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Towards cell replacement therapy in Parkinson's disease. Proteoglycans and Nogo-A as modulators of axonal growth in midbrain dopaminergic neurons

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2012

Academic dissertation

Towards cell replacement therapy in Parkinson's disease

Proteoglycans and Nogo-A as modulators of axonal growth in midbrain dopaminergic neurons

by

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With the approval of the Faculty Medicine at Lund University
this thesis will be defended
on September 21st, 2012 at 9.15 in Sagerfalksalen,
Wallenberg Neuroscience Center, BMC A10, Lund, Sweden

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LUND UNIVERSITY
Faculty of Medicine

Organization	Document name
LUND UNIVERSITY Department of Experimental Medical Science Neural Plasticity and Repair Unit	DOCTORAL DISSERTATION Date of issue 17th August, 2012
Author(s) Zuzanna Kurowska	Sponsoring organization

Title and subtitle

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Abstract

Parkinson's disease (PD) is currently the second most common neurodegenerative disorder (after Alzheimer's disease). PD is diagnosed on its motor symptoms, which include akinesia, bradykinesia, rigidity, postural imbalance and resting tremor. It is well established that the motor symptoms develop due to lack of dopamine in the striatum. This is caused by progressive degeneration of dopaminergic neurons (DNs) in the midbrain (substantia nigra pars compacta), which innervate the striatum. Although initially efficacious, the therapeutic effect of various anti-parkinsonian pharmacological treatments is limited to five to ten years. Hence, the new therapeutic strategies for PD are highly warranted.

The concept of cell replacement therapy in PD is based on the idea that the diminished dopamine levels in the striatum would be restored by the functional DNs, obtained from donated, electively aborted fetuses, and subsequently injected into the patients' striatum. In several clinical trials, about 400 patients received such therapy worldwide. Although the therapeutic effects varied, some individuals experienced major motor improvement even for up to 16 years. Other PD patients did not respond so favorably and even developed adverse effects, i.e. graft-induced dyskinesia. The clinical trials are currently on hold.

In the first project, we have analyzed the survival and integration of the graft in a patient who underwent the transplantation in Lund, in 1987, as one of the first transplanted cases in the world. Clinically, the patient did not show the symptomatic relief in response to the transplantation. Examining the postmortem brain, we observed a very small surviving graft. In addition, we also detected signs of PD-like pathology in the transplanted DNs. Nonetheless, the graft survived in this brain for 22 years and such a long graft-life has never before been reported.

It is certain that embryonic tissue will not be routinely used as a cell source for transplantation therapy in PD. Yet, stem cells bear a great potential for future PD therapy. Recent publications show that DNs of the midbrain subtype could be differentiated from stem cells in a time-efficient manner. Moreover, those DNs could form well surviving tumor-free grafts which reverted the PD-like motor symptoms in animal models. Nonetheless, the differentiation of embryonic stem cells to DNs is still not fully understood and therefore difficult to control. Proteoglycans may be engaged in differentiation of DNs, as they govern the formation of the nervous system in developing vertebrate embryo. My second project aimed at defining the genes encoding proteoglycans and the enzymatic machinery fine-tuning their structure during the DN differentiation. From around 2000 proteoglycan-related genes, we identified two (neurocan and HS3ST5) that potentially could enhance the differentiation efficiency of DNs from stem cells. Our results can serve as a starting point for the further functional studies.

After transplantation, the grafted DNs have to survive, and also integrate with the host brain, i.e. grow neurites, form synapses and release dopamine in a regulated fashion. In the third project, we studied how Nogo-A protein affects the survival and neurite growth in DNs. Nogo-A is a strong growth-inhibitory protein in the brain and spinal cord. Interestingly, in recent years, the growth- and survival-supportive role of Nogo-A in various types of neurons has been shown. We have demonstrated here for the first time the Nogo-A roles in the substantia nigra pars compacta DNs.

A careful analysis of all data coming from initial clinical trials, studies on animal parkinsonian models, and the emergence of recent safe and effective dopaminergic differentiation protocols, collectively imply that the cell replacement approach in PD holds a great therapeutic potential. Understanding of the mechanisms governing the DNs differentiation, survival and neurite growth will help develop safe and efficient cell replacement therapies in PD. The work presented in this thesis will hopefully contribute to such advancement.

Key words: Parkinson's disease, cell replacement therapy, grafting, embryonic stem cells, proteoglycans, glycosaminoglycans, Nogo-A, midbrain dopaminergic neurons, neurocan, heparan sulphate 3-*O*-sulfotransferase, microarray, substantia nigra pars compacta, LUHMES cells

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language
English

ISSN and key title
165-28-22-0

ISBN
978-91-87189-36-4

Recipient's notes

Number of pages
106

Price

Security classification

Distribution by (name and address)

Towards cell replacement therapy in Parkinson's disease

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Cover description
The artwork at the front page is a modified version of a photograph taken by Olle Lindvall during the first clinical trial of ventral mesencephalon tissue transplantation into brains of patients with Parkinson's disease, at Lund University Hospital in 1987. The photo was taken before the transplantation and depicts a patient (‘Patient 1’) performing one of the motor tasks, and a camera operator (Patrik Brundin) recording it. The graft in the brain of Patient 1, post mortem, was one of the subjects of study in this thesis (paper 1). The artwork was prepared together with Mariusz Libel.

Faculty of Medicine, Department of Experimental Medical Science
Doctoral Dissertation Series 2012:73
ISBN 978-91-87189-36-4
ISSN 165-28-22-0

Printed in Sweden by Media-Tryck, Lund University
Lund 2012

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Lay summary

Parkinson's disease (PD) is the second most common, after Alzheimer's disease, neurodegenerative disease in man. Stiffness in joints, shaky hands, and slowness of movements make it difficult for people with PD to complete normal daily tasks like buttoning up a shirt. Additionally, PD affects memory, mood, sleep and the function of the gastro-intestinal tract. Over time, the disease progresses gradually and worsens.

We still do not understand fully the mechanisms that cause PD, but what we do know is that the gradual loss of movement control is caused by successive death of specific cells – dopaminergic neurons, in a particular region of a brain known as (from Latin) the substantia nigra pars compacta. The dopaminergic neurons produce dopamine, a neurotransmitter, which regulates a large part of the movement-governing system in the brain. The current most common therapies for PD rely on restoration of the dopamine levels in this system. Usually, a patient takes pills of a medication called L-DOPA a few times per day and can control his or her body movements again. Although initially this therapy works, the dose of L-DOPA required to bring the relief gradually increases, and eventually this treatment must be discontinued due to troublesome side effects. Hence, the new therapies for PD are warranted.

One of the potential treatments for PD is based on the idea that dopamine can be provided by young dopaminergic neurons injected directly into the patients' brain. Those neurons are taken from donated, electively aborted embryos, from the site where the substantia nigra would be formed. In several clinical trials, around 400 patients received this treatment worldwide, and although the therapeutic effects varied, some individuals experienced major improvement, even for up to 16 years, and could completely set aside L-DOPA. Other patients, however, did not experience any symptomatic relief and even developed some movement complications, so the clinical trials are currently on hold. Careful analysis of the clinical data from all of these trials, together with experiments, including ones in animal models of PD and in (dopaminergic) cell cultures, will deepen the understanding on how to improve cell replacement therapy in PD.

In my first project, we have analyzed the survival and integration of the graft in a patient who underwent the transplantation in Lund, in 1987, as one of the first transplanted cases in the world. Clinically, the patient did not show symptomatic relief in response to the transplantation. After examining the brain postmortem, we

observed only a very small surviving graft. In addition, we saw signs of PD-like pathology in the transplanted neurons. Nonetheless, the graft survived in this brain for 22 years and such a long graft-life has never been reported elsewhere.

It is certain that in the future, embryonic tissue will not be routinely used as a cell source for transplantation therapy in PD. Instead, stem cells will be differentiated to dopaminergic neurons and subsequently injected into the brain. Although research in recent years has shown that embryonic stem cells can be a safe and an efficient source for obtaining transplants that survive and reverse the PD-like symptoms in animal models, the differentiation of embryonic stem cells to dopaminergic neurons is still not fully understood, and is therefore difficult to control. Proteoglycans (proteins with long sugar chains attached) may be engaged in differentiation of dopaminergic neurons, as they regulate the process of brain development in vertebrate embryos.

My second project aimed at defining the genes encoding proteoglycans and the enzymatic machinery fine-tuning their structure that is involved in the differentiation of dopaminergic neurons. From around 2000 proteoglycan-related genes, we identified two (neurocan and HS3ST5) that could potentially enhance the differentiation efficiency of dopaminergic neurons from stem cells. Our results may serve as a starting point for further functional studies.

In order to bring back, at least partially, the control of movement to an individual with PD, the grafted dopaminergic neurons have to survive, and also integrate with the neurons of the host brain, i.e. extend neurites, form synapses and release dopamine.

In my third project, we have been studying how a protein called Nogo-A affects dopaminergic cell survival and neurite growth. Nogo-A is a strong growth-stopping agent found within the brain and spinal cord (hence the name ‘no-go’). Interestingly, in recent years, some studies showed the growth- and survival-promoting role of Nogo-A in neurons. Our results supported this notion and we were first ones to suggest Nogo-A roles in dopaminergic neurons of the substantia nigra pars compacta. Perhaps upon grafting into the PD brain, the dopaminergic neurons stop producing Nogo-A and this may be the reason why many die or do not integrate with the host neurons following the operation?

The results of initial clinical trials, studies in animal parkinsonian models and recent safe and effective dopaminergic differentiation protocols, collectively imply that the cell replacement approach in PD holds great therapeutic potential. Nonetheless, in order to develop a safe and efficient cell replacement therapy in PD, the understanding of the mechanisms governing dopaminergic cell differentiation, survival and neurite growth is needed. I hope that my work will contribute to such positive development.

Populärvetenskaplig sammanfattning

Parkinsons sjukdom (PD) är efter Alzheimers sjukdom den näst vanligaste neurodegenerativa sjukdomen. Ett framskridet stadium av sjukdomen medför stelhet i leder, darrande händer och långsamma rörelser, vilket gör att den drabbade har svårt att utföra dagliga aktiviteter. Sjukdomen påverkar även patientens minne, humör, sömn, och mag-tarmkanalens funktion. Symptomen förvärras gradvis vartefter sjukdomen fortskrider.

Det saknas fortfarande kunskap om de mekanismer som orsakar sjukdomen, dock vet vi att den gradvisa förlusten av rörelsekontroll orsakas av att specifika celler dör. Dessa celler kallas dopaminerga neuroner och återfinns i en region av hjärnan som på latin kallas substantia nigra pars compacta. Dopaminerga neuroner producerar dopamin, en neurotransmittor, som till stor del reglerar det system i hjärnan som styr kroppsrörelser. Återställning av dopaminnivåerna i detta system utgör grunden för de idag vanligaste behandlingsformerna av PD. Vanligtvis tar patienten tabletter innehållande substansen L-DOPA ett fåtal gånger per dygn och återfår därmed kontrollen över sina kroppsrörelser. Trots att denna behandling initialt fungerar behöver dosen av lindrande L-DOPA gradvis ökas för att uppnå önskad effekt. Till sist leder den ökade dosen till oönskade biverkningar, vilket medför att behandlingen måste avbrytas. Därför finns det ett stort behov av nya typer utav behandlingar vid PD.

En av de potentiella behandlingsformerna av PD är baserad på idén att dopamin kan tillhandahållas genom att injicera dopaminerga neuron i patientens hjärna. Neuron doneras från aborterade embryon och tas från det område som normalt utvecklas till substantia nigra. Flera kliniska studier har utförts och globalt har ca 400 patienter fått denna typ av behandling. De terapeutiska effekterna har varierat. Vissa individer har upplevt en ansenlig förbättring som kvarstått under en period upp till 16 år och under denna tid helt kunnat avbryta L-DOPA-behandlingen. Andra patienter har inte upplevt någon symptomlindring och i vissa fall har rörelsekomplikationer förekommit. De kliniska prövningarna ligger för närvarande på is. Noggranna analyser av de kliniska data som finns, tillsammans med experiment utförda i djurmodeller och i dopaminerga cellkulturer, kommer att fördjupa förståelsen för hur behandlingen av PD kan förbättras och effektiviseras.

I mitt första projekt har vi studerat överlevnad och integrering av ett transplantat tillhörande en patient. Transplantationen genomfördes i Lund 1987 och var en av de första transplantationerna av sitt slag som genomförts i världen.

Transplantationen medförde ingen kliniskt positiv effekt. Postmortem undersökning av hjärnan visade att endast en liten andel utav de transplanterade neuron överlevt och att de uppvisade PD-liknande patologi. Trots detta överlevde transplantatet i hjärnan i 22 år, vilket är den längsta tid som hittills rapporterats.

I framtiden kommer inte embryonal vävnad att användas som en rutinmässig cellkälla för transplantationsterapi inom PD. Istället kommer stamceller att differentieras till dopaminerga neuroner och därefter injiceras i hjärnan. Under de senaste åren har embryonala stamceller visat sig vara en säker och effektiv källa för att få transplantat som överlever och har även i djurmodeller visat sig upphäva PD-liknande symptom. Dock är differentieringen av embryonala stamceller till dopaminerga neuron inte helt klarlagd och därför svår att styra. Proteoglykaner (proteiner med mycket långa sockerkedjor) kan vara betydelsefulla för differentieringen av dopaminerga neuron, då de reglerar processen för hjärnans utveckling i vertebrata embryon.

Mitt andra projekt har som mål att definiera gener som kodar för proteoglykaner och det enzymatiska maskineriets struktur som är involverad i differentieringen av dopaminerga neuron. Utifrån ca 2000 proteoglykan-relaterade gener, identifierade vi två gener (neurocan och HS3ST5) som potentiellt skulle kunna effektivisera differentieringen av dopaminerga neuroner från stamceller. Våra resultat ligger till grund för ytterligare funktionella studier. För att delvis återfå rörelsekontrollen hos en individ med PD måste de transplanterade dopaminerga neuronen inte bara överleva utan även interagera med nervcellerna i individens hjärna, t.ex. genom att bilda synapser och frisläppa dopamin.

I mitt tredje projekt har vi studerat hur ett protein som kallas Nogo-A påverkar den dopaminerga cellöverlevnaden och neurit tillväxt. Nogo-A verkar stark tillväxt-hämmande i hjärnan och ryggmärgen (därav namnet "no-go"). Under de senaste åren har forskare visat att Nogo-A har en tillväxt-och överlevnads-stödjande roll i neuron. Våra resultat stöder denna uppfattning och vi var de första att föreslå Nogo-A effekt på dopaminerga neuron i substantia nigra pars compacta. En hypotes till att många celler dör eller misslyckas integrera med patientens egna neuron vid transplantation kan vara att neuronen slutar producera Nogo-A.

Resultat från kliniska prövningar, djurstudier och de senaste effektiva dopaminerga differentieringsprotokollen stöder att cellersättning har en stor terapeutisk potential vid PD. Det behövs ytterligare kunskap om mekanismerna som styr den dopaminerga celldifferentieringen, överlevnad och neurit-tillväxt för att utveckla en säker och effektiv cellterapi.

Jag hoppas att mitt arbete kommer bidra till en positiv utvecklingen av cellterapi som behandlingsform för PD.

Papers included in this thesis

Kurowska, Z., Englund, E., Widner, H., Lindvall, O., Li, J.-Y. & Brundin, P. (2011) [1]

Signs of degeneration in 12–22-year old grafts of mesencephalic dopamine neurons in patients with Parkinson's disease. *Journal of Parkinson's Disease* **1**, pp. 83-92.

Kurowska, Z., Mani, K., Li, J.-Y.

Proteoglycans in differentiation of human dopaminergic neurons

Manuscript

Kurowska, Z., Brundin, P., Schwab, M.E., Li, J-Y.

Roles of Nogo-A in developing and adult midbrain dopaminergic neurons

Submitted

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Abbreviations

6-OHDA	6-hydroxydopamine
^{18}F-DOPA	L-3,4-dihydroxyphenylalanine radiolabeled with [^{18}F]
aa	amino acids
BDNF	brain-derived neurotrophic factor
CS(s)	chondroitin sulfate(s)
DAT	dopamine transporter
dbcAMP	dibutyril 3'-5'-cyclic adenosine monophosphate
DCC	deleted in colorectal cancer
DN(s)	dopaminergic neuron(s)
DS(s)	dermatan sulfate(s)
E13.5	13.5 days of embryonic life
ECM(s)	extracellular matrix (matrices)
ESC(s)	embryonic stem cell(s)
ER	endoplasmatic reticulum
FGF(s)	fibroblast growth factor(s)
GAG(s)	glycosaminoglycan(s)
GDNF	glial cell line-derived neurotrophic factor
GID(s)	graft induced dyskinesia(s)
GSK3B	glycogen synthase kinase 3 beta
HCS	high content screening
HS3ST5	heparan sulfate 3-*O*-sulfotransferase 5
KS(s)	keratan sulfate(s)
L-DOPA	L-3,4-dihydroxyphenylalanine
LUHMES	Lund University human mesencephalic
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NgR-I	Nogo receptor I
PD	Parkinson’s disease
PET	positron emission tomography
PG(s)	proteoglycan(s)
RTN	reticulon
SHH	sonic hedgehog
TGF-β	transforming growth factor beta
TH	tyrosine hydroxylase

Background

Parkinson's disease

Parkinson's disease (PD) took its name from James Parkinson, who in 1817 thoroughly described this disorder in 'An Essay on the Shaking Palsy' (Parkinson, 1817). Occurring in more than 1% of people older than 65, PD is the second most common (after Alzheimer's disease) neurodegenerative disorder. Due to aging populations in developing countries, PD prevalence is likely to increase.

Despite the fact that studies on familial PD have revealed mutations in several genes, where *SNCA (PARK1)*, *Parkin (PARK2)*, *PINK1 (PARK6)*, *DJ1* (*PARK7*) and *LRRK2 (PARK8)* have also been causally linked to the disease (Gasser *et al.*, 2011); the majority of genetic and environmental risk factors for idiopathic PD are undefined, making the cause of PD unknown in 90-95% of cases.

Currently, this chronic neurodegenerative disorder is primarily diagnosed on its motor symptoms that include akinesia (inability to initiate the movement), bradykinesia (slowness of movement), rigidity, postural imbalance and resting tremor. Nonetheless, PD patients also suffer from numerous non-motor symptoms that affect four domains: neuropsychiatric, autonomic, sensory and sleep (Lim & Lang, 2010). Moreover, there is growing evidence that some non-motor symptoms appear already many years before the patient is diagnosed with PD (Gao X. *et al.*, 2012).

Although the neuropathology in PD is distributed in many brain regions [as judged by Lewy body formation (Braak *et al.*, 2004)], the motor symptoms in PD are clearly attributed to diminishing levels of dopamine in the striatum, which is caused by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (Hornykiewicz, 1966; Riederer & Wuketich, 1976). The onset of motor symptoms is usually correlated with a 70-80% drop in dopamine levels in the striatum and death of around 50% of neurons in the substantia nigra pars compacta. The role of the dopaminergic neurons of substantia nigra pars compacta in the control of movement is briefly explained in Figure 1.

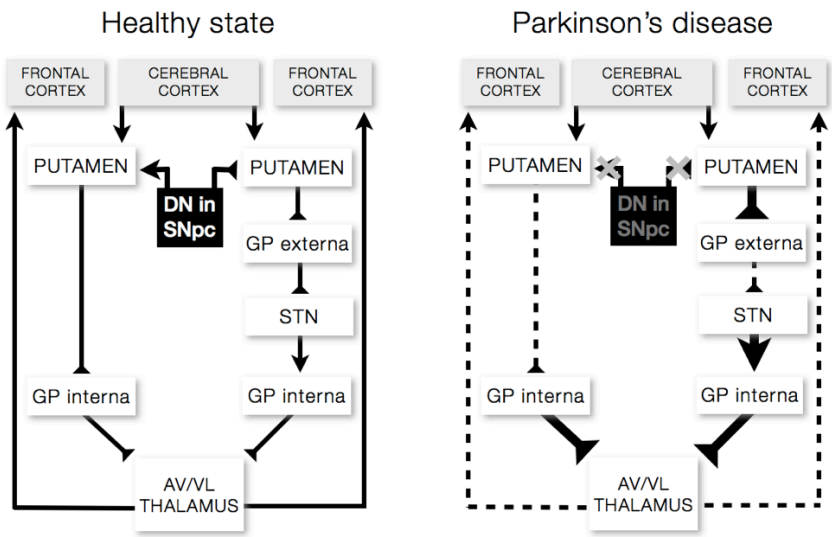

Figure 1. The simplified schema of the basal ganglia circuit and its involvement in movement control, in the healthy state and in the brain affected by PD
The black box represents dopaminergic neurons (DN) in the substantia nigra pars compacta (SNpc) of the midbrain. The basal ganglia centers (white boxes) are connected to each other with excitatory glutamatergic (→) or inhibitory GABAergic (⊣) pathways. In PD condition (to the right), the increased inputs are indicated with thick lines, and the diminished inputs with dashed lines. In the healthy brain (left), DN release dopamine in the putamen, which reduces the threshold for cortical activation there. Consequently, a series of signals in the basal ganglia occurs and finally a signal is sent to the motor centers in the frontal cortex to initiate a movement. In the parkinsonian brain (to the right), death of dopaminergic neurons in the SNpc leads to much diminished levels of dopamine in the putamen. In turn, the threshold for the impulses from the cortex is higher than in the healthy person, and consequently the initiation of a movement is more difficult. GP - globus pallidus, STN – subthalamic nucleus, AV/VL – anterior ventral/ventral lateral thalamic nuclei.

Therapies for Parkinson's disease

Patients diagnosed with PD are usually subjected to a therapy aiming to restore dopamine levels in the striatum, in order to ameliorate motor symptoms. The dopamine-replacement (pharmacological) therapy usually includes oral administration of various dopamine agonists and/or different L-DOPA (dopamine precursor) preparations, applied together with drugs that prolong the bioactivity of L-DOPA. In more advanced cases, the treatments include apomorphine, delivery of L-DOPA through continuous intestinal administration, and deep brain stimulation of the subthalamic nucleus and globus pallidus through surgically

implanted electrodes [for review of current PD treatments, please see (Fox *et al.*, 2011; Seppi *et al.*, 2011)]. None of those therapies, however, counteract the progression of the disease.

L-DOPA, to date, is the gold standard in therapeutic treatment of PD. Still, it remains unknown how to efficiently overcome the disabling side effects of L-DOPA, including motor fluctuations and dyskinesias, which are experienced by all patients at some point during L-DOPA treatment.

Although the range of different approaches to treat PD motor and non-motor symptoms are now richer than it has ever been before, the current therapeutic options are still not sufficient to sustain the quality of life of individuals affected with PD. Hence, the development of new therapeutic strategies for PD is highly warranted.

Cell replacement therapy in Parkinson's disease

The concept of cell replacement therapy in PD is based on the idea that the diminished dopamine levels in the striatum of a PD patient would be restored by the functional dopaminergic neurons (obtained from midbrains of donated, electively aborted fetuses). The first results of neuronal grafting in animal models of neurodegenerative disease were published in the late 1970s (Bjorklund & Stenevi, 1979; Perlow *et al.*, 1979). Those two independent studies employed 6-OHDA-induced parkinsonian models in rats. They showed that grafting rat embryonic dopaminergic neurons can revert motor symptoms caused by dopamine depletion. In turn, this therapeutic effect deteriorated when the graft was destroyed. Similarly, transplantation of fetal midbrain cells into striata of non-human primates, subjected earlier to MPTP or 6-OHDA, led to improvement of some motor deficits caused by those neurotoxins (Dunnett *et al.*, 1991; Annett, 1994).

The positive outcome of fetal transplantation in the rat parkinsonian model was encouraging and led to the first open-label trial at Lund University Hospital (Lindvall *et al.*, 1989; Lindvall *et al.*, 1990). Two patients with PD (referred to as patient 1 and patient 2) received intrastriatal grafts of human fetal ventral midbrain tissues that were obtained from therapeutic abortions. The clinical effects of transplantation were modest in those two cases. Nonetheless, revision and optimization of some parameters of the grafting technique on rat models (for example using a needle with a smaller diameter and more tissue material), resulted in the positive therapeutic outcome in some patients in the next Lund series and in some patients grafted in other centers (Dunnett *et al.*, 2001; Lindvall & Bjorklund, 2004; Politis *et al.*, 2010; Politis *et al.*, 2011). The grafts could survive in the striatum, as indicated by increased ^{18}F-DOPA uptake in the transplanted regions,

and provided symptomatic relief as measured by increased efficacy of L-DOPA and less rigidity, which could last for as long as 16 years following transplantation (Dunnett *et al.*, 2001; Lindvall & Bjorklund, 2004; Politis *et al.*, 2010; Politis *et al.*, 2011). In the most successful cases, the patients were able to withdraw from L-DOPA therapy completely. Although the results from those small open-label trials were promising, the outcomes of two following double-blinded placebo-controlled trials hindered clinical application of transplantation therapy in PD. Those trials showed limited functional improvement in motor function rating scales, despite the increased ^{18}Fluoro-DOPA uptake in grafted striata (Freed *et al.*, 2001; Olanow *et al.*, 2003; Ma *et al.*, 2010). The dramatic differences in the outcome between the initial open-label studies and the later clinical trials could be attributed to fundamental procedural differences between the clinical centers (Bjorklund *et al.*, 2003). In addition, further development of the fetal cell replacement approach was hampered by the appearance of adverse effects, so called graft-induced dyskinesias (GIDs), in a subgroup of patients (Freed *et al.*, 2001; Hagell *et al.*, 2002; Olanow *et al.*, 2003).

To date, it is estimated that around 400 PD patients worldwide have received transplants with human fetal ventral tissue, nonetheless, cell replacement therapy in PD is currently on hold. Careful examination of the procedural nuances, together with the analysis of the clinical and post-mortem data from the grafted individuals (ultimately undergoing autopsy), shed light on the critical factors influencing the success of cell replacement therapy in PD (for example age of patient and the stage of PD); it also raised some questions which need to be answered by pre-clinical studies, before cell replacement therapy can re-enter the clinic.

Challenges in cell replacement therapy in Parkinson's disease

Previous clinical trials in cell replacement therapy for PD lacked homogeneity of outcomes and were hindered by the development of troublesome graft-induced dyskinesias in some patients. The successful re-launch of clinical trials now face three major challenges: how to avoid graft induced dyskinesias; how to increase the efficiency and reduce the variability of the grafts; how to generate and standardize dopaminergic neurons in large numbers. There are several factors at the root of each of those issues and they have been analyzed in detail in a number of reviews (Lindvall & Bjorklund, 2004; Piccini *et al.*, 2005; Lane *et al.*, 2010).

Some concerns in cell replacement therapy are related to the use of electively aborted human embryos (aged 6-8 weeks post conception) as the donors of

dopamine neuron-rich midbrain tissue, which is transplanted into the brains of PD patients. In the future, due to ethical and logistical reasons, as well as variability, this cell source will most probably be replaced by sources that are easier to standardize: stem cells. Possible stem cell types and their sources are described in Figure 2, and their potential usefulness in the transplantation therapy in PD have been previously reviewed (Li *et al.*, 2008a; Brundin *et al.*, 2010; Politis & Lindvall, 2012).

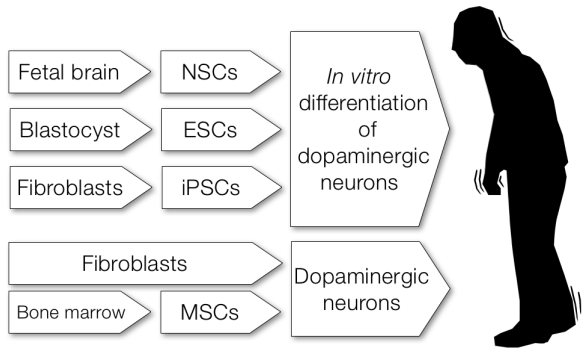

Figure 2. Potential stem cell sources for cell replacement therapy in Parkinson's disease
The dopaminergic neurons derived from (stem) cell sources indicated here show at least partial reversal of motor symptoms in animal parkinsonian models [for review, please see (Politis & Lindvall, 2012)]. Neuronal stem cells (NSCs) isolated from fetal brain; embryonic stem cells (ESCs) derived from blastocysts; induced pluripotent stem cells (iPSCs) obtained from (patients' own) fibroblasts – such cells could be differentiated *in vitro* to dopaminergic neurons, and subsequently transplanted into the brain of a person with PD. Fibroblast and bone marrow derived mesenchymal stem cells (MSCs) can be trans-differentiated to dopaminergic neurons directly, which also makes them a potential source for cell replacement therapy in PD.

Regardless of the cell source and type of stem cell applied, the challenges of stem cell replacement therapy in PD appear to be similar. The differentiation protocols often rely on undefined ingredients, for example serum, that may display batch-to-batch variability. Although stem cells differentiate into dopaminergic neurons *in vitro,* the efficiency of this process is usually relatively low. Eventually, the cell suspension contains different types of neurons, for example serotonergic neurons that may give rise to graft-induced dyskinesias after transplantation (Politis *et al.*, 2010; Politis *et al.*, 2011; Shin *et al.*, 2012). Moreover, the dopamine neuron survival in the brain is modest, and additionally a subpopulation may lose the ability to express tyrosine hydroxylase and other elements of molecular dopaminergic machinery (Paul *et al.*, 2007; Kordower *et al.*, 2008; Li *et al.*, 2008b). Additionally, the host-graft innervation also presents a challenge, as the adult brain environment is non-permissive to axonal growth. Furthermore, the

residual pluripotent cells that have not undergone differentiation pose a large risk to the grafted patients. Those division-prone cells can give rise to tumors and teratomas in the host brain (Li *et al.*, 2008a).

Recently, by using novel differentiation approaches, a significant step forward has been achieved: the efficiency of dopaminergic differentiation and survival after transplantation was significantly improved and tumor/teratoma formation after grafting was avoided in animal PD models (Kriks *et al.*, 2011; Kirkeby *et al.*, 2012). Nevertheless, the usefulness of novel differentiation approaches needs to be confirmed longitudinally and verified in other stem cell types, if they are to be considered as a cell source for transplantation in PD patients.

Further, I will focus on two challenging aspects in experimental cell therapy in PD, namely, the dopaminergic differentiation of embryonic stem cells and axonal growth inhibitory environment of the host brain.

Dopaminergic differentiation of embryonic stem cells

In the last decade, new stem cell sources have emerged and have shown to be useful in diminishing (at least in part) the motor symptoms in parkinsonian animal models after intrastriatal transplantation. Nonetheless, knowledge of embryonic stem cells has been accumulating for many years and this cell type remains the most intensively studied kind of stem cell.

The initial strategies aiming to obtain dopaminergic neurons from embryonic stem cells employed the ability of pluripotent cells to spontaneously give rise to the neuroectoderm (Reubinoff *et al.*, 2001). Additionally, several factors have been identified which promote lineage-specific differentiation including co-cultures with stromal cells, low-density cultures and addition of fibroblast growth factors (FGFs) (Zeng *et al.*, 2004; Brederlau *et al.*, 2006). In more recent neural differentiation protocols, those strategies were combined with more refined paradigms for regionalization of induced neurons, based on developmental principles (Pankratz *et al.*, 2007; Chambers *et al.*, 2009; Kriks *et al.*, 2011; Kirkeby *et al.*, 2012; Ma *et al.*, 2012).

The dual inhibition of SMAD signaling (neutralization induction), was accompanied by inhibition of GSK3B kinase (canonical Wnt signaling pathway activation) in the latest protocols for dopaminergic differentiation (Kriks *et al.*, 2011; Kirkeby *et al.*, 2012). Combination of those factors in the specific dosage and timing, mimicked well the conditions needed for induction of Lmx1a/b/FoxA2-positive ventral midbrain dopaminergic phenotype during development (Kriks *et al.*, 2011; Kirkeby *et al.*, 2012). In the second stage of differentiation, cells were exposed to BDNF, Ascorbic Acid, GDNF, dbcAMP (Kirkeby *et al.*, 2012) and TGF-β (Kriks *et al.*, 2011). Those factors promote and

maintain the survival, plasticity and synaptic function of neurons. In both of those studies, the grafted dopaminergic neurons survived well without signs of tumor formation, and significantly relieved parkinsonian symptoms in rat (Kirkeby *et al.*, 2012), mouse and monkey PD models (Kriks *et al.*, 2011), when compared to grafts of fetal ventral mesencephalon cells or sham surgery, respectively. Those fruitful developments show that embryonic stem cell-based therapy is likely to be safe and at least as effective as its 'prototype' - the transplantation of fetal ventral mesencephalon neurons in PD brains of animal models. Indeed, those two publications bring embryonic stem cell-based therapy a step closer to the patients suffering from PD.

Proteoglycans – potential regulators of dopaminergic differentiation

During early development, locally provided endogenously signaling molecules – including RA (retinoic acid), FGFs (fibroblast growth factors), BMPs (bone morphogenic proteins), Wnts and SHH (sonic hedgehog) - are needed for neural induction in a vertebrate embryo. Spatiotemporal mosaic of those signals induces certain transcription arrays, and thereby a specific phenotype of a neuroblast progeny is acquired (LaMantia, 2012). Several protocols of embryonic stem cells dopaminergic differentiation for transplantation in PD models, including the most recent ones (Kriks *et al.*, 2011; Kirkeby *et al.*, 2012), aim to recreate the spatiotemporal signaling pattern required for development of dopaminergic neurons of a ventral midbrain phenotype. It is well established that proteoglycans (PGs) are indispensible mediators of signaling of the aforementioned factors – for example Wnts (Song *et al.*, 2005), SHH (Lum *et al.*, 2003), and FGF8 (Inatani *et al.*, 2003; Shimokawa *et al.*, 2011). Thereby PGs play a pivotal role in CNS development (Bulow & Hobert, 2006).

Proteoglycans (PGs) are a heterogeneous group of polyanionic macromolecules composed of a specific core protein covalently substituted with one or more polysaccharide linear chains called glycosaminoglycans (GAGs) (Fig. 3). PGs are generally found in all tissue types, in extracellular matrices (ECMs) and basement membranes or at the cell surface (Esko *et al.*, 2009). Depending on the GAG composition, PGs are classified into heparan sulfate PGs (HSPGs) and chondroitin sulfate/dermatan sulfate PGs (CS/DSPGs) (Fig. 3); as well as keratan sulfate PGs (KSPGs) (that have a differing linker region from HSs and CS/DSs). The GAGs have complex modification patterns, consisting predominantly of sulfations of hydroxyl groups and epimerization of specific carbon atoms in individual sugar molecules (Fig. 3).

In addition to influencing the neural inductive molecules indicated above, PGs were shown to act as axonal guidance cues in the later stages of development. Many genetic studies in various model organisms have proven HSs or HSPGs as critical modulators of Robo/Slit and DCC/Netrin signaling at the midline (Inatani et al., 2003; Steigemann et al., 2004; Kastenhuber et al., 2009).

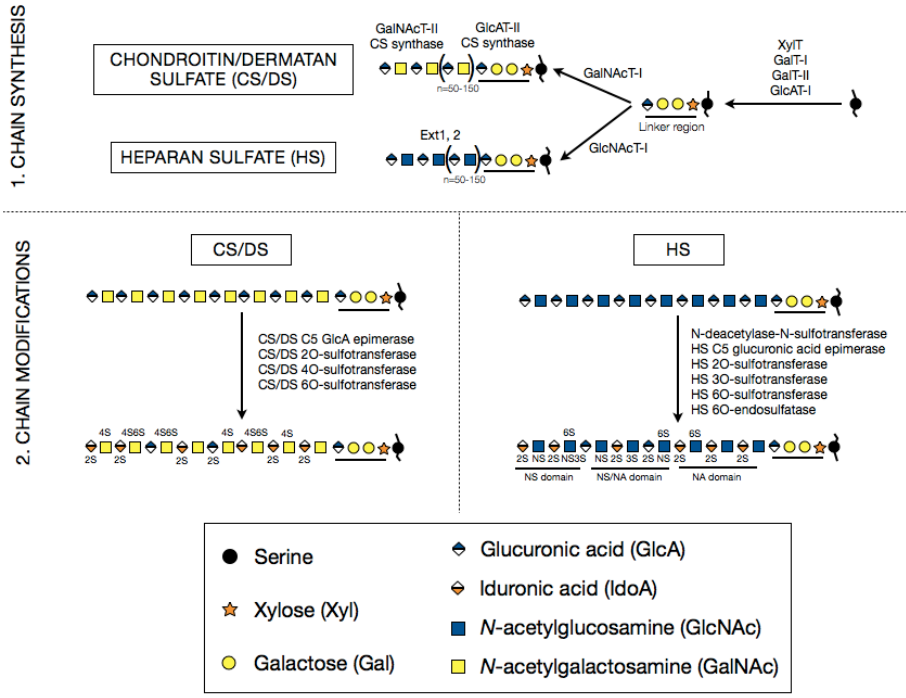

Figure 3. Synthesis of CS/DS and HS glycosaminoglycans (GAGs), a simplified schema
Chondroitin sulfate/dermatan sulfate (CS/DS), or heparan sulfate (HS) GAG chains are assembled on an initially formed common linkage region, GlcA-Gal-Gal-Xyl, which is attached to serine residues in certain consensus sequences. The addition of GlcNAc or GalNAc by a unique glucosaminyltransferase (GlcNAcT-I) or galactosyltransferase (GalNAcT-I) to the non-reducing terminal of GlcA residue, determines whether HS, or CS/DS is initiated (respectively). The linker tetrasaccharide then becomes elongated by addition of the repeating disaccharides by specific enzyme complexes. The polymerised chains are modified by a number of Golgi resident epimerases and sulfotransferases that are specific to CS or HS, creating distinctly modified domains. Names of enzymes taking part in reaction steps are listed in the schema. NA – N-acetylated; NS – N-sulfated; XylT – xylosyltransferase, GalT – galactosyltransferase, GlcAT – glucuronyltransferase, Ext – exostosin; all other abbreviations used in the figure are explained in the frame at the bottom of the figure.

Chondroitinase ABC treatments (destruction of the CSs and other elements of extracellular matrix) in the developing CNS lead to various pathfinding errors of axons, including growing retinal axons and spinal motor neurons (Bernhardt & Schachner, 2000; Chung et al., 2000). From the spatial correlation of CSPGs expression with axonal outgrowth, it was concluded that CSPGs act as a repulsive cue during development (Chung et al., 2000; Ichijo, 2006), and further, it was argued that various axonal guidance molecules such as Slit2, Netrin1, EphrinA1, EprinA5 and Semaphorin5B bind to specific CS sequences (Kantor et al., 2004; Shipp & Hsieh-Wilson, 2007).

In adult animals, the levels of CSPGs become increased in glial scar tissue after CNS injury, and this serves as a barrier for regenerating axons in many experimental animal models (Kwok et al., 2008). On the other hand, many reports have demonstrated that CSPGs, and CS/DSs themselves, promote neurite extension (Maeda et al., 1995; Hikino et al., 2003).

Although the roles of different GAG species in developing midbrain dopaminergic neurons *in vivo* and *in vitro* have been investigated previously, it was done by employing different (mostly *in vitro*) models and GAGs with unknown sulfation patterns and from different sources. Therefore the question about the specific functions of GAGs in those neurons remains open. In particular, *in vitro* studies employing various rodent primary culture models suggested that polarization, adhesion and neurite growth of developing midbrain dopaminergic neurons can be influenced by specific GAGs in different ways (Lafont *et al.*, 1992; Gates *et al.*, 1996; Mendes *et al.*, 2003). Additionally, it has been speculated that GAGs of CS-4 and KS types may navigate axonal pathfinding of nigrostriatal neurons during development and stabilize the organization of the striatosomes in the adult brain (Charvet *et al.*, 1998a; b).

During the last decade, many reports have revealed that GAGs with their enormous structural diversity (Fig. 3), have the capacity to encode functional information in a sequence-specific manner (analogous to DNA, RNA and proteins). It has been documented that specific GAG structure motifs serve as molecular recognition elements for growth factors, cytokines, morphogenes and other types of molecules (Deepa *et al.*, 2004; Gama *et al.*, 2006; Nadanaka *et al.*, 2011; Miyata *et al.*, 2012), including those promoting embryonic stem cell proliferation and differentiation (Sasaki *et al.*, 2009; Kraushaar *et al.*, 2010; Pickford *et al.*, 2011).

Non-permissive environment of the adult CNS

Upon intracerebral transplantation, damage of the host tissue cannot be avoided, causing partial destruction of local neurons, their axonal myelination and leading to activation of microglia, astrocytes and formation of a glial scar. Myelin contains many growth inhibitory molecules that interact with the receptors on the axon under normal conditions and therefore stabilize the neuronal circuits in the mature brain. Upon injury, those molecules, including Nogo-A (Chen *et al.*, 2000; GrandPre *et al.*, 2000), Oligodendrocyte myelin glycoprotein - OMgp (Wang *et al.*, 2002a), myelin-associated glycoprotein - MAG (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994), ephrinB3 (Benson *et al.*, 2005) and semaphorin4D (Moreau-Fauvarque *et al.*, 2003), make up debris that creates a growth non-permissive environment for the axons.

Nogo-A - inhibitor of axonal growth

Nogo-A is a 1200 amino acid (aa) transmembrane protein originally discovered in the adult CNS. The 200 aa C-terminal of Nogo-A classifies this protein as part of the reticulon (RTN) family – ubiquitous transmembrane proteins which are enriched in the endoplasmic reticulum (ER), but can also reach other cellular compartments including the plasma membrane (Schwab, 2010) (Fig. 4). Reticulon proteins share a RTN homology domain, which contains two transmembrane regions separated by a 66-70 aa loop and a short C-terminus (Fig. 4). The biological functions of this RTN domain, and also of most of the four reticulon gene families, are unknown at present (Schwab, 2010). Nogo-A, in contrast to the shorter isoforms Nogo-B and –C, or other reticulon proteins, is highly enriched in the nervous system. In the adult CNS, Nogo-A is primarily expressed by oligodendrocytes and myelin and by subpopulations of neurons, in particular those that maintain high plasticity in adulthood (Caroni & Schwab, 1988; Huber *et al.*, 2002; Wang *et al.*, 2002b).

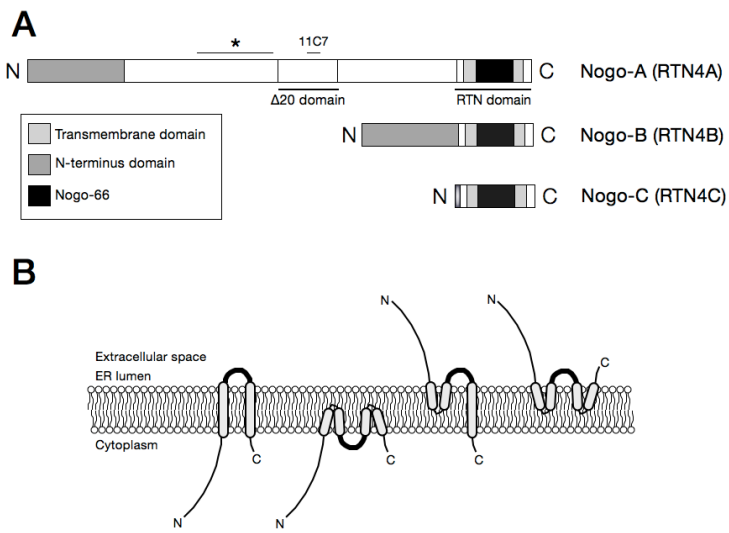

Figure 4. Domains and localisation of Nogo proteins
Nogo-A, -B and -C, the members of the reticulon (RTN) protein family, share the C-terminal RTN domain (A). This domain consists of a Nogo-66 loop region flanked by two transmembrane fragments. Additionally, Nogo-A and -B share the N-terminal domain. In Nogo-C, the N-terminal domain is encoded with use of a different promoter, and is much shorter (A). Shown in A (black) and B (thick black lines) Nogo-66 loop, together with Δ20 domain, are the main mediators of the neurite growth inhibitory action of Nogo(s); the site of binding of 11C7 (the monoclonal anti-Nogo-A antibody used in paper 3) is marked in A; the star (A) indicates the 272 aa region (aa 290-562) which is probably involved in intracellular scavenging of reactive oxygen species (Mi *et al.*, 2012). The topology of RTN proteins in plasma membrane and endoplasmatic reticulum (ER) is debatable. In B the possible topologies of reticulons are depicted (Yang & Strittmatter, 2007; Schweigreiter, 2008; Schwab, 2010).

The neurite growth inhibitory effects of Nogo-A are mediated by a receptor complex, which includes NgR-1, p75/TROY and Lingo1 as well as additional, yet uncharacterized components. The receptor(s) activate(s) the small GTPase RhoA and its effector ROCK (Fournier *et al.*, 2003; Yiu & He, 2006; Montani *et al.*, 2009). Activation of this cascade leads to growth cone collapse and inhibition of axonal sprouting and growth after injury in the CNS, but also in intact adult CNS (Schwab, 2010). These are the best-studied effects of Nogo-A.

In addition to mature oligodendrocytes, Nogo-A is also expressed by subpopulations of neurons, particularly during development (Huber *et al.*, 2002; Mathis *et al.*, 2010; Petrinovic *et al.*, 2010). Nogo-A is expressed by some types of migrating neurons, which are restricted by Nogo-A expression (Mingorance-Le Meur *et al.*, 2007; Mathis *et al.*, 2010). Constitutive genetic ablation or antibody-mediated neutralization of Nogo-A during development causes aberrant growth of the peripheral neurites *in vitro* and nerves *in vivo* (Petrinovic *et al.*, 2010). Later in development, Nogo-A affects the plasticity of the visual cortex and other parts of the CNS (Kapfhammer & Schwab, 1994; McGee *et al.*, 2005). Nogo-A/NgR-1 signaling also negatively regulates LTP at hippocampal synapses (Raiker *et al.*, 2010; Delekate *et al.*, 2011), as well as axonal and dendritic sprouting and plasticity (Zagrebelsky *et al.*, 2010).

The presence of Nogo-A protein in the midbrain region has been evaluated previously. In the adult mouse substantia nigra, Nogo-A expression was ranked as ‘medium’, yet the staining was not shown (Wang *et al.*, 2002b). In turn, colorimetric labeling in human neuromelanin-enriched nigral neurons did not definitively confirm the presence of Nogo-A in this region (Buss *et al.*, 2005). Hence, the roles of Nogo-A in developing and adult midbrain dopaminergic neurons have not been yet addressed.

Aims of the thesis

The overarching aim of this thesis is to define the molecules, which may improve the axonal growth of dopaminergic neurons after transplantation in the parkinsonian brain.

The specific aims of this thesis intend to answer the following questions:

1. Can the grafted human midbrain dopaminergic neurons survive and extend axons for 22 years in the brain of a patient with Parkinson's disease? (paper 1)

2. Which proteoglycans and glycosaminoglycans affect the process of differentiation and axonal growth in dopaminergic neurons *in vitro*? (paper 2)

3. Does Nogo-A, a potent neurite-growth modulator, play a role in survival and axonal growth of developing midbrain dopaminergic neurons *in vitro*, and in adult midbrain dopaminergic neurons? (paper 3)

Materials and Methods

Throughout this thesis I have been working with several different methods and models. Here I will discuss briefly the considerations for selected procedures. For a detailed description of all methods, I will kindly refer the reader to the respective papers.

Nogo-A knock-out and wild type mice

Nogo-A knock-out mice and wild type mice were employed in paper 2 in order to establish the effect of Nogo-A protein on the midbrain dopaminergic neurons survival and neurite growth *in vitro* and after 6-OHDA lesion.

We used Nogo-A homozygous knock-out mice in 129X1/SvJ background (Dimou *et al.*, 2006) and 129X1/SvJ 000961 wild type controls (The Jackson Laboratory, USA). Nogo-A knock-out mice lack Nogo-A protein expression, but do express the -B and -C isoforms of Nogo. A detailed description of the construct has been published previously (Simonen *et al.*, 2003; Dimou *et al.*, 2006). The animals were housed under standard conditions with free access to food and water under a 12-h light–dark regime. The Ethical Committee For the Use of Laboratory Animals at Lund University approved all experiments described in this thesis, that were conducted on mice. Mice were 12-13 weeks old in case of 6-OHDA experiment and the mouse embryos used for immunelabeling were E13.5 (13.5 days post conception).

Ventral mesencephalon primary cell culture

The adherent primary culture models give a unique advantage to study molecular and physiological events at a single cell level. It is common to use the embryonic tissue as the starting material for cell culture because the cells display relatively high potential for divisions and survival, compared with cells from a mature organism. In case of ventral mesencephalic dopaminergic neurons of mice, the culture is usually set up from mouse fetuses at E11-E13 (Pruszak *et al.*, 2009).

In paper 2, we have employed a classical ventral mesencephalon *in vitro* model to evaluate the differences in neurite growth between the wild type and Nogo-A knock-out midbrain neurons. We followed the protocol by Pruszak (Pruszak *et al.*, 2009), with slight modifications.

The procedure was performed aseptically in a timely manner and the embryos or the embryonic tissues were always kept on ice in sterile HBSS without Ca^{2+} and Mg^{2+} (Sigma). At E13.5 the pregnant wild type or Nogo-A knock-out mice were killed and the embryos were immediately aseptically dissected out from the uterine horns. After removing the uterine sac and amniotic membranes, embryos were moved to fresh HBSS solution and decapitated. The skull layer was removed from the brain and the rostral forebrain and caudal hindbrain regions were removed resulting with a remaining tube-like structure of midbrain (Fig. 6 A-B).

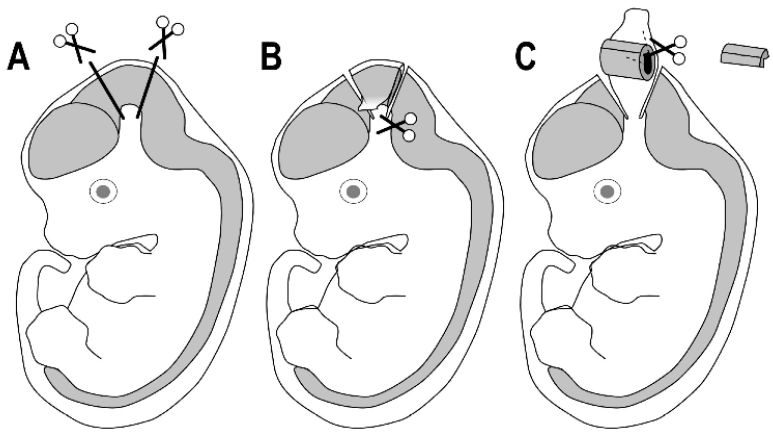

Figure 6. Schema of the ventral mesencephalon dissection from an embryo
The first incision is made on the mesencephalon-diencephalon border and the next one, ca. 30^{o} posterior to it (A). Next, the skull and meningeal tissues are removed (B) and the dissected fragment of neuronal tube can be isolated from the embryo (C). Following, the incisions along the dorsal midline are made (C) to obtain ventral mesencephalon structure, which can be subsequently cut into small pieces and trypsinized (as described in the text above).

Special care was taken to avoid dissection of the serotonergic neurons, which are located caudally to the dopaminergic progenitors. Cutting the mesencephalon tube along the dorsal midline (Fig. 6 C), the flat butterfly-structure was obtained, which was then trimmed along the dorsal edges to remove 2/3 of dorsal tissue. Following the additional mechanical dissociation of ventral midbrain tissue pieces into small chunks (<1 mm), trypsin was applied to obtain a single cell suspension: 0.1% Trypsin (Gibco), 0.05% DNAse (Sigma), 20 min, 37^{o}C. Tissues were then additionally dissociated by gentle pipetting, centrifuged (300 RCF, 5 min, 4^{o}C),

and finally dissolved in DMEM medium (Gibco) containing 5% of FCS (Gibco). 7.0×10^4 cells per cm^2 were plated on coated glass slides (0.01% Poly-L-Lysine and 5 µg/mL Fibronectin, Sigma) in 24-well plates and after 24 h the initial medium was replaced by serum-free medium: DMEM/F12 with Glutamax (Gibco), B27, 10 ng/mL of human recombinant GDNF (R&D Systems). Serum-free medium was changed every other day, and after 6 days, the cells were fixed (with 4% PFA, 20 min in room temperature) and after brief washing with PBS, kept in the fridge at 4^oC until staining was performed. Typically, in each experiment tissues from 5-7 embryos from one pregnant mouse were combined and the cells were plated in 4 wells per treatment.

6-OHDA model

The 6-hydroxydopamine (6-OHDA) model is the most widely used tool for replicating the PD-like death of dopaminergic neurons in the substantia nigra pars compacta (Ungerstedt, 1968). The neurotoxin 6-OHDA has a high affinity to dopamine transporter (DAT), which carries toxin inside the cell. Because 6-OHDA is injected in site(s) along the nigrostriatal pathway, it affects mainly dopaminergic neurons (as only DAT-positive in this tract). 6-OHDA causes cell death by creating oxidative stress and by inhibition of respiratory chain complexes I and IV in mitochondria (Glinka *et al.*, 1997). It is also known that microglia participate in the neurodegenerative process evoked by 6-OHDA (Rodriguez-Pallares *et al.*, 2007). The big advantage of the 6-OHDA model is its versatility. Depending on the site(s) of delivery and dosage, varying degrees of nigrostriatal lesions that develop with different temporal profiles can be achieved (Kirik *et al.*, 1998), mimicking different stages of PD.

In paper 2 we employed the partial unilateral 6-OHDA lesion model. The toxin was injected into the dorso-lateral striatum in order to cause partial degeneration of fibers coming from the nigral neurons. By doing so, we aimed to observe temporal differences in dopaminergic neuron death and differences in regeneration of nigrostriatal fibers, between Nogo-A knock-out and wild type mice.

In this procedure 6-OHDA (Sigma) was injected into the right striatum under isoflurane anesthesia and analgesia (1.5-2% isoflurane in 1:2 of oxygen: nitrous oxide), using a stereotaxic mouse frame (Stoelting) (Fig. 5) and a 5 µL Hamilton syringe fitted with a fine glass capillary. The toxin was used at a concentration of 8 µg/µL, dissolved in a solution of 0.02% ascorbic acid in 0.9% sterile saline. A total volume of 1 µL was injected using the stereotaxic coordinates: A/P = +0.5 mm, M/L = –2.0 mm, D/V = –3.0 mm, with a flat skull position and all coordinates measured from bregma. Injections were made at a rate of 0.25 µL/15 seconds. We

maintained the capillary in place for an additional 3 min to allow the toxin to diffuse. After removing the needle, the wound was cleaned and sutured and the mouse was injected with 75 µg of bupivacaine around the wound, to diminish pain. We performed the injections being blinded for the genotype. Experimental groups were composed of balanced numbers of male and female adult mice.

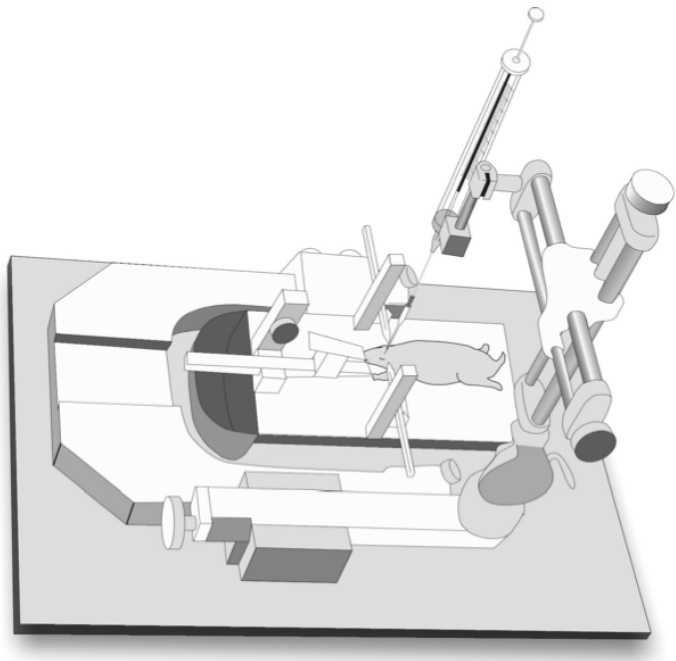

Figure 5. Schematic drawing of a mouse in a stereotactic frame
The head of an anesthetized mouse is stabilized by two earbars and the frontal snout holder.

LUHMES cell culture

In papers 2 and 3, the Lund human mesencephalic (LUHMES) cell line was employed to study the expression of different proteins in maturing human midbrain dopaminergic neurons.

LUHMES cells have been subcloned from the MesC2.10 cell line (Lotharius *et al.*, 2005), which originated from the human embryonic midbrain. This cell line was immortalized with *c-myc* gene (Lotharius *et al.*, 2002). At the undifferentiated state, LUHMES cells bear features of neuroepithelial neuroblasts, expressing

Sox2, *Pax2* and *Otx2* (Scholz *et al.*, 2011). Switching off *c-myc* with tetracycline induces exit from mitosis to differentiation. During the first 5-6 days of differentiation, the midbrain (A9) dopaminergic phenotype is acquired (Scholz *et al.*, 2011). Expression of *Nurr1*, *Pitx3* and *TH* transcripts, as well as genes involved in dopamine storage and release: *DRD2*, *DAT*, *VMAT-2*, *AADC* are enhanced then. Moreover, the specific A9 substantia nigra dopaminergic phenotype is also acquired during that period, as expression of *Girk2* (G-coupled inward potassium channel) gene - *KCNJ6* - becomes augmented at that time (Scholz *et al.*, 2011). Almost all differentiated LUHMES cells produce tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis (and a common marker of dopaminergic phenotype) (Schildknecht *et al.*, 2009). At 8-11 days of differentiation, the expression of dopamine phenotype related genes is stable while the cells continue to acquire neuronal electrical features (Scholz *et al.*, 2011).

LUHMES cells maintenance and differentiation was performed as previously described (Scholz *et al.*, 2011) and it is schematically shown in Figure 7. Cells were grown on glass or plastic dishes, pre-coated with 50 µg/mL poly-L-ornithine and 1 µg/mL fibronectin (Sigma-Aldrich). Proliferation medium consisted of Advanced Dulbecco's modified Eagle's medium/F12 (Gibco), N-2 supplement (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich) and 40 ng/mL recombinant bFGF (R&D Systems). For differentiation, medium consisting of Advanced Dulbecco's modified Eagle's medium/F12, N-2 supplement, 2 mM L-glutamine, 1 mM dibutyryl cAMP (Sigma-Aldrich), 1 µg/mL tetracycline (Sigma-Aldrich) and 2 ng/mL recombinant human GDNF (R&D Systems) was used. For enzymatic detachment and dissociation, LUHMES cells were incubated with ATV trypsin (138 mM NaCl, 5.4 mM KCl, 6.9 mM $NaHCO_3$, 5.6 mM d-Glucose, 0.54 mM EDTA, 0.5 g/L trypsin from bovine pancreas type-II-S; Sigma-Aldrich) for 3 min at 37 °C and then spun down in 300 RCF for 5 min.

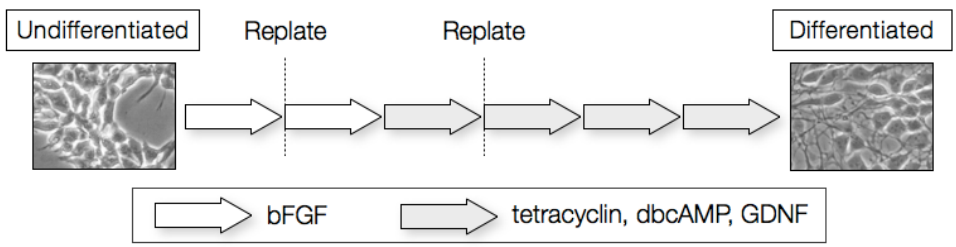

Figure 7. Schema of LUHMES cell culture differentiation protocol
Each arrow indicates a 24 h period and the arrow color shows what additives the medium was supplemented with (in addition to L-glutamine and N-2 supplement); bFGF – basic fibroblast growth factor, dbcAMP - dibutyril 3'-5'-cyclic adenosine monophosphate, GDNF – glial cell line-derived neurotrophic factor.

Differentiation of LUHMES cells comprised two steps: 24 h after plating, the differentiation was initiated by changing from proliferation to differentiation media. After an additional 48 h, we enzymatically detached and re-plated the cells to new dishes for 3 additional days of differentiation (medium was changed 48 h after replating) (Fig. 7). In the replating step, we usually plated 1.5×10^5 cells/cm^2 for imaging and 2.5×10^5 cells/cm^2 for protein harvest or radiolabeling. We seeded undifferentiated LUHMES cells with density of 2.0×10^4 cells/cm^2, and proteins were harvested 2 days later.

According to our experience, the critical points in successful differentiation of LUHMES cells are: two-step protocol (better control over final cell number than without the replating step), coating mixture, using Advanced DMEM/F12 medium (with defined serum-like components) and using cells up to 20th passage.

Metabolic radiolabeling and isolation of PGs and GAGs

The metabolic radiolabeling of the sulfated glycosaminoglycan (GAG) chains of proteoglycans (PGs) is obtained when the cells in culture are given an isotope of sulfur [^{35}S] as the only source of sulfur in the medium. After an incubation period, the PGs and GAGs can be isolated and purified by ion exchange chromatography and gel permeation chromatography. By applying metabolic radiolabeling and isolation of sulfated PGs and GAGs, we aimed to compare the levels of GAGs and PGs expressed and released by the cells before and after the dopaminergic differentiation (paper 2).

For metabolic radiolabeling, LUHMES cells cultured as monolayers were incubated in low-sulfate, $MgCl_2$-labeling medium supplemented with 50 μCi/mL [^{35}S] sulfate, N-2 supplement, 2 mM L-glutamine (for undifferentiated cells) and 1 mM dibutyryl cAMP, 1 µg/mL tetracycline (for differentiated cells). After an incubation period of 24 h, culture medium was collected and the cells were extracted with 0.1-0.2 mL/cm^2 dish of 0.15 M NaCl, 10 mM EDTA, 2%(v/v) Triton X-100, 10 mM KH_2PO_4, pH 7.5, 5 µg/mL ovalbumin containg 10 mM N-ethylmaleimide, and 1 mM diisopropylphosphoro-fluoridate on a slow shaker at 4°C for 10 min. The protein concentration in the cell lysates was determined using the bicinchoninic acid protein assay (Pierce Biotechnology). The substances indicated in the standard differentiation protocols that contained sulfate salts were omitted when the $MgCl_2$-labeling medium with [^{35}S] sulfate was carried out.

Microarray analysis

The DNA microarray is a high throughput method, which gives an opportunity to establish the relative transcription levels of thousands of genes at once. In paper 2, to study the difference in proteoglycan (PG) and glycosaminoglycan (GAG) expression between undifferentiated and differentiated dopaminergic neurons (LUHMES cells), we employed the non-commercial focused affymetrix-based microarrays containing the selection of ~2000 so-called glyco-genes - the genes related to the cellular glycome. Those microarrays contain human and mouse highly annotated genes coding for proteins responsible for glycan synthesis and glycan binding, including enzymes relating to PG/GAG synthesis, metabolism and recognition. The genes are represented on the array by the ~25 nucleotide-long 3' gene fragments (probes), present as picomole DNA spots on a chip. Glyco-gene arrays are available free of charge at the website of Consortium for Functional Glycomics.

First, the RNA sample was amplified to obtain fluorescently-labeled anti-sense total RNA (target) and was then hybridized with a chip. Basic Affymetrix Chip Analysis and Experimental Quality Analysis, as well as probe summarization and data normalization, were performed using the Expression Console Software v1.1.2 (Affymetrix Inc). We used the Robust Multi-array Average (RMA) analysis (Irizarry *et al.*, 2003) for summarization and normalization of the microarray data. Further, to identify the significantly differentially expressed genes, we employed Significance Analysis of Microarrays (SAM) (Tusher *et al.*, 2001)(TMEV v4.0 software). Clustering was carried out using HCA (Hierarchical Clustering Analysis /TMEV). Gene annotation information was extracted from UCSC Human Genome Browser Data: Human Build HG19 and Mouse Build MM9 Databases. The RNA quality control and amplification, as well as microarray data analyses were performed at SCIBLU Genomics and Affimetrix Unit at Lund University (ID C00081), Lund, Sweden. Four independent total RNA extractions from independent cell culture condition (undifferentiated and differentiated) were performed in this experiment. The raw data from the microarray experiment will be submitted online, and will be available at Consortium for Functional Glycomics website.

High content screening - Cellomics analysis

High content screening (HCS) is a type of phenotypic screen to analyze whole cells or their components with a simultaneous readout of several parameters. The HCS Cellomics platform consists of automated digital

microscopy in combination with software for the analysis and storage of data. This system provides rapid, automated and unbiased assessment of experiments.

In paper 3, we employed vHigh Content Screening (Thermo Fisher Scientific, Cellomics, Pittsburgh, PA, USA) software to perform optimization and analysis of the neurite growth in ventral mesencephalon cells obtained from wild type and Nogo-A knock-out embryos. Our protocol was optimized based on the NeuronalProfiling.V4 algorithm, assay version 6.0.0.4008. The thresholds were adjusted so that the program recognizes the majority of the stained cells together with their processes, despite minor differences in staining derived from some variety in each experimental repeat. By configuration of the assay parameters, we have adjusted the following thresholds: for DAPI staining - nucleus area, total intensity, average intensity; for beta-III tubulin staining – cell body nucleus count, cell body total/average intensity, neurite length (22-400 μm); for tyrosine hydroxylase staining – total/average intensity. There were up to 1772 cells sampled per experiment, and on average about 400 beta-III tubulin positive neurons recognized per well.

Of note is that the primary culture experiments were initiated before this HCS platform was available, so the photos were taken with the use of Eclipse 80*i* microscope (Nikon) and then analyzed with HCS Cellomics software. In order to do so, all the 'non-native photos' were split to single RGB channels and renamed using NameChanger 2.3.2 © MRR Software, 2007-2011. For splitting the channels, ImageJ 1.42q and the following macro was used.

```
dir=getDirectory("Choose a Directory");
print(dir);
splitDir=dir + "\Split\\";
print(splitDir);
File.makeDirectory(splitDir);
list = getFileList(dir);

for (i=0; i<list.length; i++) {
  if (endsWith(list[i], ".tif")){
          print(i + ": " + dir+list[i]);
        open(dir+list[i]);
        imgName=getTitle();
    baseNameEnd=indexOf(imgName, ".tif");
    baseName=substring(imgName, 0, baseNameEnd);

    run("Split Channels");
    selectWindow(imgName + " (blue)");
    rename(baseName + "-blue.tiff");
    saveAs("Tiff", splitDir+baseName + "-blue.tif");
```

```
    close();
    selectWindow(imgName + " (green)");
    saveAs("Tiff", splitDir+baseName + "-green.tif");
    close();
    selectWindow(imgName + " (red)");
    saveAs("Tiff", splitDir+baseName + "-red.tif");
    close();
}}
```

Immunolabeling

In order to detect selected proteins in the fixed tissues, cell cultures and cell extracts in papers 1, 2 and 3, we employed standard immunelabeling techniques: immunocytochemistry and Western-blot. Those methods were performed according to the standard protocols. Table 1 lists the primary antibodies that we used for immunolabeling.

Table 1. The antibodies employed for immunolabeling

Epitope	Dilution	Source
Tyrosine hydroxylase	1:1000	P40101-0, Pel-Freez
Alpha-synuclein	1:1000	LB509, Zymed
Dopamine transporter (DAT)	1:500	AB1766, Chemicon
Ionized calcium binding adaptor molecule 1 (IBA-1)	1:500	019-19741, Wako
Phosphorylated S129 alpha-synuclein	1:1000	AB51253, Abcam
Glial fibrillary acidic protein (GFAP)	1:500	20334, Daco
G-protein regulated inward-rectifier potassium channel (Girk2)	1:100	Almone Labs
Nogo-A (11C7)	1:1000-5000	Novartis for M. Schwab's laboratory
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	1:5000	AB9485, Abcam
Beta III-Tubulin	1:200	T2200, Sigma
Neurocan	1:50	AB31979, Abcam
Heparan sulfate 3 *O*-sulfotransferase 5 (HS3ST5)	1:200	HPA021823, Sigma

Results and discussion

Graft can survive over two decades

In paper 1, we have compared post-mortem brain specimens from two transplanted patients, called 'patient 1' and 'patient 3' according to grafting. Patient 1, who died at age of 69, was grafted unilaterally in the putamen 22 years prior to death. She never exhibited any significant clinical benefit (Lindvall *et al.*, 1989) probably due to poor graft survival caused by suboptimal surgical technique (Lindvall *et al.*, 1989; Lindvall *et al.*, 1990). Patient 3 was implanted bilaterally 12 and 16 years before death, which first occurred at an age of 65. Clinically, he improved substantially for several years, which has been previously reported (Lindvall *et al.*, 1990; Lindvall *et al.*, 1992; Lindvall *et al.*, 1994; Wenning *et al.*, 1997; Hagell *et al.*, 1999; Li *et al.*, 2008b; Li *et al.*, 2010).

We have demonstrated that grafted human fetal mesencephalic neurons can survive and extend axons for 22 years in the brain of a patient with PD. In this patient, the overall survival and fiber outgrowth of the grafts were, however, relatively poor (Fig. 8) - when compared to other grafts (Fig. 8). This was consistent with the lack of significant clinical graft-induced benefit in patient 1.

We have also compared the morphology of neurons in the 22-year old grafts with those in two younger grafts (16- and 12-year old) sequentially transplanted in patient 3. In the case with the 22-year-old transplant, a high proportion (up to 38%) of the grafted dopaminergic (neuromelanin-granule containing) neurons did not express tyrosine hydroxylase and dopamine transporter, and their cell bodies appeared atrophic (Fig. 9). The proportion of pigmented neurons not expressing these markers was lower in the 12-16 year old grafts (Fig. 9). Furthermore, in the 22-year-old graft, 49% of the pigmented neurons showed α-synuclein in the cell body and 1.2% of them contained Lewy bodies.

Our findings support the notion that the magnitude of motor improvement following neural transplantation in PD is dependent on the survival of the graft and its integration with the host tissue (Hagell & Brundin, 2001). In patient 3, a rich and extensive dopaminergic innervation of the putamen derived from both the 12- and 16-year old grafts, demonstrated *in vivo* by ^{18}F-dopa PET and using histopathology at post-mortem, was consistent with the positive clinical outcome (Lindvall *et al.*, 1990; Hagell & Brundin, 2001).

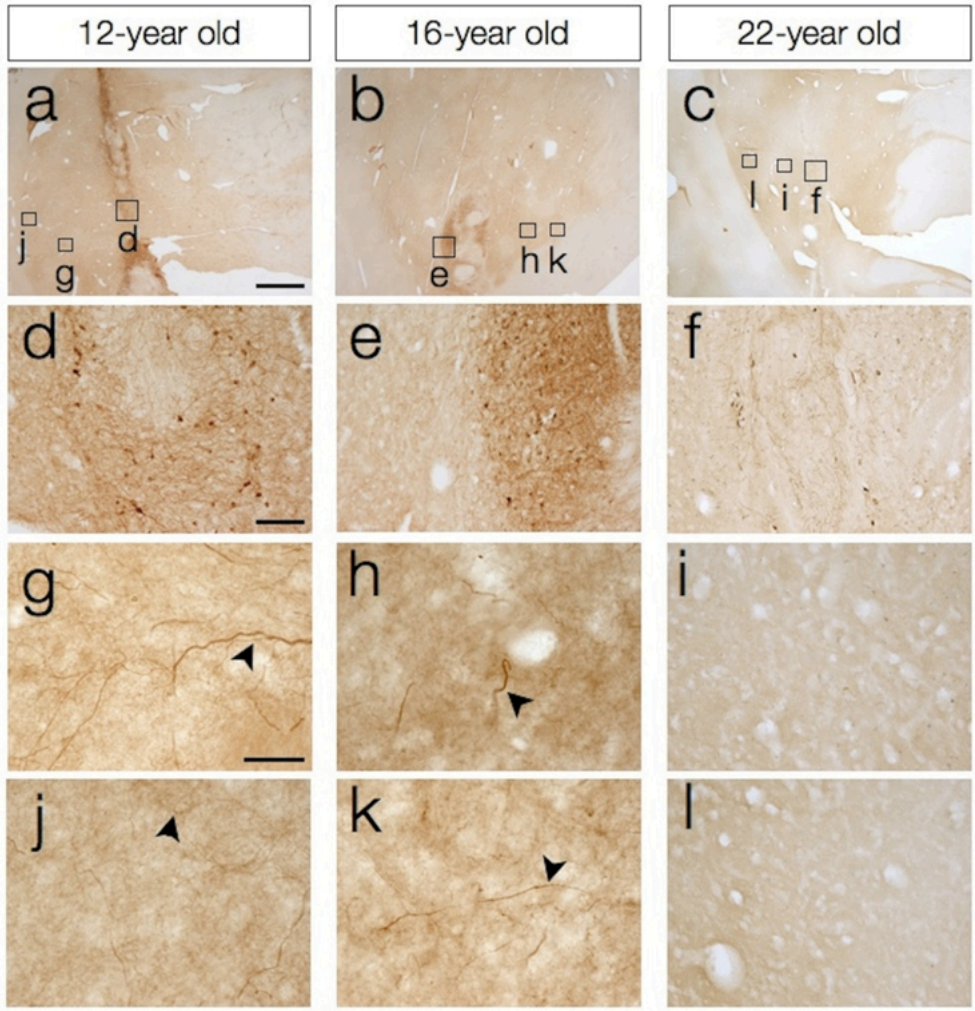

Figure 8. Survival of grafted dopaminergic neurons and fiber extension into the host putamen by 12-22-year old grafts
The left panel shows the 12-year old grafts with good cell survival (a, d) and extensive fiber extension into the host brain (g, j). The middle panel shows the 16-year old graft with similar degree of cell survival and fiber integration to the 12-year old one (b, e, h, k). The right panel shows very poor cell survival (c, f) and fiber extension (i, l) of 22-year old dopaminergic neurons in the graft. Scale bars: 2 mm (a-c), 100 µm (d-f) and 50 µm (g-l).

Conversely, the relatively poor graft survival and virtual lack of striatal dopaminergic reinnervation in patient 1 were in line with the lack of changes in ^{18}F-dopa uptake and modest and transient improvement of motor function (Lindvall *et al.*, 1989). The low number of surviving, grafted cells in patient 1 was probably due to a suboptimal transplantation technique, which caused significant tissue damage at the implantation sites (Lindvall *et al.*, 1989). The ability of the

grafts to integrate with the host brain circuitry might be affected by local inflammation and the presence of a glial scar (Sofroniew, 2009). A host microglial reaction in association with the grafts in patient 3 (Li *et al.*, 2008b) has been reported before, and in this study we observed activated astrocytes locally, around the 12- and 16-year-old grafts. In contrast, the small, poorly integrated 22-year-old transplant in patient 1 was not surrounded by any astro- or microgliosis. This could be due to the small initial size of the surviving transplant and/or the long time that had elapsed after surgery.

In conclusion, the results in paper 1 show that grafted dopaminergic neurons can survive and grow axons for more than two decades after grafting. However, over time an increasing proportion of grafted neurons exhibit signs of degeneration.

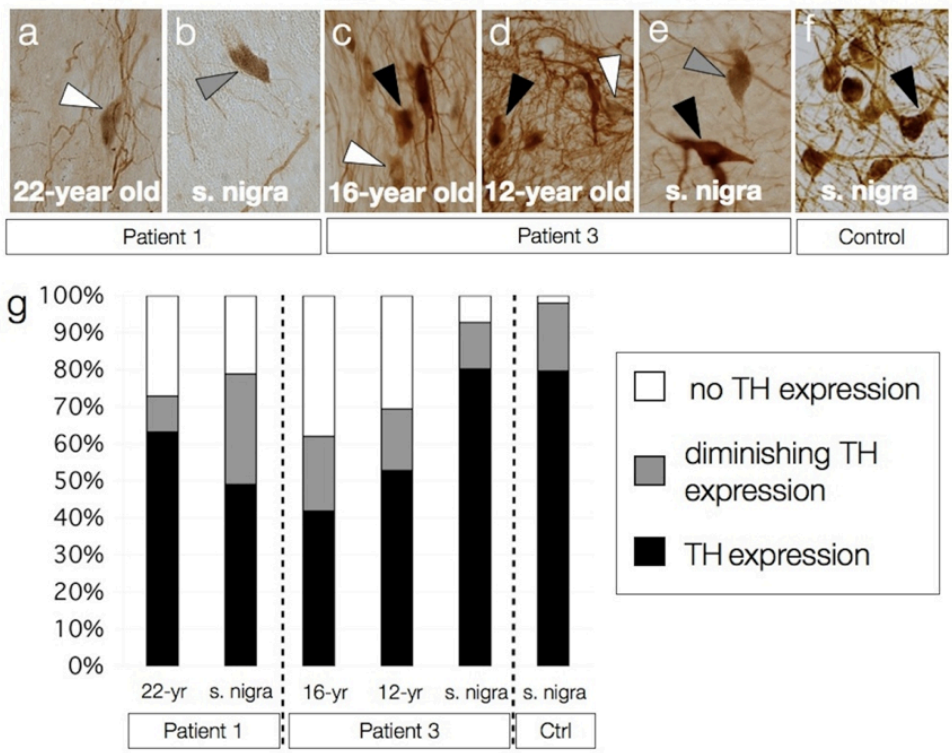

Figure 9. Alterations in morphology and TH expression in 12-22-year old grafts
A-F exemplify representative TH-positive/NM-granule containing cells seen in examined tissues of patient 1 (a - 22-year old graft, b - substantia nigra), patient 3 (c - 16-year old graft, d - 12-year old graft, e - substantia nigra) and the healthy control subject (f - substantia nigra). Colors of arrows correspond to different morphological characteristics of grafted cells, quantified in g. The chart (g) shows the proportions of three morphologically characterized subgroups of neurons.

Neurocan and 3-*O*-sulfotransferase 5 are upregulated in maturing midbrain neurons *in vitro*

In paper 2, we have focused on one of the central issues in development of embryonic stem cell replacement therapy in Parkinson's disease, namely dopaminergic differentiation.

In the central nervous system, various heparan sulfate (HS) and chondroitin/dermatan sulfate (CS/DS) PGs modulate ligand-receptor interactions of molecules involved in neuronal patterning and axonal pathfinding during development. Although it is recognized that differentiation of dopaminergic neurons is driven by specific patterning codes during development, the expression and function of PGs and GAGs in this process has not yet been addressed, particularly in midbrain neuron maturation. In paper 2, we have employed the human and mouse midbrain-derived cell lines LUHMES and MN9D, respectively. First, using metabolic radiolabeling we demonstrated that expression and extracellular release of PGs and GAGs increased markedly upon differentiation of both human and mouse dopaminergic neurons (Fig. 10 A-B, C-D, respectively).

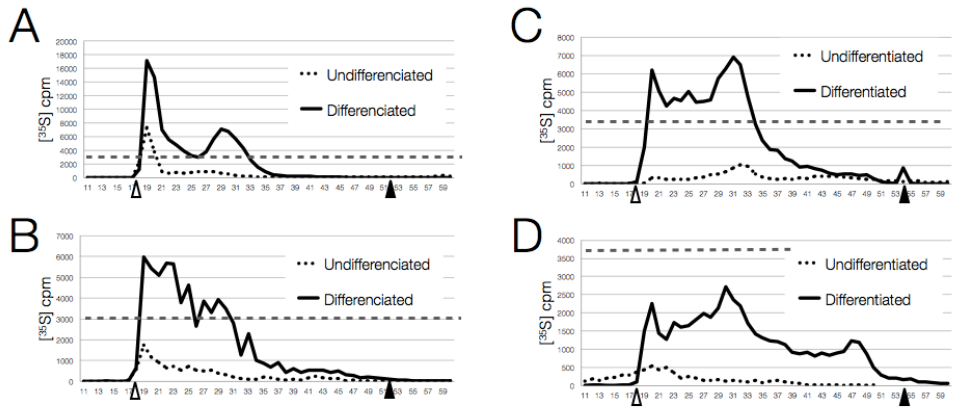

Figure 10. Dopaminergic neurons produce and release sulfated GAGs upon differentiation

The charts show results of gel chromatography on Superose 6 of products present in conditioned media (A and C) and cell extracts (B and D) of undifferentiated (dashed line) and differentiated (solid line) LUHMES cells (A-B) and MN9D cells (C-D). The cpm values were corrected for protein concentration and [^{35}S] sulfate concentration; as a reference, the horizontal dotted lines indicate [^{35}S] cpm=3000. V_0, void volume (empty arrow head); V_t, total volume (full arrowhead).

Then, using a targeted microarray approach, we investigated transcription of around 2000 glycome-related genes, which included a set of PG- and GAG-associated transcripts, in undifferentiated and differentiated LUHMES cells. This strategy yielded a list of 29 genes robustly up- or down-regulated upon dopaminergic differentiation. In response to dopaminergic differentiation, the two most strongly upregulated glyco-genes were CSPG3, neurocan and HS 3-O-sulfotransfetase, HS3ST5. The qPCR results confirmed the microarray results that the given gene was overexpressed in the differentiated dopaminergic neurons, compared to the undifferentiated cells (Fig. 11 A-E), although the magnitude of the differences detected by the methods varied. Such variation has been previously reported (Ton et al., 2002; Koria et al., 2003; Smith et al., 2005) and it is perhaps related to a different dynamic range and normalization methods in those two techniques. Importantly, at the protein level we could also confirm neurocan and HS3ST5 differential expression upon LUHMES cell differentiation (Fig 11 B-D, F-H, respectively).

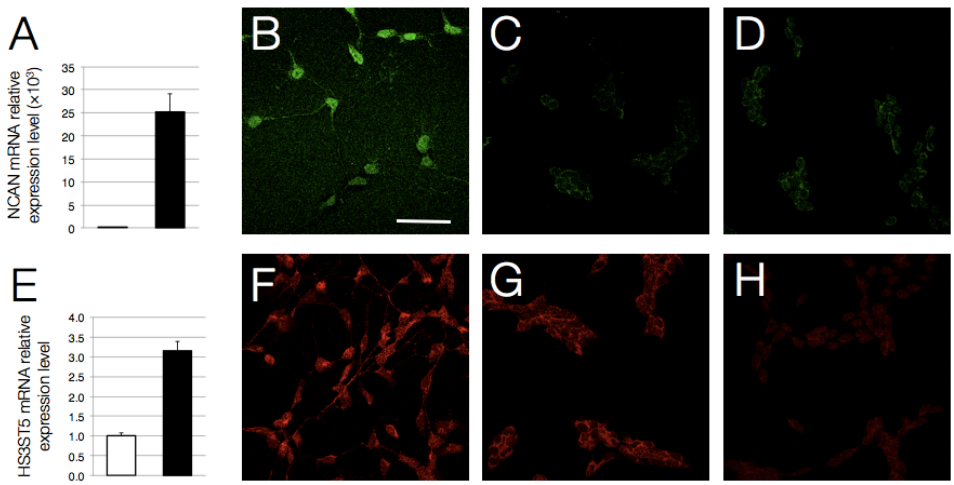

Figure 11. Upregulation of NCAN and HS3ST5 (mRNA and proteins) upon differentiation

The figure depicts mRNA relative levels (A, E) and immunohistochemical staining of LUHMES cells before and after differentiation (B-D, F-H) in the case of two genes most highly upregulated upon differentiation: NCAN (neurocan, A-D) and HS3ST5 (Heparan sulfate [glucosamine] 3-O-sulfotransferase 5, E-H). The relative expression of neurocan (A) and HS3ST5 (E) was upregulated in differentiated LUHMES (black) when compared to undifferentiated (white). In the case of both proteins, much stronger staining was observed after differentiation (B, F), than before (C, G). Labeling in undifferentiated cells (C, G) had similar intensity as control staining of neurocan and HS3ST5 protein (the secondary antibody only, D and H). Average ±S.E.M., n=4 (A, E), scale bar equals 100 µm (B-D, F-H).

Neurocan is a CSPG found exclusively in the CNS and its spatiotemporal expression has shown to be increased in the developing rat brain but diminished postnatally (Meyer-Puttlitz *et al.*, 1995; Engel *et al.*, 1996). During development, neurocan may regulate axonal growth in rodents by binding to different cell adhesion molecules, for example N-CAM, Ng-CAM/L1 (Grumet *et al.*, 1993; Friedlander *et al.*, 1994), tenascin-C (Grumet *et al.*, 1994), axonin-1 (Milev *et al.*, 1996), and pleiotropin (Rauvala & Peng, 1997). In the adult rat brain, neurocan and versican, are components of a glial scar, repressed re-growth of nigrostraital fibers, as revealed by application of chondroitinase ABC or hyaluronidase treatments (Moon *et al.*, 2001; 2002; Moon *et al.*, 2003).

In various *in vitro* models, including embryonic midbrain neurons (Mace *et al.*, 2002), neurocan appeared to be inhibitory for neuronal attachment and neurite extension (Asher *et al.*, 2000; Inatani *et al.*, 2001; Ughrin *et al.*, 2003). On the other hand, ESC differentiated to neuronal progenitors showed enhanced production of neurocan, versican, aggrecan and hyaluronan (Abaskharoun *et al.*, 2010), suggesting rather a positive role of neurocan in cell survival or the maintenance of neuroectodermal phenotype. Interestingly, in differentiated human midbrain dopaminergic neurons *in vitro*, the transcription and translation of neurocan, but not other ECM-associated molecules (including PGs), was highly upregulated. Whether or not enhanced neurocan production plays a role in the adhesion and neurite growth in our experimental setup remains to be elucidated. Nevertheless, it can be hypothesized that such a high increase of a single ECM-associated molecule may indicate its function as a specific factor promoting dopaminergic differentiation.

The enzymatic activity of the heparan sulfate 3-*O*-sulfotransferases family results in sulfation at the 3-OH position of a glucosamine residue of HS to form 3-*O*-sulfated HS. Currently 7 isoforms of 3-*O*-sulfotransferases are known and they are differentially expressed in human tissues (Shworak *et al.*, 1999; Xia *et al.*, 2002; Xu *et al.*, 2005). HS3ST5 was cloned in 2002 (Xia *et al.*, 2002) and its activity was recognized as being critical for creating antitrombin-binding sequence – GlcNs3S±6S and also as gD-binding site for the herpes simplex virus 1 (HSV-1)(Xia *et al.*, 2002). Early studies on HS3ST5 have shown the highest mRNA expression levels in extracts from human CNS: developing brain, spinal cord and adult brain (Mochizuki *et al.*, 2003). This suggests a CNS-specific role of the 3-*O*-sulfotransferase isoform 5. Moreover, another member of the HS 3-*O*-sulfotransferase family, HS3ST3, which also creates gD-binding site for HSV-1, has been suggested to be a modulator of the Notch signaling pathway in Drosophila (Kamimura *et al.*, 2004), a pathway critical in the proper development and function of the nervous system.

Interestingly, some previous evidence suggests a functional connection between neurocan and HS chains. The interactions of neurocan with heparin (Feng *et al.*, 2000), as well as with heparin-binding factors like pleiotropin, amphoterin

(Milev *et al.*, 1998a) and FGF-2 (Milev *et al.*, 1998b) have been reported. It has been postulated that those heparin-binding factors are mediators in interaction between CS chains of neurocan and HSPGs (Milev *et al.*, 1998a; Milev *et al.*, 1998b). Moreover, it was proven that the neurite outgrowth of mouse cerebral granule cells *in vitro* is highly enhanced when the cells grow on dishes coated with C-terminal fragments of neurocan (Akita *et al.*, 2004). Further, based on the experiments involving heparitinase I and chondroitinase ABC treatments, the authors proposed that HSPGs syndecan-3 and glypican-1 expressed by those cells can bind neurocan substrate and that the core proteins, but not CS chains of neurocan, are the mediators of this interaction (Akita *et al.*, 2004).

Taken together, in paper 2, we have shown that, upon differentiation, neurocan and HS3ST5 gene expression is enhanced, concomitantly with increased protein synthesis. The results suggest that the specific CS/HSPGs, and enzymes involved in PGs synthesis, enable unique interactions of PGs with extracellular space components, thereby modulating the differentiation of dopaminergic neurons.

Nogo-A promotes neurite growth of midbrain neurons *in vitro*

In paper 3, we have evaluated the role of Nogo-A in dopaminergic neurons in mice, and specifically we were interested in studying if this potent axonal growth inhibitor affects the growth and survival of midbrain dopaminergic neurons.

Nogo-A induces growth cone collapse and inhibition of axonal growth in the injured adult CNS. In the intact CNS, Nogo-A functions as a negative regulator of growth and plasticity. Although originally discovered as a myelin-bound protein, Nogo-A is also expressed by certain neurons. In paper 2 we show that Nogo-A is present in neurons derived from human midbrain (LUHMES cell line), as well as in embryonic and postnatal mouse midbrain dopaminergic neurons. In LUHMES cells, Nogo-A was upregulated 3-fold upon differentiation and neurite growth. Cultured midbrain dopaminergic neurons from Nogo-A knock-out mice, but not the anti-Nogo-A treated wild type mice, exhibited decreased numbers of neurites and branches, compared with the wild type neurons (Fig. 12 B-C, E-F). *In vivo*, neither the density of striatal tyrosine hydroxylase immunolabeling, nor the regenerative capacity of nigrostriatal neurons after partial 6-hydroxydopamine (6-OHDA) lesions, were affected by Nogo-A deletion (Fig. 13 A). However, two months after the lesion, we observed a trend where slightly fewer tyrosine hydroxylase-positive dopaminergic neurons survived in the substantia nigra pars compacta of Nogo-A knock-out mice than in wild type controls (Fig. 13 B).

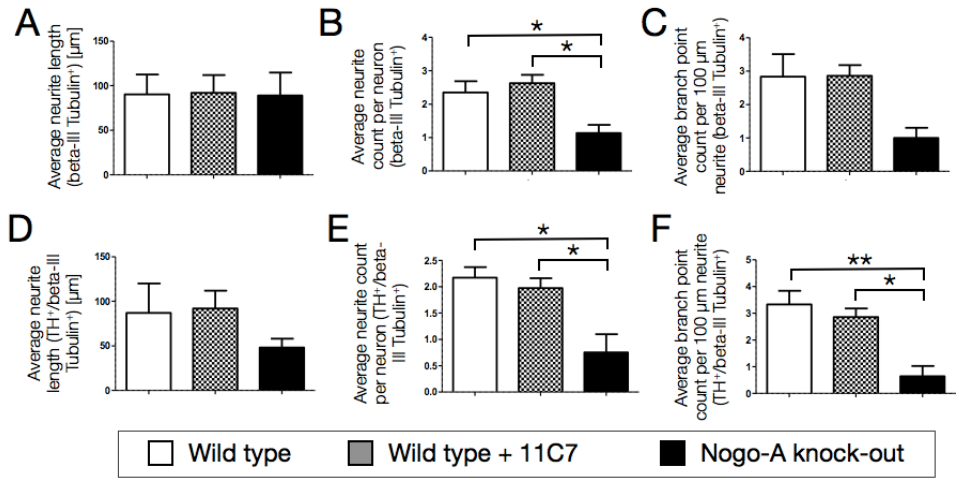

Figure 12. Nogo-A knock-out, but not neutralizing antibody, hinders neurite growth and branching in mouse ventral mesencephalon (dopaminergic) neurons in vitro
The figure shows quantification of neurite parameters of beta-III-tubulin-positive neurons (A-C) and tyrosine hydroxylase-positive/beta-III tubulin-positive (dopaminergic) neurons from wild type (white bars), anti-Nogo-A antibody (11C7) treated wild type (grey bars) and Nogo-A knock-out (black bars) ventral midbrain cell cultures. Average neurite length was similar in wild type, 11C7-treated wild type and Nogo-A knock-out neurons (A), likewise in dopaminergic neurons (D). The average neurite count per neuron was significantly decreased in Nogo-A knock-out cultures, when compared with the wild type (non-treated or 11C7-treated), both in dopaminergic (E) as well as all types of neurons labeled with beta-III tubulin (B). In C, the difference in the average branch point count per 100 µm of neurite length was not statistically significant between the three groups of beta-III-Tubulin-positive neurons. Though, in case of TH-positive neurons only, Nogo-A knock-out neurons had significantly less branches per neurite fragment, than the wild type, both in non-treated and 11C7-treated cells (F). Above parameters were assessed only for neurites longer than 25 µm, all bars represent mean of three-four independent experiments (+S.E.M.), one-way ANOVA and Tukey post-hoc test, A (p=0.996, F=0.003), B (p=0.016, F=7.872), C (p=0.042, F=4.83), D (p=0.395, F=1.09), E (p=0.0144, F=7.546), F (p=0.0043, F=13.15). For all groups Df=2.

Intracellular Nogo-A facilitates initiation and branching of neurites in midbrain dopaminergic neurons *in vitro*

Nogo-A's enrichment in different types of maturing neurons with growing processes has been documented in previous reports. Growing axons of the olfactory tract expressed Nogo-A (Tozaki *et al.*, 2002), suggesting its positive role in axonal extension. Likewise, in an *in vitro* model, Nogo-A accumulated at axonal branch points and the central domain of the growth cones, co-localized with growth-associated proteins (Richard *et al.*, 2005). Similarly, in embryonic cortical neurons, Nogo-A was enriched in the axonal varicosities and growth cones

in vitro (Hunt *et al.*, 2003). Functional studies where Nogo-A was deleted or its activity was neutralized with specific antibodies showed that the effects of Nogo-A suppression on migrating neurons and growing neurites differed according to cell type and probably also age. Thus, Nogo-A/B/C knock-out slowed down the tangential migration of interneurons into the embryonic cortex, and the cortical neurons *in vitro* showed earlier polarization and increased branching, when compared with the wild type condition (Mingorance-Le Meur *et al.*, 2007). Radial migration of the cortical neurons themselves along the radial glial fibers was enhanced *in vivo* and *in vitro* by anti-Nogo-A antibodies or knock-out (Mathis *et al.*, 2010). In dorsal root ganglion cells *in vitro* and peripheral nerves *in vivo* Nogo-A neutralization or ablation led to longer neurites, increased fasciculation and decreased branching (Petrinovic *et al.*, 2010). Growth cones of dorsal root ganglia dissected from new-born and adult mice showed increased motility and size in the absence of Nogo-A (Montani *et al.*, 2009). Finally, in regenerating retinal ganglion cells *in vivo*, Nogo-A overexpression in the neurons enhanced, whereas Nogo-A knock-out or knock-down diminished, regenerative sprouting (Pernet *et al.*, 2011).

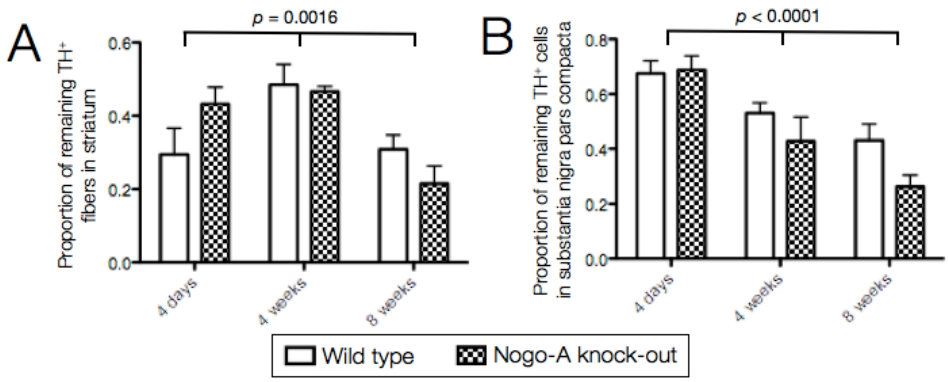

Figure 13. Dopaminergic fiber degeneration and nigral cell death in wild type and Nogo-A knock-out mice after 6-OHDA lesion

TH-positive fiber amounts (assessed by densitometry, A) and TH-positive neuron loss (assessed by neuron counts, B) were quantified in both hemispheres. The values obtained by the same kind of measurement on the non-lesioned side of the section served as a reference and values from the knock-out were expressed referring to wild type condition. The bars represent the average with S.E.M., n=5-10; 2-way ANOVA (A - time p=0.0016, F=7.55, Df=2; genotype p=0.843, F=0.040, Df=1; interaction p=0.093, F=2.516, Df=2; B - time p<0.0001, F=22.77, Df=2; genotype p=0.0497, F=4.08, Df=1; interaction p=0.195, F=1.70, Df=2).

In paper 3, we observed that midbrain neurons lacking Nogo-A had fewer processes and fewer branches, when compared to their wild type counterparts. Though, the neutralization of the plasma membrane-bound Nogo-A only, did not duplicate this effect. This suggests that intracellular Nogo-A has a neurite-growth promoting role in dopaminergic neurons of ventral midbrain *in vitro*. In the adult mice, however, the density of TH immunolabeling in the striatum was not different between wild type and Nogo-A knock-out mice. This may be due to compensatory mechanisms, which occur frequently in different models of gene knock-out. Indeed, increased levels of certain semaphorins and ephrins and their receptors have been seen in the CNS of mice lacking Nogo-A (Kempf *et al.*, 2009), and also Nogo-B was shown to be up-regulated in Nogo-A knock-out mice (Simonen *et al.*, 2003; Dimou *et al.*, 2006). The molecular mechanisms causing the different effects of Nogo-A or Nogo-A deletion in developing neurons and neurites are currently unknown. Surface Nogo-A interacts with at least 3 binding partners/receptors, NgR-1, PirB, and the yet uncharacterized Nogo-A specific receptor (Schwab, 2010). Though, the effects seen in our primary culture model are most likely mediated by intracellular interactions of Nogo-A; Nogo-A is enriched in the ER, where it has structural roles (Voeltz *et al.*, 2006), but a number of other molecular interactions have been observed, some of which could influence neuronal metabolism, neurite growth, branch formation or cell survival (Yang *et al.*, 2009; Schwab, 2010; Pernet *et al.*, 2011).

Nogo-A may play a pro-survival role in adult nigral dopaminergic neurons

It has been reported that Nogo-A knock-out mice show behavioral alterations, which include higher sensitivity to amphetamine compared to wild type mice (Willi *et al.*, 2009). Correspondingly, D2 receptor expression was increased and dopamine levels were lowered in the striatum of Nogo-A knock-out mice (Willi *et al.*, 2010). These findings suggest that Nogo-A influences properties of the nigrostriatal pathway.

In the study described in paper 3, we observed an expected progressive loss of dopaminergic neurons 4 to 8 weeks after a 6-OHDA injection into the striatum of Nogo-A knock-out and wild type mice. The effects were slightly more severe in the Nogo-A knock-out than in the wild type mice (trend). This suggests that Nogo-A is neuroprotective in adult dopaminergic neurons challenged with 6-OHDA.

The mechanism behind the pro-survival role of Nogo-A in the nigral neurons is obscure. Kilic and co-workers showed a pro-survival role of Nogo-A in response to cellular stress caused by ischemia: increased neuronal death was seen in the striatum of Nogo-A knock-out mice (or mice pretreated with Nogo-A antibody) after transient medial cerebral artery occlusion, mediated via a Rac1/RhoA signaling pathway (Kilic *et al.*, 2010). In addition, in a superoxide

dismutase (SOD) mutant mouse model of amyotrophic lateral sclerosis genetic deletion of Nogo-A and Nogo-B accelerated disease progression and decreased spinal motor neuron survival (Yang *et al.*, 2009). This effect was correlated with the interaction of Nogo-A with the chaperone protein disulfide isomerase (Yang *et al.*, 2009). Because elevated reactive oxygen species probably mediate the neurotoxic effects in all the three models (SOD mutation, cerebral artery occlusion and 6-OHDA), similar mechanisms of a probable intracellular action of Nogo-A may be at work. Intracellular interactions of Nogo proteins with the survival promoting protein bcl-2 (Tagami *et al.*, 2000; Zhu *et al.*, 2007) and with the ROS-scavenging enzyme peroxiredoxin 2 (Prdx2) leading to enhanced survival of cortical neurons have also been described (Mi *et al.*, 2012).

Taken together, the results presented in paper 3 indicate that during maturation of midbrain dopaminergic neurons, intracellular Nogo-A supports neurite growth and neurite branch formation. In the adult brain, Nogo-A related mechanisms might protect nigral dopaminergic neurons against the deleterious toxic effect of 6-OHDA.

Concluding remarks

The grafted dopaminergic neurons derived from the human ventral mesencephalon can survive over twenty years in the striatum of a patient with PD (as we have shown in paper 1). The microenvironment of the aging parkinsonian host brain, which is full of misfolded α-synuclein, may promote aggregation formation in the grafted cells and cause loss of dopaminergic phenotype. Results of this study indicate that downregulation of TH and DAT occurs in proportions of grafted neurons, 12-22 years after intrastriatal transplantation, and the transplants become dysfunctional in a progressive manner, which will have a potential impact on the clinical usefulness of neural transplantation in PD.

Although the therapeutic usefulness of cell replacement therapy was marginal for patient 1 (paper 1), this therapy brought beneficial effects to some individuals suffering from PD, even for up to 16 years after the transplantation (Politis *et al.*, 2010). As shown in animal models, one of the critical conditions for such positive development is the delivery of a sufficient number of postmitotic dopaminergic neurons of a midbrain (A9) phenotype (Grealish *et al.*, 2010; Kirkeby *et al.*, 2012).

The production of dopaminergic neurons from embryonic stem cells relies on the combination of different signaling cues, to which midbrain cells are exposed during development. Sensitivity to those developmental molecular cues (i.e. Wnts, SHH, FGFs) is modulated by proteoglycans (PGs).

In paper 2, we have shown that production of sulfated proteoglycans is a phenomenon associated with differentiation of human midbrain neuroblasts into mature dopaminergic neurons. It is likely that specific expression of the CS/HSPGs, and perhaps more importantly, synthesis of the enzymes specifically regulating the HS sulfation codes (such as HS3ST5), enables unique interactions of PGs with extracellular space components (including morphogenes and growth factors), modulating the differentiation of dopaminergic neurons. Our results provide a starting point to identify PGs/GAG and their structural changes that are influencing the differentiation of midbrain dopaminergic neurons. New knowledge about this process is required for better understanding of midbrain neuron maturation and, ultimately, for improving cell replacement therapies for Parkinson's disease.

The success of transplantation therapy in PD relies not only on the efficient dopaminergic differentiation and cell survival after grafting; dopaminergic

neurons need to grow axons to appropriate targets, form synapses and release dopamine in a regulated fashion.

In paper 3, we have focused on neurite growth of midbrain dopaminergic neurons and the role of growth inhibitory protein Nogo-A in this process. Although Nogo-A is mostly expressed in oligodendrocytes and myelin in the adult CNS, neuronal expression is prevalent during development and includes midbrain dopaminergic neurons. Nogo-A influences neurite outgrowth, branching and fasciculation in different types of neurons in either a positive or negative manner, probably dependent on different types of neurons, stage of differentiation, and site and mechanisms of action. Our experiments indicated that neuronal ER-bound Nogo-A supports the neurite initiation and neurite branching in the ventral mesencephalon dopaminergic and non-dopaminergic neurons *in vitro*. In adult Nogo-A knock-out dopaminergic substantia nigra neurons, the neurotoxin 6-OHDA has a slightly more deleterious effect than in wild type cells (trend), consistent with the intracellular pro-survival action of Nogo observed in other neuronal cell types. All these results make Nogo-A an interesting target for further studies, in particular in the context of Parkinson's disease.

The results of initial clinical trials, studies in animal parkinsonian models, recent safe and effective dopaminergic differentiation protocols and the new stem cell sources emerging, collectively imply that the cell replacement approach in PD holds a great therapeutic potential. Nonetheless, in order to develop safe and efficient cell replacement therapy in PD, further understanding of the mechanisms governing the dopaminergic cell differentiation, survival and neurite growth is needed. The results presented in this thesis suggest that Nogo-A, neurocan and HS3ST5 may contribute to those processes and therefore may serve as targets for molecular manipulation when embryonic stem cells are considered as a source of cells for transplantation in PD.

Acknowledgements

First, I would like to thank my supervisor - Jia-Yi Li and my co-supervisor - Patrik Brundin, for giving me an opportunity to join your research group, back in October 2008. Jia-Yi, thank you for your peacefulness and for providing space for my scientific and personal development. Patrik, thank you for your in-depth understanding of leadership idea and your vibrant sense of humor at all times.

Next, I would also like to send my gratitude to our collaborators: Martin Schwab (also my co-supervisor), Katrin Mani, John Couchman and Marcel Leist. It was very inspiring to receive your insights on the projects; thank you for your kindness and support.

Dear friends and colleagues from TBN and NESU, I have learned something from each and every of you and I will never forget the friendly and cheerful atmosphere accompanying our JC, ShaSup and TBN meetings. I would like to extend my special thanks to Miguel Burgillos, Marcus Davidsson, Tomas Dejeborg, Martha Escobar, Phil Gaughwin, Sonja George, Oktar Guloglu, Birgit Haraldsson, Staffan Holmquist, Martina Holst, Adnan Husic, Shorena Janelidze, Laura Jensen, Wen Li, Anna Magnusson-Lind, Andy McCourt, Ulrika Mundt-Petersen, Anna-Karin Oldén, Reena Prity Murmu, Srikanth Ranganathan, Laurent Roybon, Edina Silajdzic - thanks for our discussions and your voluntary intellectual and mental support. Christian Hansen, Jordi Boix and Ilknur Özen - many thanks for all the laughs we had together in A1041, and for being inspiring professional role models for me.

Here, I would also like to send my warmest appreciations to Tomas Björklund, Maria Björkvist, Ulrikka Nördström and Maria Swanberg - your enthusiasm and encouragement were empowering for me, especially in the last year of my Ph.D..

I must also express gratitude to all my friends and colleagues from different sites of BMC, other than my own lab: Ulrika Aili, Anna Appelberg, Cecilie Bay-Richter, Karthik Devaraju, Erika Elgstrand, Veronica Francardo, Shane Grealish, Anna Hammarberg, Hanna Iderberg, Zuzana Kolkova, Bengt Ljungkvist, Kajsa Löfgren-Söderberg, Bengt Mattsson, Jenny Nelander, Mariena van der Plas, Andreas Puschmann, Justyna Rak, Daniella Rylander, Rohit Sahdeva, Jenny Shin, Gabriel Svensson, and Filip Ventorp. I appreciate all your helpfulness and optimism.

I would like to use this opportunity to acknowledge here Ulrikke Voss and all my colleagues from *Medicinska Doktorandrådet* (MDR, The Medical Ph.D. Student Union). Thanks for speaking English at our gatherings; I hope you will continue doing the great job that you do. I am grateful to MultiPark board members who also agreed to speak English, so that I could take part in their meetings as a Ph.D. student representative, which was a wonderfully enriching experience.

My Ph.D. was partially funded by EU, being a part of Marie Curie Initial Training Network, 'Axregen'. I owe many thanks to my dear Axregen-roommate, and great friend, Iben Lundgaard, and also to all who organized and participated in our workshops: thanks for our interesting discussions, as well as for all the great time we had together.

On a personal note, I would like to send my gratitude to the Flakowicz family and the Begier family. Thank you for your helpfulness throughout the years here, in Lund. Finally, I am sending love to my family, my *sambo* and my dear non-work-related friends. I deeply appreciate all your love, support and encouragement.

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Paper I

Journal of Parkinson's Disease 1 (2011) 83–92
DOI 10.3233/JPD-2011-11004
IOS Press

Signs of Degeneration in 12–22-Year Old Grafts of Mesencephalic Dopamine Neurons in Patients with Parkinson's Disease

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Abstract. We demonstrate that grafted human fetal mesencephalic neurons can survive and extend axons for 22 years in the brain of a patient with Parkinson's disease (PD). In this patient, the overall survival and fiber outgrowth of the grafts were, however, relatively poor, which is consistent with the lack of significant clinical graft-induced benefit. We have compared the morphology of neurons in the 22-year old grafts with those in two younger grafts (16- and 12-year old), which were sequentially implanted in another PD patient. In the case with the 22-year-old transplant, a high proportion (up to 38%) of the grafted dopaminergic (pigment-granule containing) neurons do not express tyrosine hydroxylase and dopamine transporter and their perikarya appear atrophic. The proportion of pigmented neurons not expressing these markers is lower in the 12–16 year old grafts. Furthermore, in the 22-year-old graft, 49% of the pigmented neurons display α-synuclein immunoreactivity in the cell body and 1.2% of them contain Lewy bodies. In conclusion, our results show that grafted dopaminergic neurons can survive for more than two decades. However, over time an increasing proportion of grafted neurons exhibit signs of degeneration.

Keywords: Neural, transplantation, graft survival, dopamine, phenotype, tyrosine hydroxylase, alpha-synuclein, Lewy bodies

INTRODUCTION

Grafted human fetal dopaminergic neurons can survive, extend axons, release dopamine and functionally integrate in the striatum of patients with Parkinson's disease (PD) [1]. The reported functional outcome has varied greatly between patients, ranging from major improvement, allowing some patients to withdraw L-dopa medication for more than a decade, to no functional benefit in other cases. Based on positron emission tomography studies, these differences in outcome appear to be partly explicable by differences in graft survival and function at the neurochemical level [2], and partly by the extent of dopaminergic denervation in brain areas not reached by the grafts [3]. Detailed morphological analyses of post-mortem samples might shed more light on why some grafts function well while others do not.

Post-mortem studies have shown two abnormalities occur in neurons grafted to subjects with PD, namely down-regulation of tyrosine hydroxylase (TH) and dopamine transporter (DAT) [4–6] normally expressed in dopaminergic neurons, and abnormal han-

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dling of α-synuclein. Kordower and coworkers have reported reduced DAT expression in dopaminergic neurons already 4 years after transplantation, whereas decreased TH expression was apparent after 14 years [4]. α-Synuclein gradually accumulates in grafted dopaminergic neurons several years after surgery [5, 7] and eventually contributes to the formation of Lewy bodies (LBs) and Lewy neurites [4–8]. The LBs appear in a subpopulation (2–8%) of grafted dopaminergic neurons and they also stain for several other LB markers (i.e., phosphorylated α-synuclein, ubiquitin, Thioflavine-S) [5–9]. The generation of LBs in grafted neurons has been suggested to take place in two steps. First, soluble α-synuclein slowly accumulates in the cytoplasm of neurons, including dopaminergic neurons. Second, α-synuclein aggregates in an insoluble form, possibly triggered by a "seed" of misfolded α-synuclein that is transmitted from host cells to the grafted neurons [10–14].

Here we have compared post-mortem brain specimens from two transplanted patients, called 'patient 1' and 'patient 3' according to grafting. Patient 1, who died at age of 69, was grafted unilaterally in the putamen 22 years prior to death. She never exhibited any significant clinical benefit [15] probably due to poor graft survival caused by suboptimal surgical technique [15, 16]. Patient 3 was implanted bilaterally 12 and 16 years before death, which occurred at an age of 65. Clinically, he improved substantially for several years, which has been reported in earlier papers [7, 8, 16–20].

We had four objectives in the present study. First, to determine if dopaminergic neurons can survive and remain integrated in the Parkinsonian brain up to 22 years. Second, to examine whether the degree of graft survival and integration is associated with the widely different functional outcomes in our two patients. Third, to analyze whether the dopaminergic neurons contain Lewy bodies in the 22 year-old grafts and if the occurrence of Lewy bodies increases between 16 and 22 years after transplantation. Fourth, to monitor whether the dopaminergic phenotype of the grafted neurons, in terms of TH and DAT expression, is more compromised at 22 years after grafting than at 12 and 16 years.

MATERIALS AND METHODS

Patient history

Patient 1

This female patient was aged 47 years and had 14 years history of PD when she in 1987 received transplants of human fetal ventral mesencephalon (fresh tissue, cell suspension with small aggregates) unilaterally into left putamen (2 injection tracts) and left caudate nucleus (one injection tract). The grafting procedure, donor tissue, neurological outcome and imaging data have all been reported previously [15]. Briefly, Patient 1 experienced only very slight symptomatic relief during the first 6 months post-surgery and positron emission tomography showed no significant changes of fluorodopa uptake. At 3 years after grafting, she received subcutaneous infusion with apomorphine, and 8 years later deep brain stimulation was started bilaterally in the subthalamic nuclei with lasting beneficial effects. At 32–34 years after PD onset, she developed, diabetes, dysautonomia and subcortical dementia. In 2009, she had a large stroke of the right middle cerebral artery territory and died of acute bronchopneumonia 4 months later.

Routine neuropathologic analysis in patient 1 revealed signs of old ischemic-hypoxic damage in several areas in the right hemisphere following an ischemic insult 4 months prior to death.

Patient 3

This male patient was transplanted bilaterally 16 and 12 years before his death. His clinical course and history have been described in detail [16, 17, 19, 20]. Briefly, in 1989, at age of 49, and 12 years after disease onset, the patient received grafts at 3 tracts into the left putamen. This intervention resulted in reduced time in off-phase, a longer duration of L-DOPA effect and a bilateral reduction of rigidity and hypokinesia in the off phase. Two years after grafting, his parkinsonian symptoms worsened on the side ipsilateral to the graft with increased rigidity and hypokinesia, whereas he continued to be improved on the contralateral side. Four years after the first transplantation, he received grafts at 5 tracts in the right putamen. This resulted in longer periods spent in the on phase and improved function in the left arm. Off-phase rigidity disappeared and timed motor tasks improved slightly contralateral to the second graft. However, after 13 months, the patient started to experience right-sided, painful off-phase related dystonia, which was L-dopa responsive. Due to multiple degenerated cervical disks, he developed a radiculopathy affecting the right hand and arm 22 months after transplantation. Regular assessments were stopped at 24 months after the second transplant.

Postmortem tissue preparation

Brains were fixed in 6% buffered formaldehyde solution for about two months, followed by standard immunohistochemistry for paraffin embedded or frozen sections, as previously described [7, 8]. Forty micrometer thick free floating sections were cut and stained with antibodies against α-synuclein, TH, DAT, G-protein regulated inward-rectifier potassium channel (Girk2), calbindin, ionized calcium binding adaptor molecule 1 (IBA-1), and glial fibrillary acidic protein (GFAP) [7, 8]. In addition to processing the graft regions, the brains were sectioned and processed for regular analysis of PD and other pathologies.

Immunohistochemistry

We stained sections from Patient 1 and 3, as well as one age-matched subject without brain disease. Antigen retrieval was performed in 10 mM sodium citrate pH 7.6, 80°C, 30 min followed by incubation with quenching solution (3% H_2O_2, 10% methanol) for 15 min to block activity of endogenous peroxidase. Then we washed and blocked tissues for 1 h with 5% of adequate serum. We incubated sections with primary antibodies over-night (TH – Pel-Freez P40101-0 1 : 1000, α-synuclein – Zymed LB509 1 : 1000, DAT – Chemicon AB1766 1 : 500, IBA-1 Wako 019-19741 1 : 500, phosphorylated S129 α-synuclein Abcam AB51253 1 : 1000, GFAP – Dako 20334 1 : 500). After washing, we applied biotin-conjugated secondary antibody (1 : 200, Vector Laboratories) for 1 h, followed by washing and 1 h incubation with ABC-solution (PK-6100, Vector Laboratories), washing and incubation with DAB-solution (SK-4100, Vector Laboratories).

Sections were analyzed using a Nikon microscope (Eclipse 80*i*) and images were processed with iPhoto (Apple Inc.) software. Transplanted dopaminergic neurons contain dark brown neuromelanin granules and can be easily distinguished from other cells in the striatum that do not contain the pigment.

Thioflavin-S staining

After mounting and drying on gelatin-coated glass slides, sections were treated with a mixture of chloroform and ethanol (1 : 1) for 2 h, hydrated through the 6-grade gradient of ethanol to distilled water, and incubated for 10 min in 0.1% thioflavin-S (Sigma) in the dark. The slides were kept in 80% ethanol for 10–12 s, rinsed with distilled water and mounted in PVA DABCO medium.

RESULTS

Long-term survival and morphological integration of grafted dopaminergic neurons

Surviving dopaminergic neurons were observed in the grafts in both patients. However, the grafts in Patient 1 (Fig. 1c) were smaller in volume and contained much fewer TH-positive neurons than those in Patient 3 (Fig. 1a and b). We estimated that about 2 700 dopaminergic neurons had survived in one injection tract in Patient 1. By comparison, in Patient 3 we previously estimated that each tract contained 12 100–29 500 TH-immunoreactive dopaminergic neurons [7].

In Patient 3, the grafted dopaminergic neurons formed a dense TH-immunoreactive network within the core of the implants (Fig. 1d and e) and extended fibers into the entire host striatum (Fig. 1g, h, j and k). By contrast, the graft-derived TH fibers were few in Patient 1 and rarely crossed the graft-host boundary (Fig. 1c, f, i and l).

Long-term morphological and phenotypic changes of grafted dopaminergic neurons

During normal aging, dopaminergic neurons down-regulate their expression of phenotypic markers such as TH [21–23]. We examined TH expression levels in grafted cells and in host dopaminergic neurons in the substantia nigra of Patients 1 and 3. In all 3 implanted striata (22-, 16- and 12-year old grafts), between 27% and 38% of the neuromelanin-containing cells exhibited no, or very weak, TH-immunoreactivity (Fig. 2a, c, d and g). The proportions of neuromelanin-pigmented dopaminergic neurons in the substantia nigra pars compacta that did not exhibit clear TH-immunoreactivity were 7% and 21% in Patients 1 and 3, respectively (Fig. 2b, e and g). In a 83-year old male control subject only a small fraction (2%) of the pigmented substantia nigra neurons lacked TH immunoreactivity (Fig. 2f and g).

We also explored whether the levels of DAT were changed in the grafted dopaminergic neurons. Most of the DAT-positive cells in 12–22 year-old grafts exhibited very weak, 'patchy' staining (Fig. 3a–c), which contrasted with the evenly distributed staining in the substantia nigra dopaminergic neurons of the control subject (Fig. 3d). In Patient 1, some DAT-positive

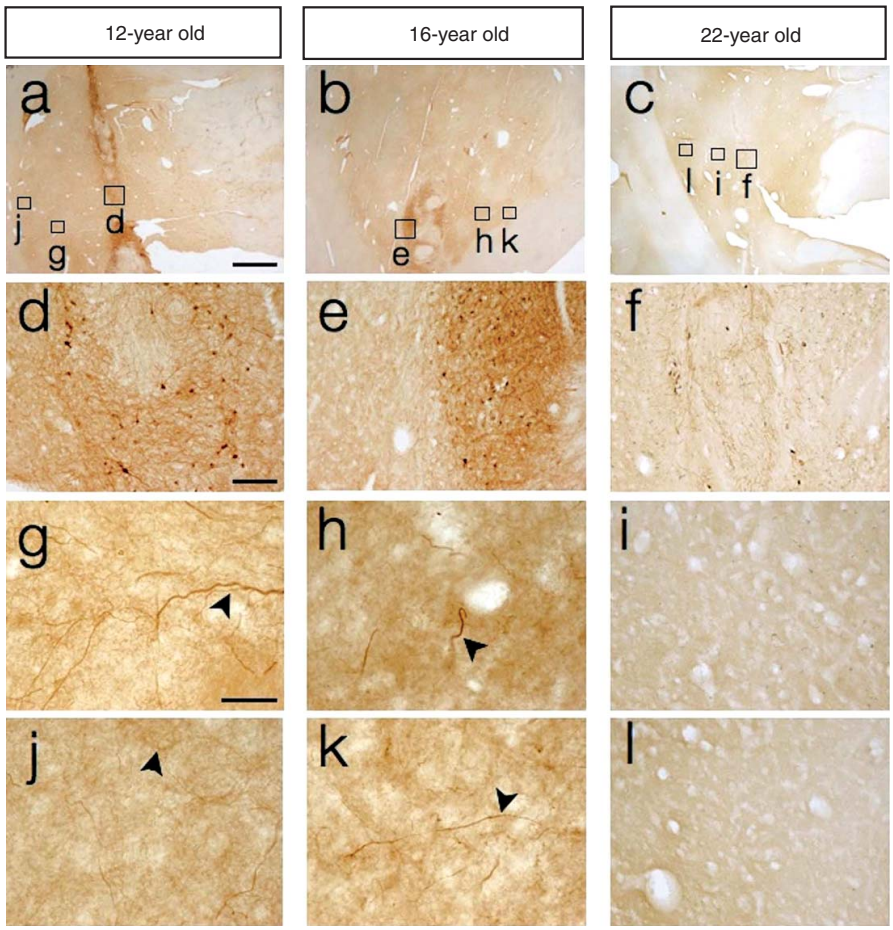

Fig. 1. Survival of grafted dopaminergic neurons and fiber extension into of the host putamen by 12–22-year old grafts. The left panel shows the 12-year old grafts with good cell survival (a, d) and extensive fiber extension into the host brain (g, j). The middle panel shows the 16-year old graft with similar degree of cell survival and fiber integration to the 12-year old one (b, e, h and k). The right panel shows very poor cell survival (c, f) and fiber extension (i, l) of 22-year old dopaminergic neurons in the graft. Scale bars: 2 mm (a–c), 100 μm (d–f) and 50 μm (g–l).

(Fig. 3) and TH-positive cells had an atrophic morphology with few processes. In addition, in Patient 1 the Girk2-, or calbindin-immunoreactive, pigmented neurons appeared atrophic (data not shown).

In summary, the phenotypic and morphological changes in the grafts suggest that part of the dopaminergic cells surviving long-term in these patients were dysfunctional, which might have contributed to attenuation of functional improvement over time after transplantation.

Accumulation of α-synuclein and Lewy bodies in grafted dopaminergic neurons

We previously reported that in Patient 3, 40% of 12-year old and 80% of 16-year old grafted, TH-positive neurons displayed diffuse α-synuclein immunoreactivity in the cell bodies [8]. Here we found that 50% of the transplanted neuromelanin-positive neurons in Patient 1 contained detectable amounts of α-synuclein (both homogeneous and punctate, pre-

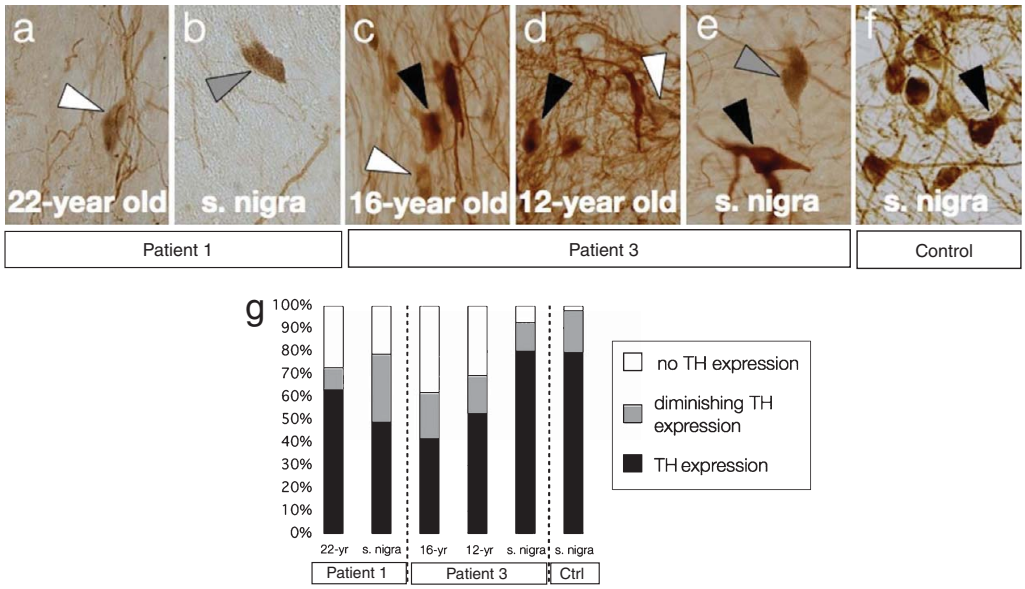

Fig. 2. Alterations in morphology and TH expression in 12–22-year old grafts. A-F exemplify representative TH-positive/neuromelanin-granule containing cells seen in examined tissues of Patient 1 (a - 22-year old graft, b - substantia nigra), Patient 3 (c - 16-year old graft, d - 12-year old graft, e - substantia nigra) and the healthy control subject (f - substantia nigra). Colors of arrows correspond to different morphological characteristics of grafted cells, quantified in g. The chart (g) shows the proportions of three morphologically characterized neurons.

sumably reflecting soluble and insoluble forms of α-synuclein). Moreover, at 22 years post-grafting 1.2% of neuromelanin-containing grafted cells manifested α-synuclein-positive LBs and Lewy neurites (Fig. 4a–c). In Patient 3, we previously reported that 2% of 12-year old and 5% of 16-year old grafted, neuromelanin-positive cells contained Lewy bodies immunoreactive for α-synuclein [8]. As previously reported for Patient 3 [8], the LBs and Lewy neurites in Patient 1 also stained for thioflavin-S (Fig. 5) and S129-phosphorylated α-synuclein (Fig. 6). The morphology of the LBs in the grafted striatum appeared similar to those in the patient's substantia nigra (Fig. 5a–d; Fig. 6a and b). Phosphorylated α-synuclein-positive LBs and Lewy neurites were also present in the striatum surrounding the graft and in the contralateral striatum (Fig. 6c and d).

Astrogliosis in long-term mesencephalic grafts

We observed clear GFAP-immunoreactive astrogliosis in both the 12- and the 16-year old grafts in Patient 3 (Fig. 7a, b, g and c, d, h). Dense GFAP-positive profiles enclosed surviving dopaminergic neurons, in contrast to host striatum where astrocytes were sparse in number (Fig. 7g and h). We observed no clear accumulation of GFAP-positive astrocytes in or around the grafts at 22-years post-operatively in Patient 1 (Fig. 7e, f and i). In addition, there were very few IBA1-positive microglial cells in and around the graft. Their morphology suggested lack of microglial activation (data not shown).

DISCUSSION

We show here that grafted fetal dopaminergic neurons can survive for more than two decades in the striatum of a PD patient who lived for 22 years following transplantation. We also find, in this and another PD patient, that long-term after transplantation, a portion of grafted neurons exhibit an abnormal phenotype suggesting that the cells have lost some of their dopaminergic features and are undergoing degenerative changes. Also, a fraction of them contain LBs, i.e., the neuropathological hallmark of PD.

Our findings support the notion that the magnitude of motor improvement following neural transplanta-

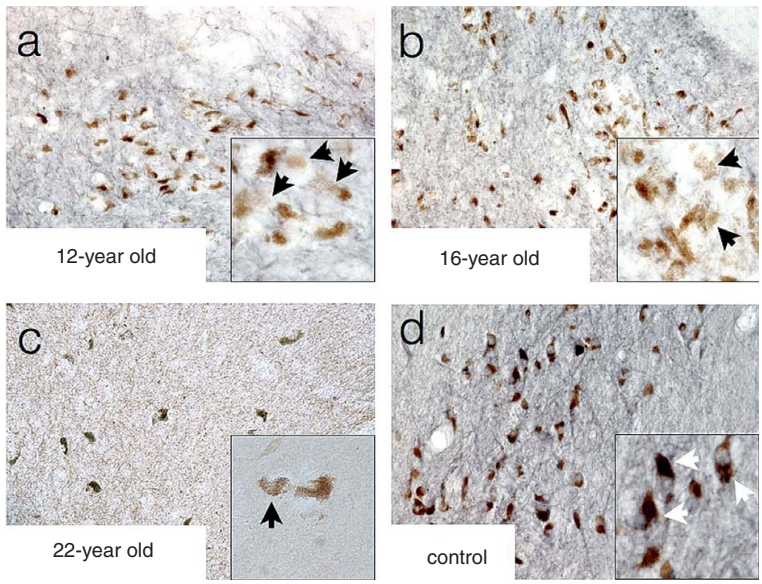

Fig. 3. Down-regulation of DAT in 12–22-year old grafts. Most of the studied cells in the 12-, and 16-year old grafts of Patient 3 (a, b) and 22-year old grafts of Patient 1 (c) displayed diminished DAT immunoreactivity (black arrows), compared with dopaminergic neurons in the substantia nigra of the control subject (d, white arrows).

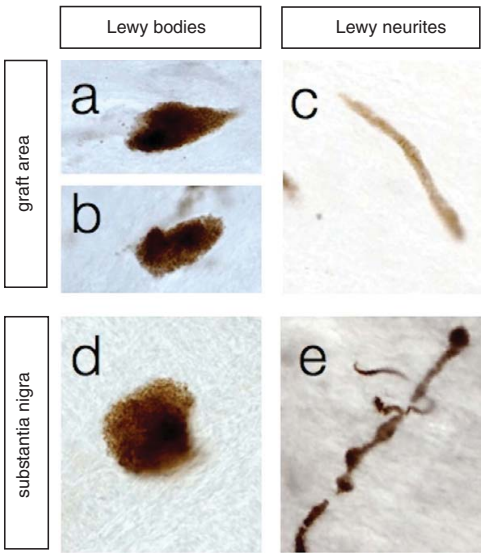

Fig. 4. Lewy bodies and Lewy neurites in 22-year old grafts. Characteristic compact, intensely α-synuclein-immunoreactive Lewy bodies (a, b) and neurites (c) in the 22-year old graft, reminiscent of that in the substantia nigra (d, e).

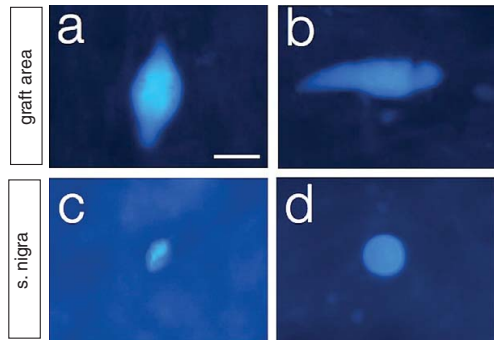

Fig. 5. Thioflavine-S positive structures in the 22-year old graft. Thioflavine-S structures were observed in Lewy bodies and Lewy neurites, both in grafted neurons (a, b) and in the host substantia nigra of Patient 1 (c, d). Scale bar = 20 μm.

tion in PD is dependent on the survival of the graft and its integration with the host tissue [2]. In Patient 3, a rich and extensive dopaminergic innervation of the putamen derived from both the 12- and 16-year old grafts, demonstrated *in vivo* by ^{18}F-dopa PET and using histopathology at post-mortem, was consistent

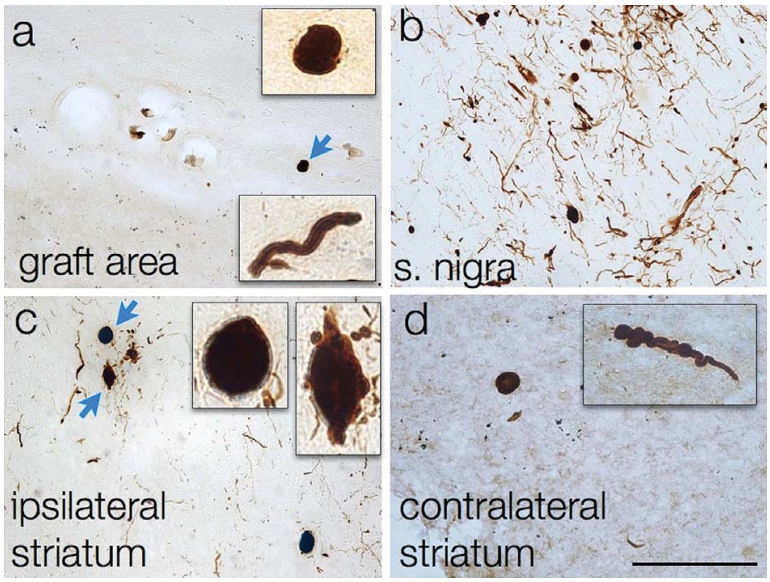

Fig. 6. Phosphorylated Ser129 α-synuclein in the 22-year old graft. Using an antibody that binds to phosphorylated (S129) form of α-synuclein we confirmed presence of Lewy bodies (arrows) and Lewy neurites, not only in the graft (a) and substantia nigra (b), but also in the host ipsilateral (c) (surrounding the grafts) and contralateral striatum (d). Scale bar = 100 μm.

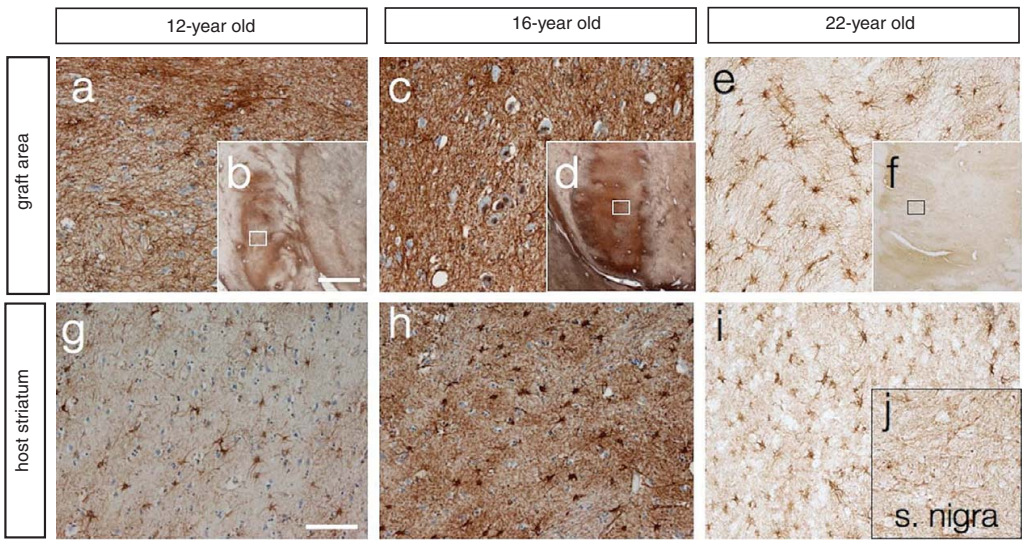

Fig. 7. Astrogliosis in 12, 16 and 22-year old grafts. Low magnified insets showed astrocyte infiltration in grafts of Patient 3 (b and d) and lack of accumulation in Patient 1 (f). Images with high magnification depict different densities of astrocytic processes in 12–16- and 22-year old grafts (Fig. a, c and e). No clear astrocytic accumulation was observed in substantia nigra of Patient 1 (j) and 3. Scale bars 2 mm (b, d and f) and 100 μm (a, c, e, g, h, i and j).

with the positive clinical outcome [2, 16]. Conversely, the relatively poor graft survival and virtual lack of striatal dopaminergic reinnervation in Patient 1 were in line with the lack of changes in ^{18}F-dopa uptake and modest and transient improvement of motor function [15]. The low number of surviving, grafted cells in Patient 1 was probably due to a suboptimal transplantation technique, which caused significant tissue damage at the implantation sites [15]. The ability of the grafts to integrate with the host brain circuitry might be affected by local inflammation and the presence of a "glial scar" [24]. We previously reported a host microglial reaction in association with the grafts in Patient 3 [7], and here we observed activated astrocytes locally, around the same 12- and 16-year-old grafts. By contrast, the small, poorly integrated 22-year-old transplant in Patient 1 was not surrounded by any astro- or microgliosis. This could be due to the small initial size of the surviving transplant and/or the long time that had elapsed after surgery.

One of the first events taking place in vulnerable substantia nigra neurons during the pathogenesis of PD is believed to be the loss of certain features of the dopaminergic phenotype [21, 23, 25, 26]. In the 83-year old control subject, most of the neuromelanin-positive, presumed dopaminergic, neurons in the substantia nigra expressed TH. One fifth (18%) of them exhibited low levels of TH immunoreactivity and only a small fraction (2%) was TH-negative, suggesting that most of the dopaminergic neurons in the 83 year-old control subject were still functional before death. In contrast, a high proportion of the neuromelanin-positive grafted neurons in Patients 1 and 3 (27–38%) lacked key features of the dopaminergic phenotype (diminished TH and DAT expression) and also exhibited signs of atrophy, despite being relatively young, i.e. 12–22 years. These changes imply that the molecular machinery required for the production and proper recycling of dopamine was impeded in the transplanted cells 12–22 years post surgery. Thus, our data provide evidence that the functional capacity and therapeutic value of fetal tissue grafts may decline over the second decade after implantation into a PD brain, which is in line with similar findings in other grafted PD patients [4–6]. In this context, it is important to highlight that TH and α-synuclein expression levels are negatively correlated with one another in the aging brain [22]. Thus, when α-synuclein levels increase in the dopaminergic cell body during normal aging, the TH levels decline. A similar relationship has been suggested after a few years in fetal dopamine neurons implanted in the PD brain [4]. Interestingly, a corresponding phenomenon was observed postmortem in Huntington patients who had received fetal striatal transplants. Ten years after transplantation, a high proportion of grafts exhibited degenerative features, with dense microglial infiltration [27, 28]. Taken together, these data indicate that fetal ventral mesencephalon or striatal tissues transplanted in Parkinsonian and Huntington patients undergo a process of degeneration in the pathological microenvironment.

It is conceivable that the loss of dopaminergic phenotype (e.g. reductions in TH and DAT) is secondary to gradual α-synuclein accumulation. Indeed in cultured neuron-like cells, α-synuclein has been reported to downregulate TH levels [29] and DAT activity [30, 31]. The reductions in TH levels may also be coupled to changes in activity of the dopaminergic neurons. Such homeostatic relationship between TH levels and neuronal activity was recently reported in mice [32], suggesting that high activity leads to upregulation of TH in directly affected neurons, whereas neighbouring neurons compensate by rapidly down-regulating TH levels. Hypothetically, the neuromelanin-positive and TH-negative subpopulation of grafted neurons in our patients may have undergone similar regulatory changes.

Previous studies demonstrated α-synuclein accumulation and LB formation in grafted cells more than 10 years after transplantation [4–8]. Based on the analysis of α-synuclein staining in two transplants (12- and 16-years old) in the same host and other reports describing grafts 18 months to 4 years after surgery [4, 5], we postulated that α-synuclein accumulation and LB formation progressively increase in the grafted dopaminergic neurons. However, in the present report, we observed that only 1.2% of the grafted dopaminergic neurons contained LBs, which is lower than in the 16-year old graft (5.0%). Possibly the accuracy of our estimates is lower in Patient 1, compared to Patient 3, due to the small number of surviving dopamine neurons. Another possibility is that the LB-bearing neurons eventually die, as has been suggested for dopaminergic neurons in the PD substantia nigra [33], and those that are seen at any one given time point developed LBs within the past 6 months. Notably, fetal mesencephalic grafts contain immature dopaminergic neurons both of the A9 substantia nigra and the A10 ventral tegmental area phenotype [34]. It can be postulated that the A9 subtype in the grafts is more susceptible to generate LBs and die, as is the case in the midbrain of PD patients [35–37]. Therefore, the A10 phenotype will predominate among the neurons remaining in the grafts after 22 years, and as the pool of

grafted A9 neurons has been depleted, a lower proportion of the overall TH neuron population exhibits LBs.

In conclusion, our study shows that grafted dopaminergic neurons derived from the human ventral mesencephalon can survive over twenty years in the striatum of a parkinsonian patient. The microenvironment of the aging host parkinsonian brain, which is full of misfolded α-synuclein, may promote aggregation formation in the grafted cells [10–14] and cause loss of dopaminergic phenotype. Our data indicate that downregulation of TH and DAT occurs in proportions of grafted neurons 12–22 years after intrastriatal transplantation, and the transplants become dysfunctional in a progressive manner, which will have a potential impact on the clinical usefulness of neural transplantation in PD.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Research Council, Swedish Parkinson Foundation, the Nordic Center of Excellence on Neurodegeneration, the Strong Research Environment of the Swedish Research Council (NeuroFortis) and Linné grant (BAGADILICO). The study was performed within the Strategic Research Area Multipark at Lund University. The authors wish to thank B.-M. Lindberg and A. Persson for their excellent technical support.

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Paper II

PROTEOGLYCANS IN DIFFERENTIATION OF HUMAN DOPAMINERGIC NEUROS

Zuzanna Kurowska[1], Katrin Mani[2] and Jia-Yi Li[1]

Proteoglycans (PGs) constitute a diverse family of macromolecules characterized by a core protein to which one or more glycosaminoglycans (GAGs) chains are attached. In the central nervous system, various heparan sulfate (HS) and chondroitin/dermatan sulfate (CS/DS) PGs modulate ligand-receptor interactions of different growth factors, axonal guidance molecules and morphogenes. Although it is recognized that differentiation of dopaminergic neurons is driven by specific patterning codes during the development, the expression and function of PGs and GAGs in this process has not been yet addressed, particularly in midbrain neuron maturation. In this study, we employed the human and mouse midbrain-derived cell lines LUHMES and MN9D, respectively. First, using metabolic radiolabeling we demonstrated that PG and GAG expression and extracellular release increased markedly upon differentiation of both, mouse and human dopaminergic neurons. Then, using targeted microarray approach, we investigated transcription of around 2000 glycome-related genes, which included a set of PG- and GAG-associated transcripts, in undifferentiated and differentiated LUHMES cells. This strategy yielded a list of 29 genes robustly up- or down-regulated upon dopaminergic differentiation. A CSPG, neurocan, and the HS modifying enzyme, heparan sulphate 3-*O*-sulfotransferase 5 (HS3ST5), transcripts were most highly upregulated upon differentiation, and their enhanced expression was further confirmed by reverse transcription, quantitative PCR and immunohistochemical stainings. We showed that upon differentiation, neurocan and HS3ST5 gene expression is enhanced, concomitantly with increased protein synthesis. The results suggest that the specific CS/HSPGs, and enzymes involved in PG synthesis, enable unique interactions of PGs with extracellular space components, thereby modulating the differentiation of dopaminergic neurons

INTRODUCTION

Proteoglycans (PGs) are polyanionic macromolecules composed of a specific core protein covalently substituted with one or more unbranched polysaccharide chains called glycosaminoglycans (GAGs). The PG core proteins are synthesized on ribosomes attached to the endoplasmatic reticulum (ER). Then, the core proteins are translocated into the ER where they become either luminal, inserted into the membrane, or transferred to glycosylphosphatidyl-inositol (GPI)-anchors. During transport through the secretory pathway GAG chains are assembled on an initially formed common linkage region, GlcA-Gal-Gal-Xyl, which is attached to serine residues in certain consensus sequences. The addition of GlcNAc or GalNAc to the nonreducing terminal GlcA residue determines whether heparan sulfate (HS) with a HexA-GlcNAc repeating motif, or chondroitin sulfate/dermatan sulfate (CS/DS) with a HexA-GalNAc repeating motif is initiated. The linker tetrasaccharide then becomes elongated by addition of the repeating disaccharides and the growing chain undergoes further modifications by sequential and coordinated series of sulfation and epimerization reactions, resulting in extensive structural diversity of GAG chains attached to PGs. PGs are generally found in extracellular matrices (ECMs) and basement membranes or at the cell surfaces (Esko et al., 2009).

Brain development is remarkably dependent on HSPGs. HSPGs support activity of various growth factors, receptors, and guidance molecules during development, including FGFs and Robo-Slit signaling (Van Vactor et al., 2006). Neural specific conditional knockout of *Ext1*, encoding the enzyme that catalyzes HS polymerization, resulted in severe guidance errors in the major commissural tracts indicating that HS GAGs are vital for brain

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patterning and axon scaffold formation in the developing forebrain (Inatani et al., 2003). Genetic studies in various model organisms have proven that HSs or HSPGs are critical modulators of Robo/Slit and DCC/Netrin signaling at the midline, both in the lateral (Inatani et al., 2003, Johnson et al., 2004, Lee et al., 2004, Steigemann et al., 2004) and longitudinal positioning of growing axonal tracts (Kastenhuber et al., 2009). Moreover, in the subventricular zone of the lateral ventricle walls, which is a major adult stem cell niche, Mercier with colleagues identified the HS-rich extracellular matrix structures (fractones) which had high FGF2 binding potential (Mercier et al., 2002, 2003, Kerever et al., 2007). Further, by careful analysis of spatial relationships between the proliferating neuroblasts and the *N*-sulfated HSPGs expression sites, the group suggested that the neuronal mitogenesis in many adult brain stem cell niches might relay on *N*-sulafated HSs (Mercier and Arikawa-Hirasawa, 2012). Recently it has also been shown that HSPG, syndecan-3, serves as a receptor for GDNF, neurturin, and artemin and that GDNF induction of neurite growth *in vitro* was mediated via HSPG (Bespalov et al., 2011).

CSPGs in the CNS are relatively well-described components of the ECM. Although the cellular origins of the ECM are not fully understood, different cells in the brain, including astrocytes, neurons, oligodendrocytes and microglia, are thought to be capable of CSPG supply and aiding in the arrangement of the extra-cellular environment of CNS (Carulli et al., 2006). It has been shown that chondroitinase ABC treatments of the developing CNS leads to various pathfinding errors of axons, including growing retinal axons and spinal motor neurons (Bernhardt and Schachner, 2000, Chung et al., 2000, Corvetti and Rossi, 2005). From the spatial correlation of CSPGs expression with axonal outgrowth it was concluded that CSPGs act as a repulsive cue during development (Chung et al., 2000, Ichijo, 2006), and further it was argued that they stabilize semaphorin5A in the extracellular matrix (Kantor et al., 2004). The microarray analysis indicated that various axonal guidance molecules such as Slit2, Netrin1, Ephrin A1, EprinA5 and Semaphorin5B bind to specific CS sequences (Shipp and Hsieh-Wilson, 2007). In adult animals, the levels of CSPGs are increased in glial scar tissue after the CNS injury, and this serves as a barrier for regenerating axons in many experimental animal models (Kwok et al., 2008). On the other hand, many reports demonstrate that CSPGs and CS/DS themselves promote neurite extension (Maeda et al., 1995, Hikino et al., 2003, Bao et al., 2004). CSPGs contribute also to neural plasticity. The digestion of CSPGs in the visual cortex re-activated the plasticity in the adult mice, after the closure of the critical period (Pizzorusso et al., 2002, Dityatev et al., 2007) when the experience dependent plasticity may occur. Miyata et al. recently reported that such effect was mediated through inhibition of binding of transcription factor Otx2 to specifically sulfated CSPG sequences on the surface of the parvalbumin-expressing interneurons, which induce the exit from the critical period (Miyata et al., 2012). During the last decade, many reports revealed that GAGs, similarly like DNA, RNA or proteins have the capacity to encode functional information in a sequence-specific manner. It has been documented that specific GAG structure motiffs serve as molecular recognition elements for growth factors, cytokines, morphogenes and other types of molecules (Deepa et al., 2004, Gama et al., 2006, Nadanaka et al., 2008, Mikami et al., 2009, Nadanaka et al., 2011, Miyata et al., 2012), including those promoting embryonic stem cell proliferation and differentiation (Johnson et al., 2007, Sasaki et al., 2009, Kraushaar et al., 2010, Pickford et al., 2011).

Dopaminergic neurons located in the ventral midbrain are critical for cognitive and motor behavior, and are associated with multiple psychiatric and neurodegenerative disorders. Neurons in substantia nigra pars compacta (A9) have been extensively studied, as the progressive degeneration of the nigrostriatal pathway is responsible for the motor disturbances observed in patients suffering from Parkinson's disease (Hornykiewicz, 1966, Riederer and Wuketich, 1976). One of the potential therapies for Parkinson's disease is cell replacement therapy. The grafts of fetal embryonic midbrain neurons could alleviate the motor symptoms of many Parkinson's disease patients, even up to 16 years after transplantation (Dunnett et al., 2001, Lindvall and Bjorklund, 2004, Politis et al., 2010, Politis et al., 2011). Still, before entering the clinic, many parameters in this therapeutic approach remain to be optimized (Politis and Lindvall, 2012). One

of the critical issues is to define the conditions assuring efficient, homogenic and stable dopaminergic *in vitro* differentiation of cells to be used as a source in replacement therapies in future: human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC).
In this study, we employed the human dopaminergic cell line LUHMES, which acquires a robust and uniform dopaminergic phenotype upon differentiation (Schildknecht et al., 2009, Scholz et al., 2011). First, we elucidated alterations in PG and GAG expression upon differentiation using metabolic radiolabeling. Further, using targeted microarray approach, we investigated transcription of about 2000 glycome-related genes, which included a set of PG- and GAG-associated transcripts, in undifferentiated and differentiated LUHMES cells. This strategy enabled us to pinpoint the gene candidates with potentially critical roles in the dopaminergic differentiation process. We finally validated the most upregulated transcripts, CSPG neurocan (CSPG-3) and heparan sulphate 3-*O*-sulfotransferase 5 (HS3ST5), by using reverse transcription, quantitative PCR and immunohistochemical stainings.

MATERIALS AND METHODS

LUHMES cell culture. The Lund human mesencephalic (LUHMES) cell line was kindly provided by Dr. M. Leist (Konstanz, Germany). LUHMES cell maintenance and differentiation were performed as previously described (Scholz *et al.*, 2011). Briefly, for LUHMES cells maintenance and differentiation, plastic T25/75 flasks (Nunc™, Danmark) or glass Nunc™ Lab-Tek™ chambered coverglass slides (Thermo Scientific, USA) pre-coated with 50 µg/mL poly-L-ornithine and 1 µg/mL fibronectin (Sigma-Aldrich) were used. Proliferation medium consisted of Advanced Dulbecco's modified Eagle's medium/F12 (Gibco), N-2 supplement (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich) and 40 ng/mL recombinant bFGF (R&D Systems)(if not indicated otherwise). For differentiation, medium consisting of Advanced Dulbecco's modified Eagle's medium/F12, N-2 supplement, 2 mM L-glutamine, 1 mM dibutyryl cAMP (Sigma-Aldrich), 1 µg/mL tetracycline (Sigma-Aldrich) and 2 ng/mL recombinant human GDNF (R&D Systems) was used, if not stated otherwise. For enzymatic detachment and dissociation LUHMES cells were incubated with ATV trypsin (138 mM NaCl, 5.4 mM KCl, 6.9 mM $NaHCO_3$, 5.6 mM d-Glucose, 0.54 mM EDTA, 0.5 g/L trypsin from bovine pancreas type-II-S; Sigma-Aldrich) for 3 min in 37 °C and then spun down in 300 RCF for 5 min. Differentiation of LUHMES cells had two steps: one day after plating, the differentiation was initiated by changing from proliferation to differentiation media. After additional 2 days we enzymatically detached and re-plated the cells to new dishes for 3 additional days of differentiation (medium was changed 48 h after re-plating). We plated 1.5×10^5 cells/cm^2 for imaging and $2\text{-}3 \times 10^5$ cells/cm^2 for radiolabeling and RNA isolation in the re-plating step.

MN9D cell culture. The MN9D cell line was kindly provided by Dr. A. Heller (University of Chicago, Chicago, IL, USA). Originally, the MN9D cell line was obtained by hybrydoma fusion of cells from murine mesencephalic with neuroblastoma cells (Choi et al., 1991). MN9D cells, when differentiated have properties of midbrain dopaminergic neurons, i.e. they express dopaminergic markers and release dopamine (Choi et al., 1991, Choi et al., 1992, Rick et al., 2006). The cells were maintained as previously described (Choi 1991). Briefly, the cells were grown in DMEM/F12 medium with L-glutamine (Gibco), supplemented in 10% FCS (Gibco) and penicilin/streptomycin (100 U/mL/100 mg/mL, Gibco). For differentiation, the cells were seeded in density of 3×10^3 cells/cm^2 and after 24 hours the sodium butyrate (1 mM) was added and the cells were cultured for the next 7 days.

Metabolic radiolabeling and extraction procedure. LUHMES cells cultured as monolayers were incubated in low-sulfate, $MgCl_2$-labeling medium supplemented with 50 μCi/mL [^{35}S] sulfate, N-2 supplement, 2 mM L-glutamine (for undifferentiated cells) and 1 mM dibutyryl cAMP, 1 µg/mL tetracycline (for differentiated cells). After an incubation period of 24 h, culture medium was collected and the cells were extracted with 0.1-0.2 mL/cm^2 dish of 0.15 M NaCl, 10 mM EDTA, 2%(v/v) Triton X-100, 10 mM KH_2PO_4, pH 7.5, 5 µg/mL ovalbumin containg 10 mM N-ethylmaleimide, and 1 mM diisopropyl-phosphoro-fluoridate on a slow shaker at 4°C for 10 min. The protein concentration in

the cell lysates was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). The same procedure was followed in case of the MN9D cells, with the difference that $MgCl_2$-labeling medium supplemented with 25 μCi/mL [^{35}S] and butyric acid was added to differentiated cells. In case of both LUHMES and MN9D cells, the substances indicated in the standard differentiation protocols that contained sulfate salts (for example GDNF, antibiotics) were omitted when the $MgCl_2$-labeling medium with [^{35}S] sulfate was carried out.

Isolation of radiolabeled PGs/GAGs. The procedures have been described in detail previously (Svensson and Mani, 2009). [^{35}S] Sulfate labeled polyanionic macromolecules were isolated from the culture medium and cell extracts by ion exchange chromatography on DEAE-cellulose. Samples were passed over a 0.4 mL-columns of DEAE equilibrated with 6 M urea, 0.5 M NaOAc, pH 5.8, 5 µg/mL ovalbumin, 0.1% Triton X-100. After sample application, the columns were washed successively with (a) equilibration buffer, (10 mL), (b) 6 M urea, 10 mM Tris, pH 8.0, 5 µg/mL ovalbumin, 0.1% Triton X-100 (10 mL) and (c) 50 mM Tris, pH 7.5 (20 mL). Bound material was eluted with 3 x 0.5 mL of 4 M guanidine-HCl, 50 mM NaOAc, pH 5.8. The eluted material were pooled and precipitated with 5 volumes of 95% ethanol overnight at -20°C using 100 µg of dextran as carrier. After centrifugation at 3200 RCF and 4°C for 30 min, the precipitated material was dissolved in 4 M guanidine–HCl, 50 mM NaOAc, 0.2% Triton X-100, pH 5.8 and subjected to gel permeation FPLC on Superose 6. Radioactivity was determined in a β-counter.

Enzymatic digestions. HS lyase (alias heparinase III or heparitinase, Seikegaku Corporation, Tokyo, Japan) digestion was performed in 10 mM HEPES, 3 mM $Ca(OAc)_2$, 10 mMEDTA, and 0.1% (v/v) Triton X-100 (pH 7.0) using 10 milliunits of enzyme per mL, at 37°C overnight. To monitor the reaction progress, 10 mg/mL of heparin sulfate 4 was added to the samples and the absorbance (232 nm) was measured before and 24 h after the enzymatic treatment started. Chondroitine ABC lyase digestion was performed in 0.1 M Tris-HCl, 10 mM EDTA, pH 5.8 using 10 milliunits of enzyme per mL at 37°C over night. To monitor the reaction progress, 2 mg of chondroitin sulfate 4 was added to the samples and the absorbance (232 nm) was measured before and 24 h after the enzymatic treatment initiation. Alkali treatment was performed by addition of 0.5 M NaOH, 0.05 M $NaBH_4$ at room temperature overnight. After digestions, 8 M guanidine was added to the samples that were further analyzed by gel-permeation chromatography on Superose 6.

RNA preparation and hybridization with microarrays. For microarray and PCR experiments, RNA was extracted from undifferentiated (n=4) and differentiated (n=4) LUHMES cells with the RNeasy mini Kit (Qiagen Inc.) and the purity and quality was verified with Nanodrop. The reverse transcription and production of cRNA was performed with GeneChip® 3 IVT Express Labeling Assay. Eight Microarray chips Glyco_v4a520670F were kindly gifted from the Consortium for Functional Glycomics, The Scripps Research Institute, La Jolla, CA.

Microarray results analysis. Basic Affymetrix Chip Analysis and Experimental Quality Analysis, as well as probe summarization and data normalization, were performed using the Expression Console Software v1.1.2 (Affymetrix Inc). We used the Robust Multi-array Average (RMA) analysis (Irizarry et al., 2003) for summarization and normalization of the microarray data. Further, to identify the significantly differentially expressed genes, we employed Significance Analysis of Microarrays (SAM) (Tusher et al., 2001)(TMEV v4.0 software). Clustering was carried out using HCA (Hierarchical Clustering Analysis /TMEV). Gene annotation information was extracted from UCSC Human Genome Browser Data: Human Build HG19 and Mouse Build MM9 Databases. The raw data from the microarray experiment will be submitted online, and will be available at Consortium for Functional Glycomics website. The RNA quality control and amplification, as well as microarray data analyses were performed at SCIBLU Genomics and Affimetrix Unit at Lund University (ID C00081), Lund, Sweden.

Reverse transcription and quantitative PCR. For reverse transcriptase (RT) and quantitative real-time PCR (qPCR) analysis, RNA was extracted with the RNeasy mini Kit

(Qiagen) and then from equal amounts of total RNA, we synthesized cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, 170-8891). For assessment of the optimal annealing temperatures, primer concentrations and the final qPCR reactions, we have performed the qPCRs with the primers targeting neurocan (FW 5'-CGG GCT GCA ATT TGA GAA CTG GC-3'; RV 5'-TGA GGC ATT CTC CAC TGC CGGA-3'), HS3ST5 (FW 5'GCG CCC TGC AGT TTA AGCGT-3'; RV 5'-TTC AAG CAG GGC CCT TGT GCCT-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, FW 5'-AAA CCC ATC ACC ATC TTC CAG-3', RV 5'-AGG GGC CAT CCA CAG TCT TCT-3') sequences. For those steps, we used sSoAdvanced™ SYBRR® Green Supermix (Bio-Rad, 1725260) and the thermocycler C1000 Touch CFX96 Real-Time System (Bio-Rad) and analyzed the results with Bio-Rad iCycler software. The C_q values were determined for each gene and the gene expression levels (corrected for primer efficiency) were compared to GAPDH. The melting curves analyses indicated the primers specificity.

Immunohistochemical stainings. For staining of neurocan and HS3ST5 in LUHMES cells, cells were fixed with 4% PFA for 20 min at room temperature and then the following steps were performed: triple rinse with PBST (PBS with 0.3% Triton X-100), 1 h blocking with 5% normal donkey serum in PBST at room temperature, incubation with primary antibodies – anti-neurocan (1:50, ab31979) or anti-HS3ST5 (1:200, HPA021823, Sigma) - in PBST over night at 4^{o}C, triple rinse with PBST, incubation with secondary antibodies DyLight™488 (715-485-151) and Cy3 (711-165-152, Jackson ImmunoResearch Inc., USA) (respectively) in PBST for 2 h at room temperature, 10 min incubation with 4',6-diamidino-2-phenylindole (DAPI), (1:500, Sigma) in PBST, triple PBST rinse, covering with a layer of DABCO medium (Sigma-Aldrich). The stainings were evaluated with the confocal microscope LSM 150 (Zeiss), objective Plan Apochromat 40x/1.3.

RESULTS

Dopaminergic neurons produce and release sulfated PGs/GAGs upon differentiation

To investigate the level of PG and GAG production in undifferentiated and differentiated dopaminergic neurones, we employed human and mouse dopaminergic neuronal cell lines, LUHMES and MN9D cells, derived from embryonic mesencephalon of human (Lotharius et al., 2005) or mouse (Choi et al., 1991). The LUHMES cells (Fig. 1A) express neuroblast and stem cell markers and possess neuronal characteristics. Upon differentiation, these cells develop an extensive neurite network (Fig. 1A), reveal spontaneous electrical activity, and express most midbrain dopaminergic markers (Schildknecht et al., 2009, Scholz et al., 2011).

To determine the level of PG and GAG production we performed radiolabeling with [^{35}S] in undifferentiated and differentiated cells. Then, using ion exchange chromatography on DEAE cellulose followed by gel permeation chromatography on Superpose 6, we isolated the polyanionic macromolecules secreted to the culture medium and accumulated in the cells. We found that both undifferentiated and differentiated human and mouse dopaminergic cells expressed [^{35}S]-labeled macromolecules (Fig. 1 C and E). Interestingly, in case of both human and mouse dopaminergic neurons, the differentiated cells expressed and secreted higher amounts of [^{35}S]-labeled material compared to undifferentiated cells (Fig.1 B-C and D-E). Moreover, in both cell types, the amounts of secreted PGs and GAGs were higher (Fig. 1 B, D) than that accumulated on or inside the cells (Fig. 1 C, E). In order to determine the presence of PGs, the portion of large sized material (fractions 7-14) was subjected to alkali treatment followed by gel permeation chromatography on Superpose 6. The size of this material was reduced by alkali treatment (data not shown), indicating that they were PGs. In order to determine the composition of the GAG chains produced by differentiated LUHMES cells, the large sized (fractions 7-14) and medium sized (fractions 15-25) material were subjected to digestion with heparitinase and chonrdroitinase ABC

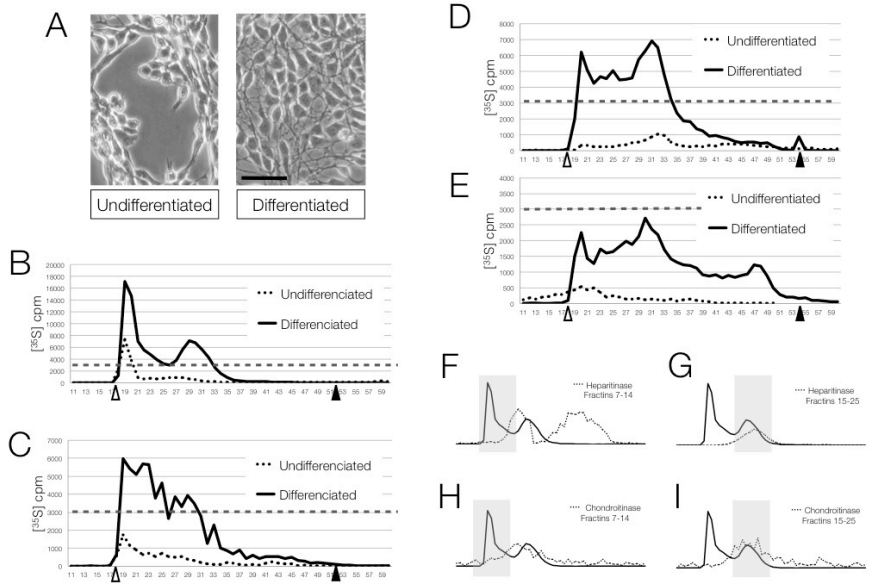

Figure 1. Dopaminergic neurons produce and release sulfated GAGs upon differentiation. Bright field images showing human mesencephalon dopaminergic cells (LUHMES) in undifferentiated and differentiated state (A). The charts show results of gel chromatography on Superose 6 of products present in conditioned media (B and D) and cell extracts (C and E) of undifferentiated (dashed line) and differentiated (solid line) LUHMES cells (B and C) and MN9D cells (D and E). In F-I, the charts show ^{35}S-labeled products isolated from conditioned medium of differentiated LUHMES cells (B, solid line), before (solid line) and after digestion (dashed line) with heparitinase (F-G) or chondroitinase ABC (H-I). The small sized and medium sized products from the undigested material were pooled, as highlighted in gray, and subjected to different digestions, as indicated in the charts and re-chromatographed on Superose 6. The scale bar in A equals 50 µm. In B-E, the cpm values were corrected for protein concentration and [^{35}S] sulfate concentration; as a reference, the horizontal dotted lines indicate [^{35}S] cpm=3000. V_0, void volume (empty arrow head); V_t, total volume (full arrowhead).

followed by gel permeation chromatography on Superpose 6 (Fig. 1 F-I). The large sized material was sensitive to both heparitinase and chondroitinase ABC treatment indicating composition of both types of GAG chains (Fig. 1 F and H). Digestions of material in fractions 15-25, which presumably contained free sulfated GAG chains, revealed that the fractions were enriched in both HS and CS species, but to a lesser extent CS (Fig. 1 G and I). In addition, the radioactivity signal shifted only slightly in case of heparitinase digestion (Fig. 1G), indicating that the fractions contained HS with sequences different than ones recognized by heparitinase i.e. highly modified HS.

These results suggest that human and mouse dopaminergic neurons increase the production and secretion of sulfated PGs and GAGs upon differentiation. In the human midbrain dopaminergic neurons, both CS and HS GAG-species decorate the core proteins released to the medium.

Differentially expressed glyco-genes in differentiated and undifferentiated dopaminergic neurons

To further study the difference in PG and GAG expression between undifferentiated and differentiated dopaminergic neurons, we employed the focused affymetrix-based microarrays containing the selection of so-called glyco-genes, the genes related to the cellular glycome. The microarray contained human and mouse highly annotated genes coding for proteins responsible for glycan synthesis and glycan binding, including enzymes relating to PG/GAG synthesis, metabolism and recognition.

First, we compared the overall differences between the expressed glyco-genes in RNA from undifferentiated and differentiated LUHMES cells. According to clustering

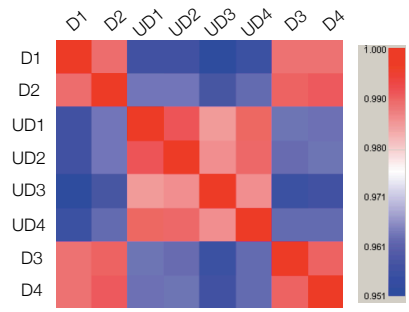

Figure 2. Overall difference in PG-related gene expression between undifferentiated and differentiated LUHMES cells. The image presents Pearson's correlation values in a form of heat-diagram, where red shades reflect high, and blue shades, low similarities between samples. 'UD1-4' – undifferentiated LUHMES, 'D1-4' – differentiated LUHMES.

algorithm, the most similarly expressed genes belonged to replicates of the same type (differentiated: D1, D2, D3, D4, or undifferentiated: UD1, UD2, UD3, UD4) (Fig. 2). The microarray experiment showed that 10374 unique transcripts were significantly altered between the undifferentiated and differentiated samples. 590 of those unique transcripts (5.68%) had false discovery rate zero (FDR=0) and further analysis was focused only on those transcripts. For each gene we averaged the fold change (FC) value from all transcripts. We presented here the genes for which expression was on average 5 fold higher (22 genes) or 5 fold lower (7 genes) between the differentiated and undifferentiated LUHMES cells (Table 1 and Table 2, respectively). Additionally, using the GoSTAT on-line software (Beissbarth and Speed, 2004), we compared the statistically overrepresented gene ontology (GO) terms between the top up-/down-regulated gene groups presented in Table 1 and 2 (Table 3). The GO terms related to location were overrepresented in the set of genes upregulated in both gene sets, from differentiated and undifferentiated LUHMES cells. However only in the maturing dopaminergic neurons, the GO terms related to metabolism of glycoproteins, oligosaccharides, proteins and carbohydrates were overrepresented (Table 3).

Together, these data indicate that during the differentiation of dopaminergic neurons, the transcriptional programs which lead to enhancement of production and processing of glycosylated and glycanated molecules become activated.

Differentially transcribed PG- and GAG-related genes in differentiating dopaminergic neurons

Previous studies have shown that GAGs and PGs may influence the survival and neurite growth of nigral dopaminergic neurons (Lafont et al., 1992, Gates et al., 1996, Charvet et al., 1998a, b, Mendes et al., 2003, Gama et al., 2006) and neuronal differentiation of stem cells (Mercier et al., 2002, Johnson et al., 2007, Sasaki et al., 2009, Kraushaar et al., 2010, Pickford et al., 2011, Mercier and Arikawa-Hirasawa, 2012). Here, we performed a biased analysis of microarray results, and aimed to establish the directions and degree of transcriptional changes in genes encoding enzymes involved in production and modification of PGs – including the core proteins and GAGs – upon dopaminergic differentiation (Table 4).

Both CSPG and HSPG genes including neurocan (CSPG), leprecan (CSPG) and glypican 5 (HSPG) were upregulated by the differentiation. Also, the galactosyltransferase genes involved in the formation of linkage region of the GAGs, B3GAALT2 and B3GAT1, were overrepresented in differentiated dopaminergic neurons. Interestingly, this balance was not maintained when the HS or CS/DS specific enzyme expression was evaluated. While only one gene involved in CS polymerisation (CSGALNACT1) was differentially regulated, six different transcripts of various HS-specific sulfotransferases (HS3ST5, HS3ST1, HS3ST2, HS6ST1, NDST3, SULF2) were overexpressed upon differentiation. At the same time, genes of some specific isoforms of sulfotransferases (HS6ST2, HS2ST1, HS3STSA1, HS3STSB1) were downregulated upon differentiation. Relatively lowered expression of glypican 4 (HSPG), syndecan 1 (CS/HSPG) and HSPG 2 (also known as perlecan) were observed in differentiated vs. undifferentiated conditions.

The data implies that the quantitative transcriptional changes of genes encoding core proteins and enzymes involved in the sulfation-code imprinting of their GAGs might have influenced the process of dopaminergic differentiation by regulating their binding to interaction partners.

Gene Symbol	Gene Name	Category	FC (Average)
NCAN	Neurocan	CBP:C-Type Lectin	47.91
HS3ST5	Heparan sulfate (glucosamine) 3-O-sulfotransferase 5	Glycan-transferase	38.79
HS3ST1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	Glycan-transferase	27.53
NCAM1	Neural cell adhesion molecule 1	CBP:I-Type Lectin	19.70
JAG1	Jagged 1	Notch pathway	12.07
B3GALT2	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	Glycan-transferase	11.83
CXCR4	Chemokine (C-X-C motif) receptor 4	Chemokine	10.50
CSGALNACT1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	Glycan-transferase	10.34
CHGA	Chromogranin A (parathyroid secretory protein 1)	Miscellaneous	8.38
FGF18	Fibroblast growth factor 18	Growth Factors & Receptors	8.26
SPOCK1	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	Proteoglycan	8.10
SPOCK2	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	Proteoglycan	7.14
BMPR2	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	Growth Factors & Receptors	6.48
B3GAT1	Beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)	Glycan-transferase	6.47
ST8SIA3	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 3	Glycan-transferase	6.35
SIGLEC11	Sialic acid binding Ig-like lectin 11	CBP:I-Type lectin	6.30
ST6GALNAC5	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5	Glycan-transferase	6.03
GAL3ST4	Galactose-3-O-sulfotransferase 4	Glycan-transferase	5.99
GPC5	Glypican 5	Proteoglycan	5.70
HS3ST2	Heparan sulfate (glucosamine) 3-O-sulfotransferase 2	Glycan-transferase	5.63
IDS	Iduronate 2-sulfatase	Glycan Degradation	5.34
NRG1	Neuregulin 1	Growth Factors & Receptors	5.30

Table 1. Genes relatively upregulated in differentiating LUHMES cells. The microarray experiment results presented as a list of genes in which expression was significantly upregulated in differentiated vs. undifferentiated LUHMES cells (average fold change [FC] > 5). The columns represent (from left): gene symbol, gene name, category and average FC.

Validation of neurocan and HS3ST5 mRNA and protein overexpression

In the next step, we decided to validate the expression of the most robustly up-regulated genes, neurocan and HS3ST5 in LUHMES cells (Table 1, Table 4). At the transcriptional level, qPCR analysis showed that neurocan mRNA was upregulated 25000 times in differentiated vs. undifferentiated LUHMES cells (Fig. 3 A), which was a much higher value than indicateby the microarray experiment (average FC=47.91). Using immunohistochemical methods, we demonstrated that the neurocan protein was also increased in the differentiated dopaminergic neurons (Fig. 3 B), compared to undifferentiated neuroblasts (Fig. 3 C) or the negative control (Fig. 3 D).

Consistent with the microarray analysis, the mRNA of the enzyme HS3ST5 was also overrepresented in the differentiated dopaminergic neurons when compared to undifferentiated cells. The qPCR analysis showed a 3-fold increase (Fig. 3 E), which was of a smaller amplitude than the 38.79 increase of average fold change measured in the high throughput microchip experiment. Similarly, at the protein level, the HS3ST5 enzyme increased in the differentiated neurons (Fig. 3 F) compared to undifferentiated cells and control stainings (Fig. 3 G, H).

Taken together, these results indicate that expression of neurocan and HS3ST5 genes is upregulated upon dopaminergic differentiation. Moreover, the differences in

Gene Symbol	Gene Name	Category	FC (Average)
HS3ST3A1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1	Glycan-transferase	0.05
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	Growth Factors & Receptors	0.06
CD302	CD302 molecule	CBP:C-Type Lectin	0.12
HS3ST3B1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	Glycan-transferase	0.16
HS6ST2	Heparan sulfate 6-O-sulfotransferase 2	Glycan-transferase	0.17
GALNTL4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 4	Glycan-transferase	0.18
FUT9	Fucosyltransferase 9 (alpha (1,3) fucosyltransferase)	Glycan-transferase	0.20

Table 2. Genes relatively downregulated in differentiating LUHMES cells. The microarray experiment results presented as a list of genes in which expression was significantly diminished in differentiated vs. undifferentiated LUHMES cells (average fold change (FC) ≤0.2). The columns represent (from left): gene symbol, gene name, category and average FC.

RNA expression are also extended to the functional level, as the differentiated dopaminergic neurons produce more NCAM and HS3ST5 proteins than undifferentiated LUHMES cells.

DISCUSSION

The roles of different GAG species in developing midbrain dopaminergic neurons *in vivo* and *in vitro* have previously been investigated. *In vitro* studies employing various rodent primary culture models suggest that polarization, adhesion and neurite growth of developing midbrain dopaminergic neurons can be influenced by different GAGs (Lafont et al., 1992, Gates et al., 1996, Mendes et al., 2003). Moreover, is has been speculated that GAGs of CS-4 and KS type may navigate axonal pathfinding of nigrostriatal neurons during the development and stabilize the organization of the striatosomes in the adult brain (Charvet et al., 1998a, b). Here, we used the LUHMES cells to model differentiation of human midbrain dopaminergic neurons. LUHMES cells have been subcloned from the MesC2.10 cell line (Lotharius et al., 2005), which originate from the human embryonic midbrain.

GO terms: Undifferentiated	GO terms: Differentiated
	Golgi apparatus part (1.0×10^{-6})
Transferase activity (3.4×10^{-4})	Golgi apparatus (1.0×10^{-6})
Golgi apparatus (3.4×10^{-4})	Golgi membrane (1.0×10^{-5})
Integral to membrane (3.5×10^{-4})	Glycoprotein metabolic process (1.7×10^{-5})
Intrinsic to membrane (3.5×10^{-4})	Oligosaccharide metabolic process (2.7×10^{-5})
Membrane part (7.4×10^{-4})	Integral to membrane (2.8×10^{-5})
Golgi membrane (8.1×10^{-4})	Intrinsic to membrane (2.8×10^{-5})
Golgi apparatus part (10.6×10^{-4})	Membrane part (1.4×10^{-4})
Membrane (23.4×10^{-4})	Proteoglycan biosynthetic process (1.4×10^{-4})
	Carbohydrate metabolic process (1.4×10^{-4})

Table 3. The overrepresented gene onthology (GO) terms in groups of genes differentially regulated upon dopaminergic differentiation of LUHMES cells. The left column includes GO terms relating to genes relatively upregulated in undifferentiated LUHMES cells (another words: relatively downregulated upon differentiation). In the right column the GO terms are listed which relate to genes upregulated in differentiated vs. undifferentiated LUHMES cells. The analysis included genes listed in Tables 1 and 2 - at least 5 times up-/down-regulated (average fold change) in LUHMES cells upon differentiation. Up to 10 best GO terms are shown, with at least 2 genes per GO. *P*-values for each GO are indicated in parentheses, goa_human GO database, minimal length of considered GO path: 3, correction for multiple testing: Benjamini correction.

	Average fold change	Link enzymes	HS Enzymes	CS/DS enzymes	PGs core proteins
Genes up-regulated in differentiated cells	≥30		HS3ST5		NCAN
	10-30	B3GALT2	HS3ST1	CSGALNACT1	
	3-10	B3GAT1	HS3ST2 HS6ST1 NDST3 SULF2		Leprecan Glypican 5
Genes down-regulated in differentiated cells	≥10		HS6ST2 HS2ST1		
	3-10		HS3ST3B1 HS3ST3A1		
	2-3		SULF1		Glypican 4 Syndecan 1 HSPG 2

Table 4. PG- and GAG-related genes up- and down-regulated upon LUHMES cells differentiation The table includes differentially regulated genes of enzymes involved in synthesis and modification of PGs/GAGs and genes of core proteins. In the upper part of the table, the genes are listed, in which average fold change was ≥30, 10-30 and 3-10 times higher in differentiated vs. undifferentiated LUHMES cells. The lower part of the table contains genes, which were on average ≥10, 3-10 and 2-3 times downregulated upon differentiation of LUHMES (average fold change). B3GAT1 (Beta-1,3-glucuronyltransferase 1 or glucuronosyltransferase P), CSGALNACT1 (Chondroitin sulfate N-acetylgalactosaminyltransferase 1), HS3ST5 (Heparan sulfate [glucosamine] 3-O-sulfotransferase 5), HS3ST2 (Heparan sulfate [glucosamine] 3-O-sulfotransferase 2), HS6ST1 (Heparan sulfate 6-O-sulfotransferase 1), HSPG2 (Heparan sulfate proteoglycan 2), HS6ST2 (Heparan sulfate 6-O-sulfotransferase 2), HS2ST1 (Heparan sulfate 2-O-sulfotransferase 1), HS3ST3B1 (Heparan sulfate [glucosamine] 3-O-sulfotransferase 3B1, HS3ST3A1 (Heparan sulfate [glucosamine] 3-O-sulfotransferase 3A1), NDST3 (N-deacetylase/N-sulfotransferase [heparan glucosaminyl] 3), NCAN (Neurocan), SULF1 (Sulfatase 1), SULF2 (Sulfatase 2), B3GALT2 (UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2).

This cell line was immortalized with *c-myc* gene (Lotharius et al., 2002). At the undifferentiated state, LUHMES cells bear features of neurepithelial neuroblasts, expressing *Sox2*, *Pax2* and *Otx2* (Scholz et al., 2011). Switching off *c-myc* with tetracycline induces exit from mitosis to differentiation. During the first 5-6 days of differentiation, the midbrain (A9) dopaminergic phenotype is acquired (Scholz et al., 2011). Expression of *Nurr1*, *Pitx3* and *TH* transcripts, as well as genes involved in dopamine storage and release: *DRD2*, *DAT*, *VMAT-2*, *AADC* are enhanced then. Moreover, the specific A9 substantia nigra dopaminergic phenotype is also acquired during that period, as expression of *Girk2* (G-coupled inward potassium channel) gene - *KCNJ6* - becomes augmented at that time (Scholz et al., 2011). Almost all differentiated LUHEMS cells produce tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis (and a common marker of dopaminergic phenotype) (Schildknecht et al., 2009). Up to 8-11 days of differentiation, the expression of dopamine phenotype related genes is stable while the cells continue to acquire neuronal electrical features (Scholz et al., 2011).

In order to study the genes related to GAG/PGs production which are involved in acquiring of the dopaminergic phenotype, we have compared cells from the 'day 0' and 'day 5' time points in the differentiation protocol. Metabolic radiolabeling of PGs/GAGs showed increased expression and secretion of sulfated PGs upon differentiation. The secreted PGs were decorated with both HS and CS/DS. These results were further confirmed in the experiment involving microarrays. Gene ontology-based analysis revealed that upon differentiation, cellular mechanisms engaged in metabolism of glycoproteins, proteoglycans, and processing of the

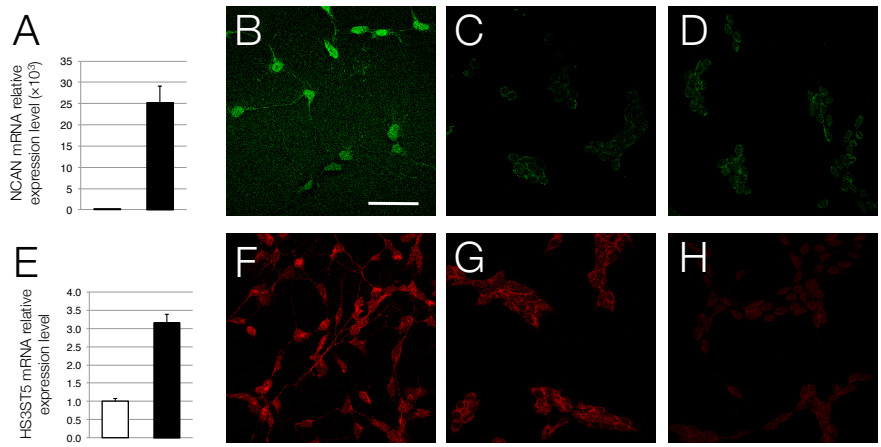

Figure 3. Upregulation of NCAN and HS3ST5 (mRNA and proteins) upon differentiation. The figure depicts mRNA relative levels (A, E) and immunohistochemical stainings of LUHMES cells before and after differentiation (B-D, F-H) in case of two genes most highly upregulated upon differentiation: NCAN (neurocan, A-D) and HS3ST5 (Heparan sulfate [glucosamine] 3-O-sulfotransferase 5, E-H). The relative expression of neurocan (A) and HS3ST5 (E) was upregulated in differentiated LUHMES (black) when compared to undifferentiated (white). In case of both proteins, much stronger staining was observed after differentiation (B, F), than before (C, G). Labeling in undifferentiated cells (C, G) had similar intensity as control staining of neurocan and HS3ST5 protein (the secondary antibody only, D and H). Average ±S.E.M., n=4 (A, E), scale bar equals 100 μm (B-D, F-H.

substrates involved in their synthesis were strongly increased. Moreover, detailed study of glyco-gene microarray results have shown that transcription of genes specific for both, to HS- and CS-decorated core proteins was augmented when cells were differentiated.

To find out which specific type of PG/GAG maturation might intrinsically stimulate dopaminergic differentiation process *in vitro*, we employed a high throughput analysis of glyco-gene transcriptomes (Smith et al., 2005, Comelli et al., 2006, Guan et al., 2010) before and during dopaminergic differentiation of LUMES cells. We found that in differentiating LUHMES cells, more PG/GAG-related genes were upregulated than downregulated (12 vs. 8 genes with average FC>2). Moreover, the degree of changes was higher in case of transcripts that were overexpressed during differentiation than in transcripts overexpressed in neuroblast stage (fold change up to 48 and 20, respectively). This observation was in agreement with the previous experiment involving radiolabeling of sulfated GAGs. The transcription of both CSPG and HSPG PGs was significantly changed during the dopaminergic differentiation. Although the maturing cells overproduced genes of certain core proteins, belonging to both HSPG and CSPG, this effect was not extended to transcription of GAG chain synthesis enzymes. Intriguingly, mRNA expression of different isoforms of all kinds of sulfotransferases engaged in HS synthesis was upregulated, and in the same time only one CS-specific enzyme was found to be enriched upon differentiation. In response to dopaminergic differentiation, the most strongly upregulated genes were CSPG, neurocan and HS 3-*O*-sulfotransfetase, HS3ST5. The qPCR results confirmed the microarray results that the given gene was overexpressed in the differentiated dopaminergic neurons, relative to the undifferentiated, although the magnitude of the differences detected by the methods varied. Such variation has been previously reported (Ton et al., 2002, Koria et al., 2003, Smith et al., 2005) and it is perhaps related to a different dynamic range and normalization methods in those two techniques. Importantly, at the protein level we could also confirm the neurocan and HS3ST5 differential expression upon LUHMES cells differentiation. Neurocan is a CSPG found exclusively in the CNS and its spatiotemporal expression has shown to be

increased in the developing rat brain but diminished postnatally (Meyer-Puttlitz et al., 1995, Engel et al., 1996). During development, neurocan may regulate the axonal growth in rodents by binding to different cell adhesion molecules, for example N-CAM, Ng-CAM/L1 (Grumet et al., 1993, Friedlander et al., 1994), tenascin-C (Grumet et al., 1994), axonin-1 (Milev et al., 1996), and pleiotropin (Rauvala and Peng, 1997). In adult rat brain, neurocan and versican, as components of a glial scar, repressed the re-growth of nigrostraital fibers, as revealed by application of chondroitinase ABC or hyaluronidase treatments (Moon et al., 2001, 2002, Moon et al., 2003). In various *in vitro* models, including embryonic midbrain neurons (Mace et al., 2002), neurocan appeared to be inhibitory for neuronal attachment and neurite extension (Asher et al., 2000, Inatani et al., 2001, Ughrin et al., 2003). On the other hand, ESC differentiated to neuronal progenitors showed enhanced production of neurocan, versican, aggrecan and hyaluronan (Abaskharoun et al., 2010), suggesting rather a positive role of neurocan in cell survival or the maintenance of ectodermal/neuronal phenotype. Interestingly, in differentiated human midbrain dopaminergic neurons *in vitro*, the transcription and translation of neurocan, but not other ECM-associated molecules (including PGs), was highly upregulated. Whether or not enhanced neurocan production plays a role in the adhesion and neurite growth in our experimental setup remains to be elucidated. Nevertheless, it can be hypothesized that such a high increase of a single ECM-associated molecule may indicate its function as a specific factor promoting dopaminergic differentiation.

The enzymatic activity of the heparan sulfate 3-*O*-sulfotransferases family results in sulfation at the 3-OH position of a glucosamine residues of HS to form 3-*O*-sulfated HS. Currently 7 isoforms of 3-*O*-sulfotransferases are known and they are differentially expressed in human tissues (Shworak et al., 1999, Xia et al., 2002, Xu et al., 2005). HS3ST5 was cloned in 2002 (Xia et al., 2002) and its activity was recognized as being critical for creating antitrombin binding sequence – GlcNs3S±6S and also as gD-binding site for the herpes simplex virus 1 (HSV-1)(Xia et al., 2002). Early studies on HS3ST5 have shown the highest mRNA expression levels in the extracts from human CNS from developing brain, spinal cord and adult brain (Mochizuki et al., 2003). This suggests a CNS-specific role of the 3-*O*-sulfotransferase isoform 5. Moreover, another member of the HS 3-*O*-sulfotransferase family, HS3ST3, which also creates gD-binding site for HSV-1, has been suggested to be a modulator of the Notch signaling pathway in Drosophila (Kamimura et al., 2004), the pathway critical in the proper development and function of the nervous system.

Some previous evidence suggests a functional connection between neurocan and HS chains. The interactions of neurocan with heparin (Feng et al., 2000), as well as with heparin-binding factors like pleiotropin, amphoterin (Milev et al., 1998a) and FGF-2 (Milev et al., 1998b) have been reported. It has been postulated that those heparin-binding factors are mediators in interaction between CS chains of neurocan and HSPGs (Milev et al., 1998a, Milev et al., 1998b). Moreover, it was proven that the neurite outgrowth of mouse cerebral granule cells *in vitro* is highly enhanced when the cells grow on dishes coated with C-terminal fragment of neurocan (Akita et al., 2004). Further, based on the experiments involving heparitinase I and chondroitinase ABC treatments, the authors proposed that HSPGs syndecan-3 and glypican-1 expressed by those cells can bind neurocan substrate and that the core proteins, but not CS chains of neurocan are the mediators of this interaction (Akita et al., 2004). The HS-decorated PGs have pivotal roles in support of signaling of FGFs, Wnts, TGF-beta, SHH and pleiotropin in the developing brain (Bespalov et al., 2011). Also, the spatiotemporal modulation of these pathways is critical in the midbrain dopaminergic phenotype specification and axonal growth (Smidt and Burbach, 2007). Different modulators of Wnt/beta-catenin, Notch and SHH- signaling pathways have been successfully employed to enhance the dopaminergic differentiation of ESC and iPSC (Ding et al., 2011, Esfandiari et al., 2012). It could be therefore hypothesized that expression of HS3ST5, perhaps orchestrated with other differentially expressed sulfotransferases indicated in this study (HS3ST1, HS3ST3A1 and HS3ST3B1), could modulate binding of growth factors, and ultimately drive the dopaminergic differentiation. Taken together, we have shown here that production of sulfated PG/GAGs is a

phenomenon associated with differentiation of human midbrain neuroblasts into the mature dopaminergic neurons. It is likely that specific expression of the CS/HSPGs, and perhaps more importantly, synthesis of the enzymes specifically regulating the HS sulfation codes (f.ex. HS3ST5) enables unique interactions of PGs with extracellular space components, modulating the differentiation of dopaminergic neurons. Our results provide an excellent starting point to identify PGs/GAG and their structural changes that are influencing the differentiation of midbrain dopaminergic neurons. New knowledge about this process is needed for better understanding of the midbrain neurons maturation process and, ultimately, for improving the cell replacement therapies for diseases affecting those neurons, such as Parkinson's disease. Currently we are working on further verification of the microarray results. We are also planning to evaluate the effect of neurocan gene-silencing on dopaminergic differentiation in our experimental model.

ACKNOWLEDGEMENTS

We would like to acknowledge financial support from the Swedish Research Council, BAGADILICO - Excellence in Parkinson and Huntington Research, MultiPark network and FP7-MC-214003-2 (Marie Curie Initial Training Network AXREGEN). We would like to thank Ulrika Aili for help with some of the radiolabeling experiments. We would like to thank Dr. Marcel Leist for providing us the LUHMES cells and Dr. A. Heller for providing us with MN9D cells in the project. We would like to acknowledge Andy McCourt for the critical reading and linguistic revision of the manuscript. The authors have no conflict of interest to declare.

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Paper III

Roles of Nogo-A in developing and adult midbrain dopaminergic neurons

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Key words: substantia nigra pars compacta, neurite outgrowth, cell survival, knock-out, nigrostriatal pathway

Nogo-A is a transmembrane protein originally discovered in myelin, produced by postnatal central nervous system (CNS) oligodendrocytes. Nogo-A induces growth cone collapse and inhibition of axonal growth in the injured adult CNS. In the intact CNS Nogo-A functions as a negative regulator of growth and plasticity. Nogo-A is also expressed by certain neurons. Neuronal Nogo-A depresses long term potentiation (LTP) in the hippocampus and modulates neurite adhesion and fasciculation during development in mice. Here we show that Nogo-A is present in neurons derived from human midbrain (LUHMES cell line), as well as in embryonic and postnatal mouse midbrain dopaminergic neurons. In LUHMES cells, Nogo-A was upregulated 3-fold upon differentiation and neurite growth. Cultured midbrain dopaminergic neurons from Nogo-A knock-out, but not the anti-Nogo-A treated wild type mice, exhibited decreased numbers of neurites and branches, compared with the wild type neurons. *In vivo*, neither the density of striatal tyrosine hydroxylase immunolabeling, nor the regenerative capacity of nigrostriatal neurons after partial 6-hydroxydopamine (6-OHDA) lesions were affected by Nogo-A deletion. However, two months after the lesion, we observed a trend where fewer dopaminergic neurons survived in the substantia nigra pars compacta of Nogo-A knock-out mice than in wild-type controls. These results indicate that during maturation of midbrain dopaminergic neurons, intracellular Nogo-A supports neurite growth initiation and neurite branch formation. In the adult, brain Nogo-A related mechanisms might protect nigral dopaminergic neurons against the deleterious toxic effect of 6-OHDA.

INTRODUCTION

Nogo-A is a transmembrane protein originally discovered in the adult central nervous system (CNS). Nogo-A belongs to the reticulon family – ubiquitous transmembrane proteins enriched in the endoplasmatic reticulum. At present, the biological functions of the majority of reticulons are unknown (Schwab, 2010). Nogo-A, in contrast to the shorter isoforms Nogo-B and –C or other reticulons, is highly enriched in the CNS. In the adult CNS Nogo-A is primarily expressed by oligodendrocytes and myelin and by subpopulations of neurons (Caroni & Schwab, 1988; Huber *et al.*, 2002; Wang *et al.*, 2002). The neurite growth inhibitory effects of Nogo-A are mediated by a receptor complex, which includes NgR-1, p75/TROY and Lingo1 as well as additional, yet uncharacterized components. The receptor(s) activates the RhoA/ROCK pathway (Fournier *et al.*, 2003; Yiu & He, 2006; Montani *et al.*, 2009), resulting in growth cone collapse and inhibition of axonal growth in injured, but also in intact adult CNS (Schwab, 2010).

In addition, Nogo-A is also expressed by subpopulations of neurons, particularly during development (Huber *et al.*, 2002; Mathis *et al.*, 2010; Petrinovic *et al.*, 2010). Migration of some types of neurons is restricted by Nogo-A expression (Mingorance-Le Meur *et al.*, 2007; Mathis *et al.*, 2010). Nogo-A function blocking during development causes aberrant growth of the peripheral neurites *in vitro* and nerves *in vivo* (Petrinovic *et al.*, 2010). Later in development, Nogo-A affects the plasticity of the visual cortex and other parts of CNS (Kapfhammer & Schwab, 1994; McGee *et al.*, 2005). Nogo-A/NgR-1 signaling also negatively regulates LTP at hippocampal synapses (Raiker *et al.*, 2010; Delekate *et al.*, 2011) as well as axonal and dendritic sprouting and plasticity (Zagrebelsky *et al.*, 2010).

ABBREVIATIONS: 6-OHDA (6-hydroxydopamine), CNS (central nervous system), DAB (3,3'-Diaminobenzidine), DAPI (4',6-diamidino-2-phenylindole), KO (knock-out), LTP (long term potentiation), LUHMES cells (Lund University Human Mesencephalon cells), PFA (paraformaldehyde), SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), TH (tyrosine hydroxylase), WT

Dopaminergic neurons located in the ventral midbrain are critical for cognitive and motor behaviour and are associated with multiple CNS disorders. Substantia nigra (A9) neurons have been extensively studied, as the slow degeneration of the nigrostriatal pathway is responsible for the motor defects observed in Parkinson's disease patients (Hornykiewicz, 1966; Riederer & Wuketich, 1976). The etiology of Parkinson's disease is 90-95% sporadic while 5-10% bear genetic mutations. Some studies showed evidence for polymorphisms and differential expression of genes involved in axonal growth in patients with Parkinson's disease (Lesnick *et al.*, 2007; Bossers *et al.*, 2009).

The roles of Nogo-A in developing and adult midbrain dopaminergic neurons have not been yet addressed. In this study we have first assessed, whether Nogo-A is present in the prenatal and postnatal nigral dopaminergic neurons. Second, we studied wild type and Nogo-A knock-out mice and employed *in vitro* and *in vivo* models to establish whether constitutive deletion of Nogo-A or application of anti-Nogo-A has an impact on survival and neurite outgrowth in midbrain dopaminergic neurons.

MATERIALS AND METHODS

LUHMES and MN9D cell cultures. The Lund human mesencephalic (LUHMES) cell line was kindly provided by Dr. Marcel Leist (Konstanz, Germany). Originally, the LUHMES cell line was obtained from a subclone of MesC2.10, a conditionally immortalized, non-transformed cell line derived from human ventral mesencephalon embryonic tissue (Lotharius *et al.*, 2002). LUHMES cell maintenance and differentiation were performed as previously described (Scholz *et al.*, 2011). Briefly, for LUHMES maintenance and differentiation plastic 6-well plates (Nunc™, Danmark) or glass Nunc™ Lab-Tek™ chambered coverglass slides (Thermo Scientific, USA) pre-coated with 50 µg/mL poly-L-ornithine and 1 µg/mL fibronectin (Sigma-Aldrich) were used. Proliferation medium consisted of Advanced Dulbecco's modified Eagle's medium/F12 (Gibco), N-2 supplement (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich) and 40 ng/mL recombinant bFGF (R&D Systems). For differentiation, medium consisting of Advanced Dulbecco's modified Eagle's medium/F12, N-2 supplement, 2 mM L-glutamine, 1 mM dibutyryl cAMP (Sigma-Aldrich), 1 µg/mL tetracycline (Sigma-Aldrich) and 2 ng/mL recombinant human GDNF (R&D Systems) was used. For enzymatic detachment and dissociation LUHMES cells were incubated with ATV trypsin (138 mM NaCl, 5.4 mM KCl, 6.9 mM $NaHCO_3$, 5.6 mM d-Glucose, 0.54 mM EDTA, 0.5 g/L trypsin from bovine pancreas type-II-S; Sigma-Aldrich) for 3 min in 37 °C and then spun down in 300 RCF for 5 min. Differentiation of LUHMES cells had two steps: 24 h after plating, the differentiation was initiated by changing from proliferation to differentiation media. After additional 48 h we enzymatically detached and re-plated the cells to new dishes for 3 additional days of differentiation (medium was changed 48 h after re-plating). We plated 1.5×10^5 cells/cm^2 for imaging and 2.5×10^5 cells/cm^2 for protein harvest in the re-plating step. Undifferentiated LUHMES we seeded with density of 2.0×10^4 cells/cm^2 and proteins were harvested 2 days after.

The MN9D cell line was kindly provided by Dr. A. Heller (University of Chicago, Chicago, IL, USA). Originally, the MN9D cell line was obtained by hybrydoma fusion of cells from murine mesencephalic with neuroblastoma cells(Choi *et al.*, 1991). MN9D cells, when differentiated have properties of midbrain dopaminergic neurons, i.e. they express dopaminergic markers and release dopamine (Choi *et al.*, 1991; Choi *et al.*, 1992; Rick *et al.*, 2006). The cells were maintained as previously described (Choi *et al.*, 1991). Briefly, the cells were grown in DMEM/F12 medium with L-glutamine (Gibco), supplemented in 10% FCS (Gibco) and penicilin/streptomycin (100 U/mL/100 mg/mL, Gibco). For differentiation, the cells were seeded in density of 3×10^3 cells/cm^2 and after 24 hours the sodium butyrate (1 mM) was added and the cells were cultured for the next 7 days.

Nogo-A Western-blot analysis. We performed eight independent protein extractions from undifferentiated (n=4) and differentiated (n=4) LUHMES cells and extractions in duplicate for undifferentiated and differentiated MN9D cells. Cells were lysed in buffer composed of: 20 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40 and protease inhibitor cocktail (1:100, P8849, Sigma-Aldrich). Protein concentration was measured using Bradford reagent (Sigma). Following boiling in Laemli buffer, eight micrograms of total proteins ware loaded onto 8% SDS-PAGE gel. After the transfer onto Immuno-Blot® PVDF membranes (Bio-Rad, Sweden), proteins were blocked with 5% milk in 0.05% PBS-Tween-20 for 2 h at

room temperature. Next, the membrane was incubated with the primary antibody over-night at 4°C, in 3% milk and PBS-Tween-20. Following washing steps, horseradish peroxidase conjugated secondary antibody was applied onto the membrane for 1 h at room temperature. For visualization, we used the ECL Western blotting luminol reagent (sc-2048, Santa Cruz Biotechnology, Inc., Sweden). We used primary antibodies against Nogo-A (11C7, 1:5000) or GAPDH (1:5000, ab9485, Abcam, UK) and secondary HRP conjugated antibodies: anti-mouse HRP and anti-rabbit (P0447 and P0448, respectively 1:10000, Dako, Sweden).
11C7 is a monoclonal antibody raised against rat Nogo-A peptide sequence 623-640 (Oertle *et al.*, 2003). This antibody does not bind to Nogo-B and –C (Oertle *et al.*, 2003; Dodd *et al.*, 2005). 11C7 has been used previously to detect Nogo-A in mouse and human samples, both in Western-blot and in immunohistochemical stainings (Bandtlow *et al.*, 2004; Buss *et al.*, 2005; Montani *et al.*, 2009; Lackner *et al.*, 2011).
Although Nogo-A has estimated molecular weight of 139 kDa, it migrates as a protein of 220 kDa on SDS-PAGE gel (Chen *et al.*, 2000; Dodd *et al.*, 2005). For densitometric measurements of luminescent signal in LUHMES cells extracts, the ImageJ 1.42q software (Version 1.42q; National Institutes of Health, USA) was used. The densitometric measurements of Nogo-A and GAPDH (39 kDa) bands were diminished by value of background and then the averaged value from 'differentiated' samples was normalized to average value of 'undifferentiated' samples.

Nogo-A knock-out and wild type mice. We used Nogo-A $^{-/-}$ (knock-out) mice in 129X1/SvJ background (Dimou *et al.*, 2006) and 129X1/SvJ 000961 wild type controls (The Jackson Laboratory, USA). Nogo-A knock-out mice lack Nogo-A protein expression, but do express the –B and –C isoforms of Nogo. The detailed description of the construct has been published previously (Simonen *et al.*, 2003;(Dimou *et al.*, 2006)). The animals were housed under standard conditions with free access to food and water under standard 12-h light–dark regime (light 07.00–19.00 h). Experimental groups were composed of balanced numbers of male and female adult mice (12-13 weeks old). All procedures in this study were approved by the Ethical Committee for the use of laboratory animals at Lund University.

Mouse embryonic tissue preparation. The morning of plug detection in wild type or Nogo-A knock-out mice was considered as the embryonic day 0.5 (E 0.5). At the E13.5 the pregnant mice were killed by cervical dislocation and the embryos were immediately isolated and post-fixed (4% paraformaldehyde, PFA) over night at 4°C, and then transferred to 30% sucrose in PB at 4°C for cryoprotection over night. The whole embryos were immersed in O.C.T. (BDH Prolabo, VWR, Sweden) and the heads were cut on cryostat in 15 µm coronal sections. The sections were collected on Super Frost Ultra Plus® slides (Thermo Scientific, USA) and dried over night, then immuno-stained.

Ventral mesencephalon primary culture preparation. The procedure of mesencephalon primary culture preparation was performed as previously described (Pruszak *et al.*, 2009), with slight modifications. The procedure was performed aseptically in a timely manner and the embryos or the embryonic tissues were always kept on ice in sterile HBSS without Ca^{2+} and Mg^{2+} (Sigma). At E13.5 the pregnant wild type or Nogo-A knock-out mice were killed and the embryos were immediately aseptically isolated from the uterine horns.
After removing the uterine sac and amniotic membranes, embryos were moved to fresh HBSS solution and decapitated. The skull layer was removed from the brain and the rostral forebrain and caudal hindbrain regions were removed resulting with a remaining tube-like structure of midbrain. Cutting the mesencephalon tube along the dorsal midline, the flat butterfly-structure was obtained, which was then trimmed along the dorsal edges to remove 2/3 of dorsal tissue. Following the additional mechanical dissociation of ventral midbrain tissues pieces into small chunks (<1 mm), trypsin was applied to obtain a single cell suspension: 0.1% Trypsin (Gibco), 0.05% DNAze (Sigma), 20 min, 37°C. Tissues were then additionally dissociated by gentle pipetting and centrifuged (300 RCF, 5 min, 4°C), and finally dissolved in DMEM medium (Gibco) containing 5% of FCS (Gibco). 7.0×10^4 cells per cm^2 were plated on coated glass slides (0.01% Poly-L-Lysine and 5 µg/mL Fibronectin, Sigma) in 24-well plates and after 24 h the initial medium was replaced by serum-free medium: DMEM/F12 with Glutamax (Gibco), B27, 10 ng/mL of human recombinant GDNF (R&D Systems). Serum-free medium was changed every other day and after 6 days the cells were fixed (with 4% PFA, 20 min in room temperature) and after brief washing with PBS, kept in the fridge until staining was performed. In order to block receptor-mediated action of Nogo-A, in some cultures with wild

type cells, the antibody 11C7 was added (10 µg/mL) together with each serum-free medium change. 11C7 was raised against an 18-amino acid Nogo-A peptide corresponding to the rat sequence of amino acids 623-640 (Oertle *et al.*, 2003) and has been previously successfully applied to neutralize Nogo-A in mice (Montani *et al.*, 2009; Kilic *et al.*, 2010; Petrinovic *et al.*, 2010). Twelve independent ventral mesencephalon primary culture experiments - wild type (n=4), wild type with 11C7 (n=4) and Nogo-A knock-out mice (n=4) - were performed. Typically, in each experiment tissues from 5-7 embryos from one pregnant mouse were combined and the cells were plated in 4 wells. After 7 days *in vitro* the percentage of differentiated tyrosine hydroxylase-positive (TH-positive) neurons in wild type, 11C7-treated wild-type and Nogo-A knock-out cultures was similar, on average 8.4±8.7, 13.3±5.5 and 1.3±2.5 (±S.D.), respectively.

6-Hydroxydopamine (6-OHDA) injections. 6-OHDA (Sigma, Sweden) was injected into the right striatum under isoflurane anesthesia and analgesia (1.5-2% isoflurane in 1:2 of oxygen : nitrous oxide), using a stereotaxic mouse frame (Stoelting, Germany) and a 5 µL Hamilton syringe fitted with a fine glass capillary. The toxin was used at a concentration of 8 µg/µL, dissolved in a solution of 0.02% ascorbic acid in 0.9% sterile saline. A total volume of 1 µL was injected using the stereotaxic coordinates: A/P = +0.5 mm, M/L = –2.0 mm, D/V = –3.0 mm, with a flat skull position and all coordinates measured from bregma. Injections were made at a rate of 0.25 µL/15 second. We maintained the capillary in place for an additional 3 min to allow the toxin to diffuse. After removing the needle, the wound was cleaned and sutured and the mouse was injected with 75 µg of bupivacaine around the wound, to diminish pain. We did the injections being blinded for the phenotype. In the first group of animals that we investigated (4 weeks time point), three out of eight mice in Nogo-A knock-out group died unexpectedly 1, 4 and 7 days following the lesion. In following experiments, we took special care of animals (glucose injections 50 mg/mL and feeding with food extruded in 30% sucrose), successfully avoiding these unexpected dropouts in 2 other groups ('4 days' and '8 weeks').

Adult mouse tissue preparation. Mice were sacrificed 4 days, 4 weeks or 8 weeks after the 6-OHDA lesions. Animals were anaesthetized with an overdose of sodium pentobarbitone i.p. (Apoteket, Sweden) and then were transcardially perfused with 15 mL of room-temperature 0.9% saline, followed by 100 mL of ice-cold 4% paraformaldehyde in 0.1 M PBS. Brains were post-fixed over night at 4°C and then transferred to 30% sucrose in PB at 4°C for cryoprotection overnight. The brains were then sectioned in the coronal plane using a microtome at a thickness of 40 µm. Sections were collected in six series and stored at 4°C in 0.01% sodium azide in PBS until free-floating immunohistochemistry was performed.

Immunohistochemical fluorescent staining. For staining of Nogo-A in LUHMES cells, cells were fixed with 4% PFA for 20 min at room temperature and then the following steps were performed: triple rinse with PBST (PBS with 0.3% Triton X-100), 1 h blocking with 5% normal donkey serum in PBST at room temperature, incubation with primary antibodies – anti-Nogo-A (11C7, 1:2000) and anti-beta III-tubulin (1:200, T2200, Sigma) - in PBST over night at 4°C, triple rinse with PBST, incubation with secondary antibodies DyLight™488 (715-485-151) and Cy3 (711-165-152, Jackson ImmunoResearch Inc., USA) in PBST for 2 h at room temperature, 10 min incubation with 4',6-diamidino-2-phenylindole (DAPI), (1:1000, Sigma) in PBST, triple PBST rinse, covering with a layer of DABCO medium (Sigma-Aldrich). In case of staining of mouse tissues, both embryonic and adult, the unspecific binding of 11C7 (raised in mouse) to mouse antigens was avoided by application of 'Mouse on Mouse' solution (M.O.M.™BMK-2202, Vector Laboratories Inc., UK) before the blocking step. We used the primary antibodies anti-11C7 (1:1000) and anti-TH (1:500, P40101-0 Pel-Freez, USA) and followed the protocol described above. In the immunocytochemical staining of primary cultures derived from mouse embryonic ventral mesencephalon we used primary antibodies anti-TH and anti-beta-III tubulin (1:1000, TuJ1 Covance) and followed the staining protocol described above.

Immunohistochemical 3,3'-Diaminobenzidine or Diaminobenzidine (DAB) staining. Sections were rinsed in PBS and then endogenous peroxidase activity was quenched in 3% H_2O_2 and 10% methanol in PBS for 20 min. After rinsing steps, the sections were incubated in a blocking solution consisting of 5% normal goat serum in PBST and 0.3% Triton X-100 for 1 h, to block nonspecific binding sites. Sections were then incubated overnight at room temperature in the same blocking solution as described above with the primary antibody, rabbit anti-TH (1:1000, P40101-0, Pel-Freez). Then the sections were

incubated in blocking solution for 20 min before 1 h incubation in a 1:200 dilution of biotinylated secondary antibody (Vector Laboratories, UK), in blocking solution. The sections were then treated with avidin–biotin–peroxidase complex (ABC Elite kit; Vector Laboratories, UK) in PBS for 1 h. The color reaction was developed by incubation in DAB (Vector Laboratories) for 1 min. Sections were mounted on gelatin coated glass slides, dehydrated in an ascending series of alcohols, cleared in xylene and cover-slipped with DPX mounting medium (BDH Chemicals, VWR, Sweden).

Imaging. For the confocal imaging of immunolabeled LUHMES cells and mouse sections the LSM510 microscope was employed (Zeiss). The objective Plan Apochromat 40x/1.3 was used and then for higher magnification 2.5x zoom was applied (Fig. 1), or the objective Plan Apochromat 20x/0.8 was used, and then for higher magnification 5x zoom (Fig. 2 B-D, F-H) or 3.1x (Fig. 3) zoom was applied. We have chosen the representative z-planes for each highest magnification image. The dimensions of images are indicated in figure legends.
We used Eclipse 80*i* microscope (Nikon) to obtain bright field images of DAB staining (objectives 1x and 4x) and images of fluorescently labeled primary culture (10x). Randomly sampled images of triple-labeled ventral midbrain neurons *in vitro* were obtained (7-9 per well) and then analyzed by the Cellomics software.

Cellomics analysis. We employed vHigh Content Screening (Thermo Fisher Scientific, Cellomics, Pittsburgh, PA, USA) software to perform optimization and analysis of the neurite growth in ventral mesencephalon cells obtained from Nogo-A knock-out, wild type untreated and 11C7 treated embryos. Our protocol was optimized based on the NeuronalProfiling.V4 algorithm, assay version 6.0.0.4008. The thresholds were adjusted so that the program recognizes the majority of the stained cells together with their processes, despite minor differences in staining derived from some variety in each experimental repeat. By configuration of the assay parameters we have adjusted the following thresholds: for DAPI staining - nucleus area, total intensity, average intensity; for beta-III tubulin staining – cell body nucleus count, cell body total/average intensity, neurite length (22-400 μm); for tyrosine hydroxylase staining – total/average intensity. There were up to 1772 cells sampled per experiment, and on average around 400 beta-III tubulin positive neurons recognized per well (in the wild type, wildtype with 11C7 and Nogo-A knock-out cultures, respectively).

Densitometry. We captured images from the TH-immunostained sections using (Eclipse 80*i*) microscope (Nikon). In all brains, we measured the extent of striatal denervation, as a consequence of lesion, by densitometry in dorsal and ventral striatum from 5-6 sections, corresponding to +1.2 mm to -1.0 mm from bregma. Using ImageJ 1.42q software (Version 1.42q; National Institutes of Health, USA), we measured intensity values. After subtracting the values of nonspecific background in the corpus callosum, values from all sections were averaged to provide a single value per animal. Optical intensity was normalized to the optical intensity value from the corresponding area of the intact hemisphere.

Cell counting in substantia nigra. We counted TH-positive neurons in substantia nigra pars compacta in ipsi- and contralateral site of the lesion using a stereological microscope. The borders defining the substantia nigra and ventral tegmental area on all levels along the rostrocaudal axis were defined using a low-power objective lens (4x). The medial border of the substantia nigra and lateral border of the ventral tegmental area were defined by a vertical line passing through the medial tip of the cerebral peduncle (and by the medial terminal nucleus of the accessory optic tract, when present in sections). The ventral border followed the dorsal border of the cerebral peduncle, thereby excluding the TH-positive cells in the pars reticulata, and the area extended laterally to include the pars lateralis in addition to pars compacta. This typically yielded up to 3-4 sections in a 1:6 series. The counting was done using a 40x objective on a Olympus BX50 microscope Olympus BX50 microscope equipped with a Marzhauser X–Y–Z step motor stage and the Visiopharm Integrator System software, Visiopharm A/S. All three axes and the input from the digital camera were controlled using the software that utilized a random start systematic sampling routine. During whole procedure we were blinded for phenotype. All the TH-positive cells seen in left and right nigra were counted. Cell survival values were obtained expressing averaged number of cells from ipsilateral as a proportion of averaged number of cells in contralateral side of an animal. The average number of TH-positive neurons counted in the intact substantia nigra was very similar in the wild type and Nogo-A knock-out mice, 163.7±50.9 and 172±41.8 (±S.D.), respectively.

Statistics. All statistical analyses we conducted using Microsoft®Excel® for Mac 2011 Version 14.1.4 (Student *t*-test) or Prism 5.0c 2009, GraphPad Software, USA (for ANOVA and post-hoc tests). Student *t*-test (2-tailed, 2 degrees of freedom) we employed for data presented in Fig. 1. We used one-way ANOVA with Tukey post-hoc test and two-way ANOVA with Bonferroni's multiple comparison to examine the data presented in Fig. 5 and 6, respectively.

RESULTS

Nogo-A is expressed in ventral mesencephalon-derived dopaminergic cell lines

First, we have employed a human dopaminergic neuronal cell line derived from embryonic mesencephalon (LUHMES cells) (Lotharius *et al.*, 2005) to investigate Nogo-A expression. The LUHMES cells express neuroblast and stem cell markers and bare neuronal characteristics. Upon differentiation, LUHMES cells develop an extensive neurite network, reveal spontaneous electrical activity and express synaptic markers (Scholz *et al.*, 2011), as well as several genes involved in dopamine uptake and release (Scholz *et al.*, 2011, Schildknecht *et al.*, 2009).

We have found that Nogo-A protein was present in LUHMES cells before and after differentiation (Fig. 1), but the expression level was three times higher in differentiated than in the undifferentiated LUHMES cells (Fig. 1 A-B). A similar expression pattern was observed in undifferentiated and differentiated mouse neuronal dopaminergic cell line, MN9D (Fig. 1 B). We found that high levels of Nogo-A were present in the cytoplasm of differentiated LUHMES cells (Fig. 1 C-E). It remains to be determined, whether Nogo-A is also present on the cell surface of these neurons at this stage of development. No intracellular labeling was observed when the same staining protocol was applied to a microglial cell line (BV2) *in vitro* (data not shown).

These results show that Nogo-A protein is present in human- and mouse-derived dopaminergic neuronal cell lines and it is upregulated upon maturation *in vitro*.

Nogo-A is expressed by dopaminergic neurons in the mouse embryo and in adult mouse substantia nigra

Tyrosine hydroxylase (TH) is the rate-limiting enzyme of dopamine synthesis and it is well established as a marker of dopaminergic neurons. TH expression in the midbrain of

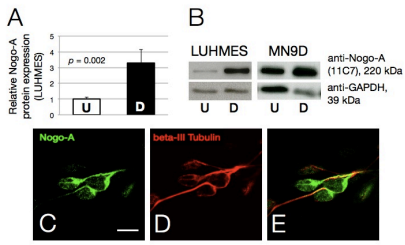

Figure 1. Nogo-A is expressed by mesencephalic dopaminergic cell lines *in vitro*. Nogo-A protein expression levels and cellular localisation was investigated. Using Western-blot, Nogo-A was identified in the protein extracts from human and mouse mesencephalon-derived (LUHMES) cell lines (220 kDa), in undifferentiated (U) and differentiated state (D), (n=4, 2-tailed *t*-test) (A). The representative Westrn-blot results for LUHMES as well as equivalent mouse MN9D cell line before and after differentiation were depicted (B). The confocal images B-D show labeling of Nogo-A (green) together with beta-III tubulin staining (red), in the differentiated LUHMES cells (B - Nogo-A single labeling, C - beta-III tubulin single labeling, D - merged B, C). X=y= 89.9 µm, z=8.17 µm, single confocal z-planes shown, scale bar equals 20 µm.

mouse begins prenatally at E11-12 (Zhou *et al.*, 1995; Maxwell *et al.*, 2005) and it is maintained throughout lifetime (Ivanova & Beyer, 2003). TH expression progresively declines in aging substantia nigra pars compacta (Emborg *et al.*, 1998; Chu *et al.*, 2002).

By performing immunohistochemical double-labeling for TH together with Nogo-A in wild type and Nogo-A knock-out mice, we were able to show the expression of Nogo-A in dopaminergic neurons of mouse embryonic midbrain (Fig. 2) and also confirm its presence in the dopaminergic neurons of adult mice midbrain (Fig. 3)(Wang *et al.*, 2002). In the ventral mesencephalon (marked on Fig. 2 A and E) of an E13.5 mouse embryo, Nogo-A labeling was present in the cytoplasm of many of the TH-positive neurons (Fig. 2 B-D) but also in some cells surrounding midbrain region. In contrast, the same double labeling performed on midbrain sections of Nogo-A knock-out embryos gave only low background fluorescent signals. The majority of neurons of the E15.5 mouse cortex were also positive for Nogo-A with slightly higher intensity than in the midbrain neurons (data not shown). Similar staining patterns as in the embryonic midbrain were observed in the corresponding dopamine neurons in the adult brain: the substantia nigra pars compacta (Fig. 3), which derives from part of the embryonic ventral mesencephalon. Here, intense Nogo-A staining again overlapped with the intense TH immunoreactivity (Fig. 3 A-C). Surrounding midbrain non-dopaminergic neurons contained lower levels or no Nogo-A. As expected, no Nogo-A labeling was present in

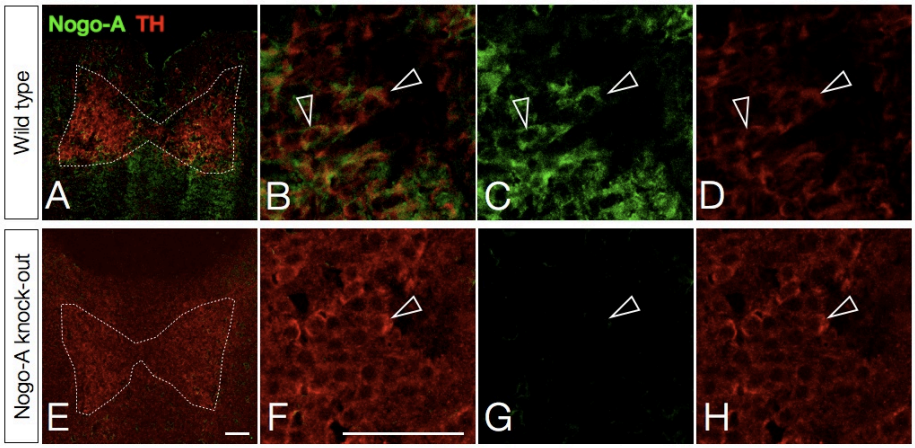

Figure 2. Nogo-A is expressed in mouse embryonic ventral mesencephalon neurons. Confocal images show double labeling of Nogo-A (green) and tyrosine hydroxylase (TH, red) in the ventral mesencephali of wild-type (A-D) and Nogo-A knock-out (E-H) mouse embryos (E13.5). Dopaminergic neurons from ventral mesencephalon (dashed lines in A and E) are shown in higher magnification (B and F, respectively) and in single-label channels, depicting Nogo-A (C and G) or TH labeling (D and H). Arrows indicate TH-positive midbrain neurons which are co-labeled with Nogo-A in the wild type (B-D), but not in the Nogo-A knock-out ventral midbrain (F-H). X=y= 449.1 µm (A, E), X=y= 89.2 µm, z=10.9 µm (B-D, F-H), single confocal z-planes shown, bar equals 50 µm.

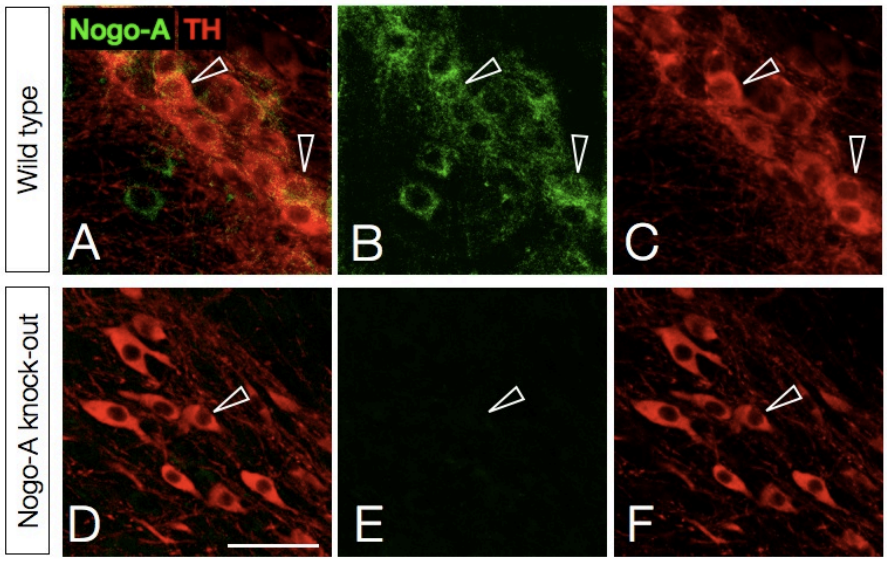

Figure 3. Nogo-A is expressed by substantia nigra neurons of adult mice. Confocal images show double labeling of Nogo-A (green) and tyrosine hydroxylase (TH, red) in the substantiae nigrae pars compactae of adult wild type (A-C) and Nogo-A knock-out mice (D-F). Images A and D are split into single channels depicting Nogo-A (B and E) or TH labeling (C and F) in single cells. Arrows indicate TH-positive nigral neurons which are co-labeled with Nogo-A in the wild type (A-C), but not in the Nogo-A knock-out mouse (D-F). X=y=144.9 µm, z=10.2 µm, single confocal z-planes shown, bar equals 50 µm.

the nigral dopaminergic neurons from the Nogo-A knock-out mice (Fig. 3 D-F).

Our results show that Nogo-A protein is expressed by dopaminergic neurons in the mouse ventral midbrain, during development (at least 2 weeks after conception). We have also confirmed the presence of Nogo-A in mature substantia nigra pars compacta in adulthood.

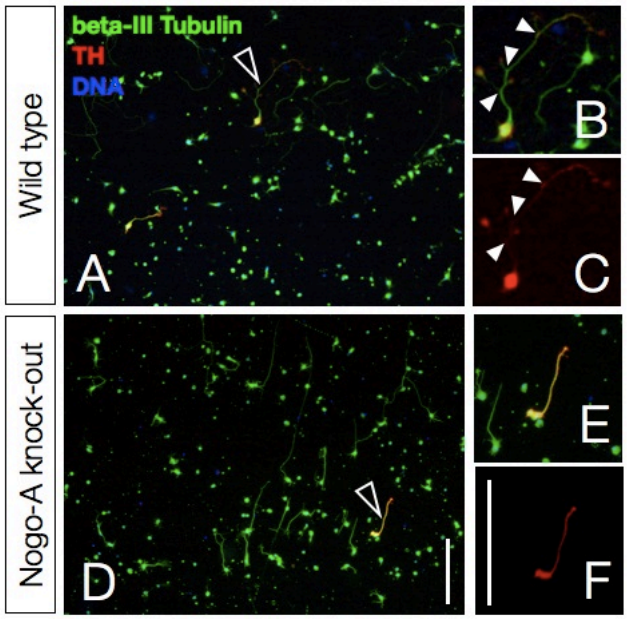

Figure 4. Neuritic differences in dopaminergic neurons from ventral mesencephalons of wild type and Nogo-A knock out mice *in vitro*. Epifluorescent images show neurons isolated from ventral mesencephalon and labeled with tyrosine hydroxylase (TH, red), beta-III tubulin (green) and DAPI (blue, DNA binding), after 7 days *in vitro*. Differences in morphology of dopaminergic (TH-positive) neurons between wild type (A-C) and Nogo-A knock-out mice (D-F) and exemplified. Individual neurons from wild type and Nogo-A knock-out mice, indicated by the empty arrows (A and D, respectively) are magnified (B and E, respectively) and shown with single TH labeling (C and F, respectively). Full arrows (B and C) indicate the branching points present in the wild type but absent in the Nogo-A knock-out dopaminergic neuron (E and F). Scale bars equal 100 µm.

Nogo-A knock-out, but not neutralization of Nogo-A on the cell surface, leads to decreased numbers of neurites and branches in ventral mesencephalon neurons *in vitro*

To investigate the role of Nogo-A in maturing dopaminergic neurons, we performed primary cultures from mouse embryonic (E13.5) ventral mesencephalon (Fig. 4 B-C and E-F, respectively). We observed differences in morphology of neurites, in particular with regard to branching, between wild type and Nogo-A knock-out neurons (Fig. 4 B-C and E-F, respectively).

This phenomenon, as well as several other neurite growth characteristics of wild type and Nogo-A knock-out ventral midbrain cells *in vitro,* was quantified with the aid of a Cellomics setup (Fig. 5). Additionally, in order to establish which poll of Nogo-A was involved (intracellular ER-bound or plasma membrane-bound), some cultures were incubated with anti-Nogo-A specific antibody (11C7), which inhibits Nogo-A/NgR1 interactions on the cells surface. We compared effects of Nogo-A on different parameters of neurite growth in conditions where intracellular and plasma membrane Nogo-A was active (wild type), only intracellular Nogo-A was active (wild type with 11C7) and where the Nogo-A was absent (Nogo-A knock-out). First, we assessed length, number and branching of neurites of ventral midbrain neurons labeled with antibody against beta-III tubulin, a pan-neuronal marker, which labeled 100% of our cells (data not shown). Although the average neurite length was similar in wild type and Nogo-A knock-out neurons (about 90 µm, Fig. 5 A), the number of neurites was markedly decreased when Nogo-A was absent (Fig. 5 B); also the branching tended to be decreased (trend)(Fig. 5 C). Nogo-A-expressing neurons had on average 2 neurites with about 3 branches each per 100 µm of neurite length, whereas Nogo-A knock-out neurons had on average only 1 neurite with just 1 branch point per 100 µm of neurite length (Fig. 5 B-C). An even stronger influence of Nogo-A on neurite length, number and branching was observed when only TH-positive/beta-III tubulin-positive (i.e. dopaminergic) neurons were analyzed (Fig. 5 D-F). In contrast to Nogo-A knock-out condition, the neutralization of Nogo-A located on cell surface only, did not exert any inhibitory effect on neurite growth parameters, which were similar to those in the wild type cells without any treatment (Fig. 5).

These results suggest that Nogo-A exerts a positive role for neurite growth initiation and branching in developing mouse ventral midbrain dopaminergic and, although less pronounced, in non-dopaminergic neurons *in vitro*. This role is most likely mediated by the ER-bound Nogo-A and not through the Nogo-A receptor (NgR1).

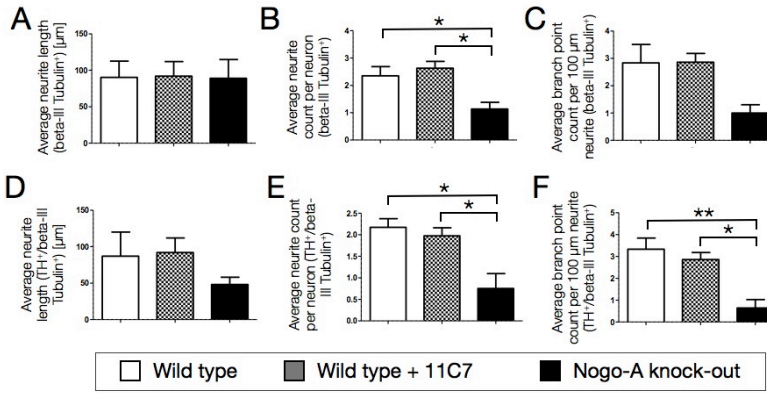

Figure 5, Nogo-A knock-out, but not neutralizing antibody hinders neurite growth and branching in mouse ventral mesencephalon (dopaminergic) neurons *in vitro*. The figure shows quantification of neurite parameters of beta-III-tubulin-positive neurons (A-C) and tyrosine hydroxylase-positive/beta-III tubulin-positive (dopaminergic) neurons from wild type (white bars), anti-Nogo-A antibody (11C7) treated wild type (gray bars) and Nogo-A knock-out (black bars) ventral midbrain cell cultures. Average neurite length was similar in wild type 11C7-treated wild type and Nogo-A knock-out neurons (A), likewise in dopaminergic neurons (D). The average neurite count per neuron was significantly decreased in Nogo-A knock-out cultures, when compared with the wild type (non-treated or 11C7-treated), both in dopaminergic (E) as well as all types of neurons labeled with beta-III tubulin (B). In C, the difference in the average branch point count per 100 µm of neurite length was not statistically significant between the three groups of beta-III-Tubulin-positive neurons. Though, in case of TH-positive neurons only, Nogo-A knock-out neurons had significantly less branches per neurite fragment, than the wild type, both in non-treated or 11C7-treated cells (F). Above parameters were assessed only for neurites longer than 25 µm, all bars represent mean of three-four independent experiments (+S.E.M.), one-way ANOVA and Tukey post-hoc test, A (p=0.996, F=0.003), B (p=0.016, F=7.872), C (p=0.042, F=4.83), D (p=0.395, F=1.09), E (p=0.0144, F=7.546), F (p=0.0043, F=13.15). For all groups Df=2.

Nogo-A knock-out may decrease survival of dopaminergic neurons upon 6-OHDA lesion in adult mice

In reference with the observations in dopaminergic cell line and primary cultures above, we explored whether the absence of Nogo-A in developing ventral mesencephalic dopaminergic neurons would have any impact on the dopaminergic nigrostriatal network formation and neuronal survival in the adult mice.

First, we compared the striatal dimensions as well as optical intensity of the TH-stained fibers (striatal axonal network, formed by the TH-positive fibers originating from the substantia nigra neurons) in their main target, the neostriatum (Fig. 6 A, B) in intact wild type and Nogo-A knock-out mice (Fig. E). We did not observe any differences between wild type and Nogo-A knock-out mice (Fig. 6 E and data not shown). We then analyzed axonal reactions and dopaminergic cell survival upon lesion with the neurotoxin 6-hydroxydopamine (6-OHDA) (specific for dopaminergic neurons). 6-OHDA was injected into the right striatum. Four days after the lesion, the optical intensity of TH-positive fibers in the neostriatum was reduced to 29%±7% (average ± S.E.M.) in the wild type and 43%±5% in the Nogo-A knock-out mice (Fig. 6 F). Four weeks after the lesion, both wild type and knock-out mice showed a reduction to about 50% of TH-positive axons in the striatum (48%±6% and 46%±1% remaining fibers, respectively; Fig. 6 F). Eight weeks after the 6-OHDA injection, the dopaminergic striatal innervation was reduced to 69%±4% in the wild-type mice and to 79%±5% in the Nogo-A knock-out mice (Fig. 6 F). The differences between wild-type and knock-out mice were not statistically significant at any time point. Furthermore, we compared optical densities of TH immunostaining separately in the 4 quadrants of the striatum: dorso-medial, medio-lateral, ventro-lateral and ventro-medial. Again, we did not observe statistically significant differences in TH-fiber denervation at any time point between wild-type and Nogo-A knock-out animals (data not shown).

Finally, we assessed cell death in substantia nigra pars compacta by counting the remaining TH-positive cells in left and right midbrain 4 days, 4 weeks or 8 weeks after the unilateral striatal 6-OHDA injection (Fig. 6 C-D and G). The proportion of remaining cells diminished gradually from 4 days to 8 weeks after the lesions (Fig. 6 G). Four days and four weeks after the lesion, the cell loss in substantia nigra was comparable between wild type and Nogo-A knock-out mice: on average 69% cells remained after 4 days while about 50% remained after 4 weeks (Fig. 6 G). Interestingly, 8 weeks after the lesion (although not statistically significant), slightly more pronounced cell death of dopaminergic cells was present in the substantia

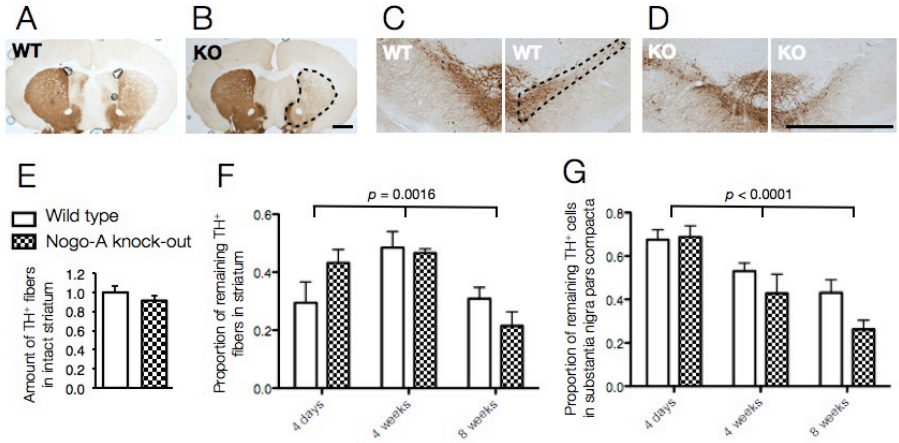

Figure 6 . Dopaminergic fiber degeneration and nigral cell death in wild type and Nogo-A knock-out mice after 6-OHDA lesion. 4 Days, 4 weeks and 8 weeks after unilateral striatal 6-OHDA lesion, brain sections from wild type ('WT,' A, C) and Nogo-A knock-out mice ('KO', B and D) were stained with tyrosine hydroxylase (TH), a marker of dopaminergic neurons. The photos exemplify the degree of dopaminergic fiber (A, B) and dopaminergic cell loss (C, D) 8 weeks after the lesion (in the right hemisphere). TH-positive fiber amounts (assessed by densitometry, E and F) and TH-positive neuron loss (assessed by neuron counts, G) were quantified in areas marked by dashed lines (B and C, respectively), in only intact (E) or both hemispheres (F and G). In E the densitometric values from KO were expressed referring to WT ('4 weeks' time point); in both F and G, the value obtained by the same kind of measurement on the non-lesioned side of the section served as a reference. The bars represent the average with S.E.M., n=5-10, in F and G: 2-way ANOVA (F - time p=0.0016, F=7.55, Df=2; genotype p=0.843, F=0.040, Df=1; interaction p=0.093, F=2.516, Df=2; G - time p<0.0001, F=22.77, Df=2; genotype p=0.0497, F=4.08, Df=1; interaction p=0.195, F=1.70, Df=2), scale bars equal 100 µm.

nigra of Nogo-A knock-out mice (26%±4% surviving neurons) compared to the wild type mice (45%±6% surviving cells; Fig. 6 G). These results suggest that in adult nigral dopaminergic neurons, Nogo-A might contribute to resistance against toxic insults.

DISCUSSION

We show that Nogo-A protein is present in developing ventral mesencephalic neurons in human (cell line) and mice, with the noticeable levels in the dopaminergic cells. Surprisingly, the role of Nogo-A varies in pre- and postnatal stages. In immature dopaminergic neurons *in vitro*, intracellular Nogo-A has a positive influence on the initiation and branching of neurites. In adult mice, this reticulon protein might play an pro-survival role in nigral neurons exposed to the long-term effects evoked by the neurotoxin 6-OHDA.

Midbrain dopaminergic neurons express Nogo-A during development

Nogo-A has previously been shown to be present in dopaminergic substantia nigra neurons of adult mice (Wang *et al.*, 2002). In turn, colorimetric labeling in neuromelanin-enriched nigral neurons did not definitively confirmed the presence of Nogo-A in this region in human (Buss *et al.*, 2005). Here we found that a ventral mesencephalon-derived human cell line expresses Nogo-A, and that the protein is upregulated during dopaminergic differentiation of these cells. We have also detected Nogo-A in ventral mesencephalic dopaminergic neurons of mice, both in the embryonic (E13.5) and adult brains. Although the exact cellular localization including the cell surface still needs to be determined, it is evident that the majority of Nogo-A in midbrain dopaminergic neurons is intracellular.

Intracellular Nogo-A facilitates initiation and branching of neurites in midbrain neurons

Upon differentiation and neurite outgrowth protein levels of Nogo-A increase (about 3-fold) in the human mesencephalic cell line. Nogo-A's enrichment in different types of maturing neurons with growing processes has been documented in previous reports. Growing axons of the olfactory tract expressed Nogo-A (Tozaki *et al.*, 2002), suggesting its positive role in axonal extension. Likewise, in an *in vitro* model, Nogo-A accumulated at axonal branch points and the central domain of the growth cones, co-localized with growth-associated proteins (Richard *et al.*, 2005). Similarly, in embryonic cortical neurons, Nogo-A was enriched in the axonal varicosities and growth cones *in vitro* (Hunt *et al.*, 2003). Functional studies where Nogo-A was deleted or its

activity was neutralized with specific antibodies showed that the effects of Nogo-A suppression on migrating neurons and growing neurites differed according to cell type and probably also age. Thus, Nogo-A/B/C knock-out slowed down the tangential migration of interneurons into the embryonic cortex, and the cortical neurons *in vitro* showed earlier polarization and increased branching, when compared with the wild type condition (Mingorance-Le Meur *et al.*, 2007). Radial migration of the cortical neurons themselves along the radial glial fibers was enhanced *in vivo* and *in vitro* by anti-Nogo-A antibodies or knock-out (Mathis *et al.*, 2010). In dorsal root ganglion cells *in vitro* and peripheral nerves *in vivo* Nogo-A neutralization or ablation led to longer neurites, increased fasciculation and decreased branching (Petrinovic *et al.*, 2010). Growth cones of dorsal root ganglia dissected from newborn and adult mice showed increased motility and size in the absence of Nogo-A (Montani *et al.*, 2009). Finally, in regenerating retinal ganglion cells *in vivo*, Nogo-A overexpression in the neurons enhanced, whereas Nogo-A knock-out or knock-down diminished regenerative sprouting (Pernet *et al.*, 2011).

In our study, we observed that midbrain neurons lacking Nogo-A had fewer processes and fewer branches, when compared to their wild type counterparts. This suggests that Nogo-A has a neurite-growth promoting role in dopaminergic neurons of ventral midbrain *in vitro*. In the adult mice, however, the denisity of TH immunoreactivity in the striatum was not different between wild type and Nogo-A knock-out mice. This may be due to compensatory mechanisms, which occur frequently in different models of gene knock-out. Indeed, increased levels of certain semaphorins and ephrins and their receptors have been seen in the CNS of mice lacking Nogo-A (Kempf *et al.*, 2009), and also Nogo-B was shown to be up-regulated in Nogo-A knock-out mice (Simonen *et al.*, 2003; Dimou *et al.*, 2006). The molecular mechanisms causing the different effects of Nogo-A or Nogo-A deletion in developing neurons and neurites are currently unknown. Surface Nogo-A interacts with at least 3 binding partners/receptors, NgR1, PirB, and the yet uncharacterized Nogo-A specific receptor (Schwab, 2010). In addition, intracellular interactions and effects are possible; Nogo-A is enriched in the ER, where it has structural roles (Voeltz et al., 2006), but a number of other molecular interactions have been observed, some of which could influence neuronal metabolism, neurite growth, branch formation or cell survival (Yang *et al.*, 2009; Schwab, 2010; Pernet *et al.*, 2011).

In adult nigral dopaminergic neurons, Nogo-A might play a pro-survival role

It has been reported that Nogo-A knock-out mice show behavioral alterations, which include higher sensitivity to amphetamine compared to wild type mice (Willi *et al.*, 2009). Corresponding to that, D2 receptor expression was increased and dopamine levels were lowered in the striatum of Nogo-A knock-out mice (Willi *et al.*, 2010). These findings suggest that Nogo-A influences properties of the nigrostriatal pathway.

Here, we observed an expected progressive loss of dopaminergic neurons 4 to 8 weeks after a 6-OHDA injection into the striatum in wild type mice. That effect was slightly more severe (trend) in the Nogo-A knock-out than in the wild type mice. This suggests that Nogo-A might be neuroprotective in adult dopaminergic neurons challenged with 6-OHDA.

The mechanism behind the hypothetical pro-survival role of Nogo-A in the nigral neurons is obscure. Kilic and co-workers showed a pro-survival role of Nogo-A in response to cellular stress caused by ischemia: increased neuronal death was seen in the striatum of Nogo-A knock-out mice (or mice pretreated with Nogo-A antibody) after transient medial cerebral artery occlusion, mediated via a Rac1/RhoA signaling pathway (Kilic *et al.*, 2010). In addition, in a superoxide dismutase (SOD) mutant mouse model of amyotrophic lateral sclerosis genetic deletion of Nogo-A and Nogo-B accelerated disease progression and decreased spinal motor neuron survival (Yang *et al.*, 2009). This effect was correlated with the interaction of Nogo-A with, and possible regulatory effects of Nogo-A on the chaperone protein disulphide isomerase (Yang *et al.*, 2009). Because elevated reactive oxygen species probably mediate the neurotoxic effects in all the three models, – SOD mutation, cerebral artery occlusion and 6-OHDA –, similar mechanisms of a probably intracellular action of Nogo-A might be at work. Intracellular interactions of Nogo proteins with the survival promoting protein bcl-2 (Tagami *et al.*, 2000; Zhu *et al.*, 2007) and with the ROS-scavenging enzyme peroxiredoxin 2 (Prdx2) leading to enhanced survival of cortical neurons have also been described (Mi *et al.*, 2012).

Overall, our data are coherent with the suggestion of an intracellular pro-survival action of Nogo-A in adult dopaminergic neurons, the detailed mechanisms of which remains to be analyzed.

Conclusion

Although Nogo-A is mostly expressed in oligodendrocytes and myelin in the adult CNS, neuronal expression is prevalent during development and includes midbrain dopaminergic neurons. Nogo-A influences neurite outgrowth, branching and fasciculation in different types of neurons in a positive or negative way, probably dependent on type of neuron, stage of differentiation, and site and mechanisms of action (cell surface vs. intracellular; Nogo-A receptor types). In adult Nogo-A knock-out dopaminergic substantia nigra neurons, the neurotoxin 6-OHDA seem to have slightly more deleterious effect than in wild type cells, in accordance with the intracellular pro-survival action of Nogo observed in other neuronal cell types. All these results make Nogo-A an interesting target for further studies in particular in the context of Parkinson's disease.

ACKNOWLEDGMENT

We would like to acknowledge financial support from the Swedish Research Council, BAGADILICO - Excellence in Parkinson and Huntington Research, MultiPark network and FP7-MC-214003-2 (Marie Curie Initial Training Network AXREGEN). We would like to thank Birgit Haraldsson and Dr. Xiaoyi Zou for excellent technical support in *in vivo* experiments and Dr. Robert C. Atwood for help with ImageJ macros. We would like to thank Dr. Marcel Leist for providing us the LUHMES cells in the project. We would like to acknowledge Dr. Andy McCourt for the critical reading and linguistic revision of the manuscript. The authors have no conflict of interest to declare.

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