatal pathway (a), reaching the caudal portion of the striatum (b). Despite the presence of lesion-spared endogenous dopaminergic fibers (hNCAM\(^+/\)TH\(^+\)), no double positive fibers were detected in dorsolateral striatum at this stage (c). (d–f) After 24 weeks, dopaminergic fibers of graft origin were seen coursing along the cerebral peduncle/nigrostriatal pathway in large numbers (d), providing dense innervation of the caudal striatum (e). Significant hNCAM\(^+/\)TH\(^+\) innervation was readily detectable in dorsolateral striatum at this stage (f). (g–i) Analysis of graft-derived dopaminergic innervation in one animal in the 24-week group with no behavioral recovery showed few hNCAM\(^+/\)TH\(^+\) fibers coursing via the cerebral peduncle/nigrostriatal pathway (g), with scattered fibers reaching the caudal striatum (h). Sparse double positive fibers can be seen reaching dorsolateral striatum (i) in this animal. (j) Quantification of hNCAM\(^+/\)TH\(^+\) positive fibers in dorsolateral striatum at 6 weeks (n = 5), 18 weeks (n = 5), and 24 weeks (n = 6) revealed a progressive increase in the density of graft-derived dopaminergic fibers in this structure over time (Kruskal–Wallis, \(\chi^2_{\text{20}} = 7.658, p < .01\), Dunn's multiple comparisons test revealed a significant difference between the 6 and 24 week groups, \(p < .005\)). (k) Assessment of amphetamine-induced rotational bias pre- and post-transplantation revealed progressive graft-mediated functional recovery at 6 weeks (n = 4; squares in (k)), 18 weeks (n = 4; triangles in (k)), and 24 weeks (n = 3; circles in (k)). Animals that received grafts of human fetal midbrain tissue (n = 6; diamonds in (k)), demonstrated significant recovery (\(G^* = 4.743, p < .01\)) at 24 weeks post-transplantation. (l) Analysis of graft-derived dopaminergic innervation of the dorsolateral striatum by fetal tissue transplants reveals several hNCAM\(^+/\)TH\(^+\) fibers (white arrow). Scale bars: [a, d, g] = 100 \(\mu\)m; [b, c, e, f, h, i] = 100 \(\mu\)m; [j] = 20 \(\mu\)m. * = \(p < .05\); ** = \(p < .01\); CP/NSP = cerebral peduncle/nigrostriatal pathway; DL = dorsolateral
The majority of transplanted GFP+ cells co-expressed mCherry, indicating efficient infection of starter neurons by ΔG-rabies (Figure 5d). At this timepoint, up to 37% of the starter neurons had matured to the stage where TH is starting to be expressed (Figure 5d–e2). Spatial reconstruction of the precise localization of the mCherry positive neurons using a 3D model of the rat brain, based on the Paxinos and Watson atlas (Paxinos & Watson, 2005) allowed us to obtain a comprehensive overview of whole brain afferent connectivity to the transplant (Figure 5c; see Methods for more details). At 6 weeks post-transplantation, traced host neurons (GFP−/mCherry+) were

**FIGURE 5** Synaptic host-to-graft integration revealed by monosynaptic tracing at 6 weeks matches endogenous connectivity. (a) Experimental design for assessment of endogenous connectivity using monosynaptic tracing. LV-helper was used to generate starter neurons in midbrain and mCherry expressing EnvA-pseudotyped ΔG-rabies was used as a retrograde transsynaptic tracer to map inputs to midbrain. (b) 3D representation of endogenous synaptic inputs to midbrain of normal un-lesioned rats, as revealed by rabies tracing. Each dot represents a traced neuron (mCherry+) in a 1:8 series of mCherry immunostained sections from a representative animal. (c) 3D representation of whole brain monosynaptic inputs to grafted neurons at 6 weeks revealed a pattern of traced neurons similar to that obtained from the mapping of endogenous inputs to the SN seen in (b). Histological analysis of transplants after 6 weeks revealed that a large proportion of grafted neurons expressed GFP and were selectively infected by mCherry expressing rabies (d). A significant proportion of starter neurons (GFP+/mCherry+) also expressed TH (e, e1, e2). (f) Heat map representing the percentage of mCherry+ labeling in each defined host brain area, divided by the total number of traced neurons per brain. Color grades represent different density of labeling, with associated values (3–18%) expressed in the legend. Data compiled from six individual animals (n = 6). Analysis of traced neurons (mCherry+) in the host brain revealed extensive synaptic inputs to the grafted neurons originating from structures such as somatosensory cortex (g), striatum (h), hypothalamus (i), and dorsal raphe nucleus (j). Scale bars: (d) = 100 μm. (e, e1, e2) = 40 μm. (g–j) = 200 μm. Amg = amygdala; Caud str = caudal striatum; Ctx = cortex; DL str = dorsolateral striatum; GP = globus pallidus; Hyp = hypothalamus; M ctx = motor cortex; PFC = prefrontal cortex; PVH = paraventricular hypothalamic nucleus; RN = raphe nucleus; SCol = superior colliculus; SN = substantia nigra; Som ctx = somatosensory cortex; Str = striatum; Tha = thalamus; VM str = ventromedial striatum; VTA = ventral tegmental area.

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readily detected in different host structures throughout the brain, including prefrontal and sensorimotor cortices (Figure 5f,g), striatum (Figure 5f,h), hypothalamus (Figure 5f,i), subthalamic nucleus (see Figure 6b), and dorsal raphe nucleus (Figure 5f,j). The overall pattern of the ΔG-rabies labeled neurons traced from the grafted neurons was similar in distribution to that obtained from the endogenous neurons (compare Figure 5b,c), indicating early and extensive establishment of anatomically appropriate synaptic inputs to the grafted human neurons (Figure 5g–j).

The regional identity of traced neurons in selected structures showing dense labeling (Figure 5f) was confirmed by performing a counterstain with NeuN to resolve anatomical boundaries (Figure 5g–j) and by immunostaining of regional phenotypic markers, expressed by neurons normally projecting to the DA neurons in the substantia nigra (Smith et al., 2016; Watabe-Uchida et al., 2012), including MOR in striatum (Figure 6a,a1), Barhl1 in the subthalamic nucleus (Figure 6b,b1) and 5-HT in the dorsal raphe nucleus (Figure 6c,c1).

In summary, the results show that the grafted neurons in the midbrain receive presynaptic inputs from the host already at 6 weeks after transplantation, and that the overall connectivity established at this early timepoint matches well the pattern of intrinsic nigral afferents, as described in the literature using this tracing method.

**FIGURE 6** Identification of the phenotype of traced host neurons. Immunostaining for specific phenotypic markers reveals the identity of host inputs to graft. Labeled neurons in striatum co-expressed μ-opioid receptor (MOR) (a, a1), neurons in the subthalamic nucleus (STN) co-expressed Barhl1 (b, b1), while those in the dorsal raphe nucleus (RN) co-expressed serotonin (5-HT) (c, c1). White arrow denotes double positive neurons for mCherry and phenotypic marker. Scale bars: (a–c1) = 20 μm. STN = subthalamic nucleus; RN = raphe nucleus; 5-HT = serotonin

### DISCUSSION

Cell-based therapy for PD was pioneered over 25 years ago, yet it is still at the stage of small-scale clinical trials due to the limited access to standardized fetal donor tissue (Barker et al., 2015). Stem cells have long been explored as an alternative cell source, and significant developments in the last decade now allow for the generation of an unlimited number of appropriately patterned VM progenitors from pluripotent stem cells (Steinbeck & Studer, 2015). For PD, several clinical trials using VM progenitors derived from hPSCs are planned (Abbott, 2014; Barker et al., 2017), and hPSC-derived DA neurons will also serve as important tools for further development with the aim to move from cell replacement to more complete circuit reconstruction.

In this study, we use hESC-derived VM progenitors transplanted to the midbrain to better understand graft functionality, reinnervation capacity, and anatomical integration of human DA neurons in the 6-OHDA lesion model of PD.

Intracerebral transplantation has been used previously to explore the capacity of grafted dopaminergic neuroblasts to extend axons over long distances toward their appropriate targets (Grealish et al., 2014; Isacson et al., 1995; Thompson, Grealish, Kirk, & Bjorklund, 2009; Wictorin et al., 1992). Here, we used this strategy to study the capacity of hESC-derived neurons to grow axons along the NSP and...
MFB and progressively innervate appropriate forebrain target structures over the course of 24 weeks. At 6 weeks post-grafting, outgrowing hNCAM+ fibers were seen to exit the graft in a rostral direction and extend along the MFB and NSP. At this timepoint, a few scattered axons were observed in the caudal striatum and NAc, but none were found in other target areas in the forebrain. By 18 weeks, we detected extensive innervation of A10 target structures including ventral striatum, NAc, septum, and PFC, and at 24 weeks graft-derived hNCAM+ fibers had expanded further to cover a large portion of the dorsolateral striatum, the main target region for A9 DA neurons.

As with fetal tissue preparations used in clinical trials, stem cell-derived DA neurons consist of a mix of both A9 and A10 DA neuron populations, and there is currently no method to identify or separate these two populations at the progenitor stage. In agreement with this, we have previously shown that long-distance axonal outgrowth from the graft is directed toward both A9 and A10 target structures (Grealish et al., 2014). Here, we observe at 18 weeks hNCAM+ fibers reaching from the graft site to the PFC and olfactory bulb over a distance of >10 mm, which is sufficient to provide full innervation of the putamen in human patients grafted with VM tissue (estimated at 5–7 mm from each graft deposit; Kordower et al., 1995). Interestingly, even though the exact graft placement varied, the fiber outgrowth pattern were similar. Thus, regardless of their dorsal/ventral location in SNc or SNr, the outgrowing fibers could be seen reaching the MFB and NSP and coursing toward appropriate forebrain target structures in a similar manner.

Our time-course analyses show that 24 weeks of graft maturation was required for the more extensive innervation of the dorsolateral striatum, that is, the functionally relevant structure for motor control in the parkinsonian rat brain. At this timepoint, we observed abundant hNCAM+/TH+ projections of graft origin progressing via the MFB and the NSP, providing a widespread innervation of the striatal parenchyma. At this stage, the presence of graft-derived dopaminergic fibers in the dorsolateral striatum correlated with functional recovery in some animals as assessed by amphetamine-induced rotations (Bjorklund et al., 1980; Dunnett et al., 1983; Grealish et al., 2010). When analyzing transplants of human fetal tissue grafted to the midbrain after 24 weeks, we observed efficient recovery in all animals which also correlated with hNCAM+/TH+ projections in the dorsolateral striatum at this point (Grealish et al., 2014). These data highlight that the long-distance hNCAM+/TH+ fibers observed at 24 weeks from transplants of hESC or fetal VM progenitors are capable of releasing DA to a sufficient extent to normalize rotational bias, highlighting this approach of intranigral grafting as a useful measure of reinervation capacity now also with a functional read-out. The validity of this approach is also highlighted by one animal in the 24-week group in which the transplant failed to induce any reduction of rotational bias despite similar graft placement, size, and TH+ neuron content as in the animals where the grafts had a functional effect. A closer analysis of this subject showed that the graft-derived fibers had failed to innervate the dorsolateral striatum despite the presence of abundant outgrowth toward ventral striatum and NAc (Figure 4g–i).

The time-course of functional recovery seen here—with the first signs observed at 18 weeks post-grafting and in some animals showing a complete recovery at 24 weeks—is very similar to that obtained with fetal-derived midbrain DA neurons transplanted to striatum, where the recovery in amphetamine-induced rotation emerges between 16 and 20 weeks post-grafting (Brundin et al., 1986; Lelos et al., 2016). It also matches remarkably well with the time-course of recovery in motor function (arm-hand movement and rigidity scores) seen in a patient with grafts of fetal VM tissue (Lindvall et al., 1990).

Clinical observations of patients transplanted with VM tissue of fetal origin reveals that grafted neurons continue to functionally mature over the course of around 3 years (Kefalopoulou et al., 2014; Politis et al., 2010). Postmortem analysis of brain tissue from these patients suggest that fibers from grafted tissue extensively innervate the host but remain confined to appropriate target structures (Li et al., 2016). We have previously shown that the functional potency of hESC-derived DA neurons is en par with that of their fetal counterparts when transplanted to the striatum (Grealish et al., 2014). The current data provide further support to the equivalence of the two cell types, in terms of potency, and axonal growth capacity and dynamics, and highlight the importance of the content of DA neurons of the A9 type for graft-induced recovery of motor function in this PD model linked to efficient reinervation of the critical striatal target—as previously demonstrated with transplants of rodent tissue (Grealish et al., 2010).

The host afferent inputs to the grafted neurons were established early, already at 6 weeks post-grafting, at a time when the outgrowing axons have yet to reach their final targets. Using monosynaptic tracing of midbrain DA neurons in the DAT-Cre mouse, it has been shown that both A9 and A10 DA neurons receive inputs from the striatum. The A9 neurons also receive relatively strong excitatory inputs from the somatosensory and motor cortices, as well as subthalamic nucleus, whereas the A10 DA neurons in the VTA receive strong inputs from the lateral hypothalamus (Watabe-Uchida et al., 2012). Using the same ΔG-rabies tracing method to map the afferent connectivity to the graft, we found substantial numbers of afferent neurons in the prefrontal and sensorimotor cortices, striatum, hypothalamus, subthalamic nucleus, and dorsal raphe nucleus already at 6 weeks post-transplantation. The overall distribution of the connections established by the host to the graft matches well the endogenous nigral afferent circuitry revealed by ΔG-rabies tracing (Watabe-Uchida et al., 2012), as well as the neuroanatomy of A9 and A10 DA neurons known from classic studies (reviewed in Gerfen & Bolam, 2010). However, despite the extent of synaptic integration observed at 6 weeks, we did not see any improvement of motor asymmetry in the amphetamine rotation test at this early timepoint, which is consistent with previous findings using fetal and hESC-derived VM progenitors (Brundin et al., 1986; Kirkeby et al., 2012; Rath et al., 2013; Wakeman et al., 2017). This finding supports the view that the primary driver of behavioral recovery in DA-dependent behavioral tests is the directed outgrowth and efficient reinervation of appropriate forebrain targets rather than the extent of host-to-graft synaptic integration.

In conclusion, we show that intranigral grafts of VM progenitors have the capacity to extensively integrate into host circuitry and grow axonal projections toward appropriate forebrain targets over time, and that the functional recovery, as assessed by amphetamine-induced rotations, matched both the timing and extent of A9 specific graft innervation of the dorsolateral part of the striatum in the animals
where recovery could be observed. Moreover, the range of afferent inputs available to grafts placed in the midbrain, as observed here, suggests that hESC-derived DA neurons grafted to the midbrain have the capacity to go beyond simple DA neuron replacement to achieve more complete circuitry repair.

ACKNOWLEDGMENTS

The authors thank Michael Sparrénus, Sol da Rocha Baez, Ingar Nilsson, Ulla Jarl, Jenny Johannson, and Marie Persson Vejlgaard for excellent technical assistance, Andreas Heuer and Jessica Giacomoni for help with surgeries and tissue processing, and Jennifer Dulin and James Conner for help with TSA. The research leading to these results has received funding from the New York Stem Cell Foundation, the European Research Council under the European Union’s Seventh Framework Programme: FP/2007-2013 NeuroStemCellRepair (No. 602278) and ERC Grant Agreement No. 30971, the Swedish Research Council (grant agreement 2016-00873 and 70662601/Bagadilico), Swedish Parkinson Foundation (Parkinsonfonden), Swedish Brain Foundation and the Strategic Research Area at Lund University Multipark (Multidisciplinary research in Parkinson’s disease). S.G. was supported by a postdoctoral stipend from the Swedish Brain Foundation (Hjärnfonden) and M.P. is a New York Stem Cell Foundation Robertson Investigator.

ORCID

Tiago Cardoso http://orcid.org/0000-0003-2686-458X

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How to cite this article: Cardoso T, Adler AF, Mattsson B, et al. Target-specific forebrain projections and appropriate synaptic inputs of hESC-derived dopamine neurons grafted to the midbrain of parkinsonian rats. J Comp Neurol. 2018;526:2133–2146. https://doi.org/10.1002/cne.24500