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Dopamine neurons in ventral mesencephalon – Interactions with glia and locus coeruleus

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Front cover: Cytoplasmatic expression of neurocan (green) in S100β-positive astrocytes (red).

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ABSTRACT

Parkinson's disease is a progressive neurodegenerative disorder, characterized by a depletion of the dopaminergic neurons in the substantia nigra. The cause of the disease is yet unknown but age, oxidative stress, and neuroinflammation are some of the features involved in the degeneration. In addition, substantial cell death of noradrenergic neurons occurs in the locus coeruleus (LC). Noradrenaline has been suggested to protect the dopamine neurons from oxidative stress and neuroinflammation. The main treatment of Parkinson's disease is Levo-dopa, although severe side effects arise from this therapy. Hence, grafting fetal ventral mesencephalic (VM) tissue into the adult striatum has been evaluated as an alternative treatment for Parkinsons's disease. However, the survival of the grafted neurons is limited, and the dopamine-denervated striatum does not become fully reinnervated. Therefore, elucidating factors that enhance dopamine nerve fiber formation and/or survival of the grafted neurons is of utmost importance.

To investigate dopamine nerve fiber formation and the interactions with glial cells, organotypic VM tissue cultures were utilized. Two morphologically different nerve fiber outgrowths from the tissue slice were observed. Nerve fibers were initially formed in the absence of migrating astrocytes, although thin vimentin-positive astrocytic processes were detected within the same area. A second, persistent nerve fiber outgrowth was observed associated with migrating astrocytes. Hence, both of these nerve fiber outgrowths were to some extent dependent on astrocytes, and appeared as a general feature since this phenomenon was demonstrated in β-tubulin, tyrosine hydroxylase (TH), and aldehyde dehydrogenase A1 (ALDH1)-positive nerve fibers. Neither oligodendrocytes (NG2-positive cells), nor microglia (Iba-1-positive cells) exerted any effect on these two neuronal growths. Since astrocytes appeared to influence the nerve fiber formation, the role of proteoglycans, i.e. extracellular matrix molecules produced by astrocytes, was investigated. β-xyloside was added to the cultures to inhibit proteoglycan synthesis. The results revealed a hampered astrocytic migration and proliferation, as well as a reduction of the glia-associated TH-positive nerve fiber outgrowth. Interestingly, the number of cultures displaying the non-glia-mediated TH-positive nerve fibers increased after β-xyloside treatment, although the amount of TH-protein was not altered. Thus, proteoglycans produced by astrocytes appeared to be important in affecting the dopamine nerve fiber formation.

The noradrenaline neurons in LC have been suggested to protect dopamine neurons from damage. Therefore, the interaction between VM and LC was evaluated. Using the intraocular grafting method, fetal VM and LC were grafted either as single grafts or as VM+LC co-grafts. Additionally, the recipient animals received 2% blueberry-enriched diet. The direct contact of LC promoted graft volume and survival of TH-positive neurons in the VM grafts. The number of dopamine neurons, derived preferably from the A9 (ALDH1/TH-positive) was increased, whereas the dopamine neurons from the A10 (calbindin/TH-positive) were not affected. A dense dopamine-β-hydroxylase (DBH)-positive innervation was correlated to the improved survival. Blueberry-enriched diet enhanced the number of TH-positive neurons in VM, although the graft size was not altered. The combination of blueberries and the presence of LC did not yield additive effects on the survival of VM grafts. The attachment of VM or the addition of blueberries did not affect the survival of TH-positive neurons in LC grafts. The number of Iba-1-positive microglia was decreased in co-grafted VM compared to single VM transplants. The addition of blueberries reduced the number of Iba-1-positive microglia in single VM transplants. Hence, the direct contact of LC or the addition of blueberries enhanced the survival of VM grafts.

Taken together, these data demonstrate novel findings regarding the importance of astrocytes for the nerve fiber formation of dopamine neurons. Further, both the direct attachment of LC or antioxidant-enriched diet promote the survival of fetal VM grafts, while LC is not affected.

Keywords: Parkinson's disease, ventral mesencephalon, nerve fiber formation, glia, locus coeruleus, grafting, antioxidant-enriched diet

POPULÄRVETENSKAPLIG SAMMANFATTNING

Parkinsons sjukdom är en allvarlig neurologisk sjukdom där de celler (neuron) som producerar signalsubstansen dopamin i mellanhjärnan bryts ner. Bristen på dopamin försämrar hjärnans styrning av kroppsrörelser. Motoriska symtom såsom muskelstelhet, skakningar och balansstörningar uppstår. Sjukdomsförloppet är långsamt och först när ungefär 50% av de dopaminproducerande cellerna är borta uppstår de första symtomen. Orsaken till sjukdomen är okänd, men drabbar främst personer över 50-60 år. I Sverige har ca 20 000 människor Parkinsons sjukdom. Sjukdomen kan inte botas men symtomen kan behandlas med läkemedel. Den vanligaste medicineringen är L-dopa som i hjärnan kan omvandlas till dopamin, men tyvärr förlorar medicinen effekt efter ett tag och mängden måste ökas. Dessutom uppkommer biverkningar, såsom ofrivilliga rörelser.

Andra behandlingsmetoder har utvärderats, till exempel att ersätta de förlorade dopamincellerna med nya celler. Denna transplantations-behandling har länge betraktats som lovande, men tyvärr är det relativt få transplanterade celler som överlever. Dessutom så växer inte alla transplanterade dopaminceller in i värdhjärnan. Det är därför viktigt att studera olika faktorer under utvecklingen som stimulerar bildandet av dopaminceller så att bättre transplantationsresultat kan uppnås. Jag har studerat hur hjärnans stödjeceller (glia celler) interagerar med dopamin-cellerna i cellodling. Mina resultat visar att en viss typ av dessa stödjeceller, s.k. astrocyter, verkar vara viktiga för nervutväxten. Dessutom har jag visat att genom att hämma tillverkningen av proteoglykaner, en viss sorts molekyl som astrocyterna producerar, så erhölls en minskad rörelse (migration) av astrocyterna från cellodlingen och därmed en minskad nervutväxt från de dopamin-producerande cellerna.

Det har längre varit känt att man vid Parkinsons sjukdom, utöver nedbrytningen av de dopaminceller som ger upphov till muskelsymtomen, även får en nedbrytning av de celler som producerar signalsubstansen noradrenalin. Studier har visat att noradrenalinceller kan agera skyddande för dopamincellerna genom att minska inflammation och oxidativ stress (fria syreradikaler som tros vara skadliga för cellerna). Både inflammation och oxidativ stress har föreslagits som möjliga orsaker till Parkinsons sjukdom. Dessutom finns det data som visar att antioxidant (motverkar fria syreradikaler)-berikad mat gynnar tillväxten av dopaminceller efter skada i djurmodeller för Parkinsons sjukdom. Med dessa nya rön i åtanke ville jag studera interaktionen mellan noradrenalinceller och dopaminceller under

utvecklingen, samt effekten av antioxidantberikad mat för dessa celler. Två procent blåbär tillsattes i maten, vilket motsvarar en kopp blåbär om dagen för en människa. Vävnadsbitar från mellanhjärnan samt ett område längre bak i hjärnan (som kallas locus coeruleus) från råttfoster transplanterades tillsammans. Bitarna från mellanhjärnan innehåller dopaminceller, och bitarna längre bak i hjärnan innehåller noradrenalinceller. Mina resultat visar att närvaron av noradrenalinceller ökade överlevnaden av de dopamin-producerande cellerna, och främst de dopaminceller som bryts ner vid Parkinsons sjukdom. Dessutom erhölls en minskning av de celler som är involverade vid inflammation (mikroglia) när noradrenalinceller transplanterades tillsammans med dopamincellerna. När blåbär tillsattes till maten erhölls samma resultat, mängden överlevande dopaminceller ökade och mängden celler involverade i inflammation minskade. Detta visar att redan under utvecklingen så har noradrenalinceller och antioxidanter en skyddande roll för dopamincellerna, celler som senare bryts ner vid Parkinsons sjukdom.

Det är viktigt att studera Parkinsons sjukdom eftersom problematiken runtomkring denna neurodegenerativa sjukdom kommer att öka då vi människor blir allt äldre. Det är viktigt att hitta faktorer som kan påverka dopamincellerna så att bättre nervutväxt erhålls, alternativt förbättra överlevnaden av de celler som transplanteras, vid ersättning av de förlorade cellerna vid Parkinsons sjukdom. Denna avhandling visar att en typ av hjärnans stödjeceller (astrocyterna), är viktiga för dopamin-cellernas nervutväxt, samt att närvaron av noradrenalin och antioxidanter ökar överlevnaden av dopamincellerna. Dessutom, det faktum att tillsats av till exempel blåbär till maten kan vara skyddande öppnar nya möjligheter för behandling av neurodegenerativa sjukdomar.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their roman numerals.

I. Berglöf E, af Bjerkén S, and Strömberg I.
Glial influence on nerve fiber formation from rat ventral mesencephalic organotypic tissue cultures. (2007)
Journal of Comparative Neurology, 501(3):431-442.
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II. Berglöf E, Plantman S, Johansson S, and Strömberg I.
Inhibition of proteoglycan synthesis affects neuronal outgrowth and astrocytic migration in organotypic cultures of fetal ventral mesencephalon. (2008)
Journal of Neuroscience Research, 86(1): 84-92.
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III. Berglöf E, and Strömberg I.
Locus coeruleus promotes survival of dopamine neurons in ventral mesencephalon. An *in oculo* grafting study. (2008)
Manuscript.

IV. Berglöf E, Bickford P, and Strömberg I.
Blueberry-enriched diet enhances the survival of fetal ventral mesencephalic intraocular grafts. (2008)
Manuscript.

ABBREVIATIONS

6-ODHA	6-hydroxydopamine
β-xyloside	methyl-umbelliferyl-β-D-xyloside
ALDH1	aldehyde dehydrogenase A1
CNS	central nervous system
DAPI	4',6-diamidino-2-phenylindole
DBH	dopamine-beta-hydroxylase
DMEM	Dulbecco's modified Eagle's medium
DIV	days *in vitro*
E	embryonic day
ECM	extra cellular matrix
FCS	fetal calf serum
GABA	γ-aminobutaric acid
GAG	glycosaminoglycan
Iba-1	ionized calcium binding adapter molecule 1
i.p.	intraperitoneal
LC	locus coeruleus
L-dopa	3,4-dihydroxy-L-phenylalanine
LGE	lateral ganglionic eminence
MPTP	1-methyl-4-phenyl-1.2.3.6-tetrahydropyridine
PBS	phosphate buffer saline
PGs	proteoglycans
SN	substantia nigra
TBS-T	tris buffer saline-tween
TH	tyrosine hydroxylase
TNFα	tumor necrosis factor alpha
VM	ventral mesencephlon
VTA	ventral tegmental area

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INTRODUCTION

Grafting of fetal tissue has been undertaken to counteract the symptoms when dopamine neurons degenerate in Parkinson's disease. Unfortunately, the survival of the grafted neurons is rather limited and the graft-derived reinnervation covers too small regions of the target area. Thus, to elucidate factors that either promote nerve fiber formation from grafted neurons and/or enhance the survival of these neurons are of utmost importance in order to achieve improved transplantation outcome. This thesis focuses on the influence glial cells exert on ventral mesencephalic (VM) dopamine nerve fiber formation, as well as the interactions between the locus coeruleus (LC) and the VM. Further, the effect of antioxidant-enriched diet on grafted tissue was investigated. The introduction of this thesis presents a brief overview on the background and pathology of Parkinson's disease, and some common experimental methods used to study Parkinson's disease.

The neurotransmitter dopamine

Fifty years ago, dopamine was identified as a distinct neurotransmitter and not only as a precursor of noradrenaline (Bertler and Rosengren, 1959; Carlsson et al., 1957; Carlsson et al., 1958). This finding, in combination with the Levo-DOPA (L-dopa; 3,4-dihydrox-L-phenylalanine) treatment, resulted in the Nobel price year 2000 given to Arvid Carlsson, together with Paul Greengard for the investigation of the molecular cascade triggered by dopamine, and Eric R Kandel who investigated memory formation and learning at the synapse level. Dopamine, noradrenaline, and adrenaline are catecholamines, i.e. molecules derived from the amino acid tyrosine (Figure 1). The catecholamines are water-soluble hormones that circulate in the bloodstream, produced either by the adrenal glands or in the postganglionic sympathetic nervous system. In the central nervous system (CNS), dopamine acts as a neurotransmitter synthesized in neurons, for example in the VM.

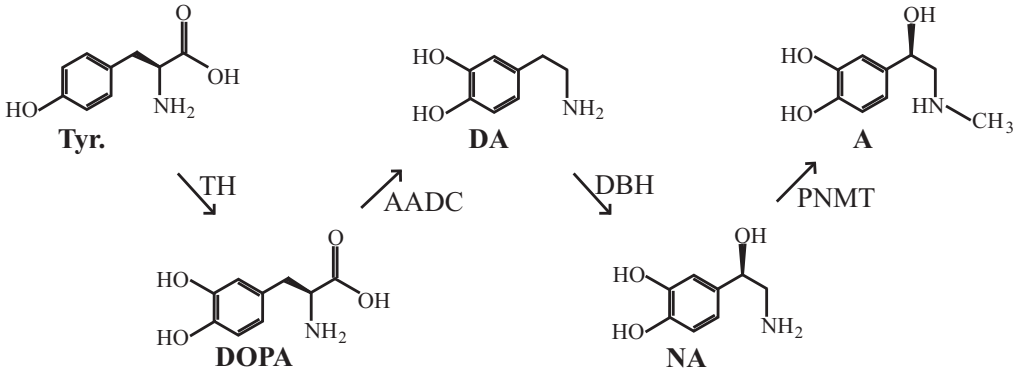

Figure 1. Synthesis of dopamine (DA), noradrenaline (NA), and adrenaline (A). Tyrosine (Tyr.) is converted to dihydroxyphenylalanine (DOPA) by the rate limiting enzyme tyrosine hydroxylase (TH). DOPA is then converted to dopamine by dopa-decarboxylase (AADC). Dopamine-β-hydroxylase (DBH) converts dopamine to noradrenaline, and phenylethanol-amine-N-methyltransferase (PNMT) converts noradrenaline to adrenaline.

The distribution of catecholamines within the brain was mapped in the beginning of the 1960s using the Falck-Hillarp catecholamine formaldehyde histoflouresence method. Dopamine and noradrenaline were converted to isoquinoline molecules, which are yellow-green fluorescent (Dahlström and Fuxe, 1964; Falck et al., 1962). Several distinct groups, named with an A and a number, were identified throughout the brain from the medulla oblongata to the hypothalamus (Dahlström and Fuxe, 1964).

Development of midbrain dopamine neurons

The nervous system derives from the ectoderm germlayer. Several transcriptions factors are involved in the early development of the midbrain neurons, e.g. sonic hedgehog (Shh), the transcription factor engrailed 1 and 2 (En1, En2), and fibroblast growth factor 8 (FGF8) (Crossley et al., 1996; Echelard et al., 1993; Hynes et al., 1994), for reviews see (Liu and Joyner, 2001; Sillitoe and Vogel, 2008). The dopaminergic neurons are generated from precursors in the ventricular zone on both sides of the midline in the neural tube. When these dopaminergic precursors become postmitotic, they start to express TH and migrate along radial glia towards their final location (Di Porzio et al., 1990; Shults et al., 1990). In the rat, the development of VM dopaminergic neurons has been thought to occur between embryonic day (E) 11-15, with the peak at E14 (Altman and Bayer, 1981; Specht et al., 1981). However, recent findings have demonstrated that the vast majority of the substantia nigra (SN) dopaminergic neurons are born already at E12

(Gates et al., 2006). When the SN neurons have reached their positions in the midbrain, they form axons projecting towards the lateral ganglionic eminence (LGE), which develops into mature striatum. In the rat, TH-positive nerve fibers are found in the LGE already at E14, however, the dopamine innervation is not completed until weeks later (Seiger and Olson, 1973). Comparing the rat with humans, the ventral migration of TH-positive cells in humans starts at approximately gestation week 7 and the first TH-positive nerve fibers are observed in the developing striatum at gestation week 9, although it takes years before the striatum is fully innervated (Freeman et al., 1991). Genes involved in differentiation of the dopamine midbrain precursors are the nuclear receptor NURR1, the LIM homedomain transcription factor Lmbx1, the paired-like homebox transcription factor Ptx3, and aldehyde dehydrogenase 1 (ALDH1) (McCaffery and Drager, 1994; Saucedo-Cardenas et al., 1998; Smidt et al., 2000; Wallen et al., 1999; Zetterstrom et al., 1997), for review see (Sillitoe and Vogel, 2008).

Midbrain dopamine projections

The vast majority of the dopamine neurons in the CNS are located in the VM and can further be divided into the retrorubral field (A8), SN (A9), and ventral tegmental area (VTA, A10) (Dahlström and Fuxe, 1964). The dopamine projections from these areas can be divided into three distinct pathways: the nigrostriatal (mesostriatal), the mesolimbic, and the mesocortical pathways. The nigrostriatal pathway arises from dopamine neurons located in the SN, which innervate the dorsal striatum (caudate-putamen), and is involved in control of motor activity (Ungerstedt, 1971). The loss of the nigrostriatal dopamine projections results in motor disturbances typical of Parkinson's disease, which will be discussed in more detail below. The mesolimbic pathway consists of dopamine neurons located in the VTA that project to the nucleus accumbens, olfactory tubercle, and amygdala and participate in motivated behavior, reward, and addiction. The VTA also projects to the frontal cortex, creating the mesocortical pathway, which is involved in memory and learning (Björklund and Lindvall, 1984; Le Moal and Simon, 1991; Ungerstedt, 1971).

A general marker used for visualization of dopamine neurons is TH (Figure 1). The dopaminergic neurons in the SN and the VTA can roughly be divided by their expression of TH together with the markers G-protein-regulated inwardly rectifying potassium channel 2 (GIRK2) and calbindin, respectively. (Gerfen et al., 1987a; Gerfen et al., 1987b; German and Liang, 1993; Schein et al., 1998) Further, the progenitor marker ALDH1 is preferably expressed in the SN dopamine neurons (Chung et al., 2005; Haque et al., 1997; McCaffery and Drager, 1994).

The basal ganglia circuit

The basal ganglia system is divided into four distinct brain nuclei; the striatum, the globus pallidus externa/interna, the subthalamic nucleus, and the SN. These nuclei participate in the control of movements, cognitive, and limbic functions. The motor behavior is controlled in the dorsal striatum, whereas the limbic and cognitive inputs occur in the ventral striatum (Björklund and Lindvall, 1984).

The dorsal striatum consists of approximately 90-95% γ-aminobutyric acid (GABA)-ergic medium-sized spiny neurons, which receives inputs from large parts of the motor cortex, thalamus, and the SN (Gerfen and Wilson, 1996; Graybiel and Ragsdale Jr, 1983). Hence, the striatum is the main input nucleus of the basal ganglia. Besides the medium-sized spiny neurons, GABAergic medium aspiny interneurons and cholinergic interneurons are also present in the striatum (Gerfen and Wilson, 1996; Graybiel and Ragsdale Jr, 1983). Striatal neurons express dopamine receptors, i.e G-protein coupled receptors that can be divided into two groups: D1-like receptors (receptor D1 and D5) stimulate adenyl cyclase, whereas D2-like receptors (receptor D2, D3, D4) inhibit adenyl cyclase (Dearry et al., 1990; Kebabian and Calne, 1979; Missale et al., 1998). GABAergic medium-sized spiny neurons expressing D1 receptors mediate the direct pathway to the globus pallidus interna, while GABAergic medium-sized spiny neurons expressing D2-receptors mediate the indirect pathway via the globus pallidus externa and the subthalamic nucleus before the signal reaches the globus pallidus interna (Gerfen and Wilson, 1996; Graybiel and Ragsdale Jr, 1983).

Normal dopaminergic input to the striatum results in an inhibition of the indirect pathway and an activation of the direct pathway. The net effect results in stimulation of the thalamus, which facilitate motor activity. However, in Parkinson's disease, the input of dopamine to the striatum is reduced, which results in a decreased stimulation of the direct pathway and an activated indirect pathway. This results in an increased output from globus pallidus interna and less thalamocortical activity and thus, impaired motor activity (Albin et al., 1989; Bjarkam and Sorensen, 2004; Blandini et al., 2000; DeLong, 1990) (Figure 2).

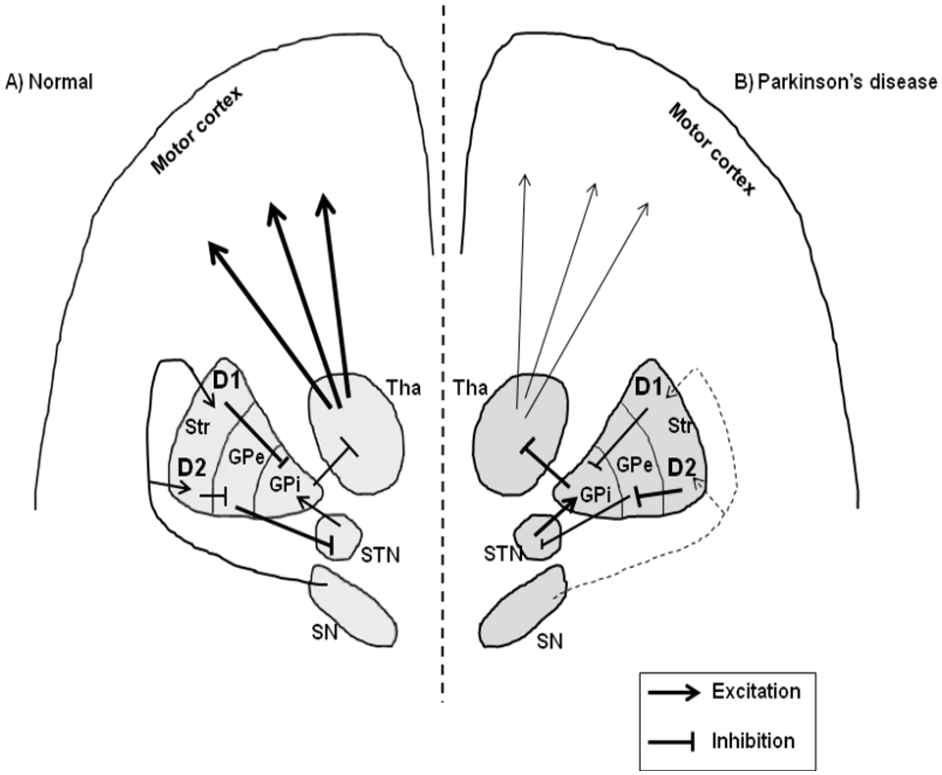

Figure 2. Schematic illustration of the motor basal ganglia system. A) Under normal conditions, dopaminergic input to the striatum facilitate motor activity. B) In Parkinson's disease, the striatum receives less dopaminergic input from the SN, which in the end results in impaired motor activity. The direct pathway is mediated by D1-receptors, and the indirect pathway is mediated by D2-receptors. Striatum (Str), globus pallidus externa (GPe), globus pallidus interna (GPi), subthalamic nucleus (STN), thalamus (Tha). Picture adapted from (Bjarkam and Sorensen, 2004).

Pathology of Parkinson's disease

The prevalence of Parkinson's disease is approximately 1-2% worldwide with a typical age-of-onset of about 60 years (Shastry, 2001). Parkinson's disease, which is a severe progressive neurodegenerative disorder, is characterized by a depletion of the dopamine neurons in the SN. As mentioned above, this loss of the neurotransmitter dopamine results in a malfunctional basal ganglia circuit giving the cardinal symptoms of the disease; muscular rigidity, reduced movement ability (hypokinesia), slowness in movements (bradykinesia), and tremor at rest (Bernheimer et al., 1973; Ehringer and Hornykiewicz, 1960; Selby, 1984; Trétiakoff, 1919). Further, a flexed posture and impaired balance are also common features (Ehringer and Hornykiewicz, 1960; Selby, 1984). The cardinal symptoms appear first when about 50% of the dopamine neurons in the SN are lost, and the levels of dopamine in the striatum are reduced by 80% (Agid, 1991; Bernheimer et al., 1973; Kish et al., 1988). Patients suffering from Parkinson's disease often display reduced spontaneity, lack of motivation as well as dementia (Braak and Braak, 2000; Jellinger, 1991; Zweig et al., 1993).

The presence of intraneuronal cytoplasmatic inclusion bodies, Lewy bodies, is another hallmark of Parkinson's disease. These eosinophilic protein aggregates composed of specific cytoplasmatic proteins, such as α-synuclein, parkin, ubiquitin, synphilin and oxidized neurofilaments, was first described in 1912 (Forno et al., 1996; Lewy, 1912). The Lewy bodies are primarily found in the SN. The presence of Lewy bodies is, however, not exclusive for Parkinson's disease since diseases such as dementia and Alzhemier's disease as well as healthy people during normal aging also display Lewy bodies (Forno et al., 1996; Gibb and Lees, 1988; Spillantini et al., 1998). However, the exact role for Lewy bodies in Parkinson's disease is not established, for review see (Harrower et al., 2005).

Neuromelanin, a polymer pigment, is present in the SN (black nuclei) and the LC (blue spot) and increases with age (Marsden, 1983). The exact function is not known, but it has been suggested to be a waste product in the metabolism of catecholamines. Extracellular neuromelanin in the SN, however, is found to correlate to areas within the SN high in immunoreactive MHC class II microglia (Beach et al., 2007). Additionally, neuromelamin can stimulate the release of tumor necrosis factor alpha (TNFα), interleukin-6, and nitric oxide from cultivated microglia (Wilms et al., 2003). The neuromelanin-pigmented neurons are the neurons undergoing degeneration in Parkinson's disease. The levels of neuromelanin are about half in brains

from Parkinson's disease patients compared to aged matched controls (Zecca et al., 2002). This loss of neuromelanin in Parkinson's disease can be monitored using magnetic resonance imaging (MRI) (Sasaki et al., 2006), for an review on neuromelanin see (Zecca et al., 2006).

In addition to the loss of VM dopamine neurons in Parkinson's disease, the noradrenaline neurons in the LC (A6) are also degenerating (Ehringer and Hornykiewicz, 1960). The loss of neurons in the LC is actually more extensive than that found in the VM (Ehringer and Hornykiewicz, 1960; German et al., 1992; Mann, 1983; Zarow et al., 2003). It has been demonstrated that noradrenaline reduces oxidative stress in VM cultures (Troadec et al., 2001). Further, the loss of noradrenergic neurons results in increased sensitivity in dopamine neurons to various insults, e.g. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Heneka et al., 2003; Mavridis et al., 1991; Srinivasan and Schmidt, 2004). One hypothesis to the cause of Parkinson's disease is actually that the degeneration of neurons in the LC precedes the loss of dopamine neurons, which might initiate the degeneration of dopaminergic neurons.

Risk factors for Parkinson's disease

Genetic discoveries in combination with epidemiologic studies have implied multiple putative causes for Parkinson's disease, thus the term "Parkinson's diseases" might be more appropriate (Galpern and Lang, 2006; Lang, 2007). Some of the features implicated to participate in the destruction of the dopaminergic neurons are age, genetic factors, environmental factors, neuroinflammation, and oxidative stress. Among these, age is the strongest risk factor for developing the disease (Semchuk et al., 1993).

About 5-10% of all cases of Parkinson's disease are familiar (Olanow and Tatton, 1999). Several genes have been suggested to participate in either dominant or recessive forms of Parkinson's disease, e.g. α-synuclein, parkin, UCH-L1, PINK1, DJ-1, and LRRK2/dardarin (Funayama et al., 2002; Maroteaux et al., 1988; Nagakubo et al., 1997; Shimura et al., 2000; Valente et al., 2004; Zimprich et al., 2004), for review see (Gasser, 2007). However, inherited factors do not participate in sporadic PD, demonstrated in twin-studies where the prevalence of the disease was not altered (Tanner et al., 1999; Wirdefeldt et al., 2004).

Environmental factors, e.g. agriculture with exposure to pesticides (rotenone, paraquat), and various heavy metals, have been associated with the disease correlated with abnormal mitochondrial activity (Baldereschi et al., 2003; Tanner et al., 1989; Tanner and Langston, 1990). A comprehensive investigation clearly suggests a correlation between pesticide exposure and Parkinsons's disease, where long exposure displayed the strongest association (Brown et al., 2006). Parkinson's disease can also be caused by various neurotoxins, where the most know example is MPTP. MPTP, a side product of synthetic heroin, was discovered in the 1980s when young drug addicts in California demonstrated features similar to Parkinson's disease (Davis et al., 1979; Langston et al., 1983). MPTP is highly lipophilic and crosses easily the blood-brain-barrier, and is converted by monoamine oxidase in glial cells to an unstable intermediate compound, followed by spontaneous oxidation to form the toxic metabolite 1-metyl-4-phenylpyridinium (MPP+) (Heikkila et al., 1984; Nicklas et al., 1985). After release, MPP+ is taken up into dopamine neurons via the dopamine transporter, and the complex I of the mitochondrial respiratory chain is inhibited (Javitch et al., 1985), for review see (Blum et al., 2001).

Neuroinflammation has been suggested to participate in the degeneration of dopamine neurons in Parkinson's disease. Activated microglia are found in the SN and anti-inflammatory drugs have been associated with reduced risks to develop Parkinson's disease (Chen et al., 2003; McGeer et al., 1988). Within the CNS, microglia can act as macrophages by remove cell debris and fight infections by producing proinflammatory cytokines, e.g. interleukin-1β (IL-1β) and (TNFα). Indeed, post mortem brains from Parkinson's disease patients reveal increased levels of TNFα, IL-1β, IL-6, and transforming growth factor β (TGFβ), and decreased levels of the neurotrophin nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) (Mogi et al., 1994; Mogi et al., 1995; Mogi et al., 1999). Microglia are associated with increased expression of iNOS and NADPH oxidase, enzymes that generate free radicals such as nitric oxide and superoxide (Langston et al., 1999).

Treatments of Parkinson's disease

The most commonly medical treatment used for Parkinson's disease is L-dopa therapy. L-Dopa is a precursor of dopamine that can cross the blood-brain-barrier. The effects of L-dopa was discovered when the impaired motor activity found after reserpin treatment, was counteracted by giving the animals L-dopa (Carlsson et al., 1957; Carlsson et al., 1958). Further studies demonstrated dramatic reversal of the symptoms, although the effect of the drug was brief (Birkmayer and Hornykiewicz, 1962). Later, trials displayed that higher doses and oral administration of L-dopa were efficient (Cotzias et al., 1967). Today, administration of L-dopa is given together with decarboxylase inhibitors to decrease the peripheral metabolism. However, after some years of treatment, the effect of L-dopa decline and the patients develop dyskinesias (involuntary movements) and/or on-off symptoms due to fluctuations in the drug dosage (Granerus, 1978; Lang and Lozano, 1998a; Lang and Lozano, 1998b; Rinne, 1981). Dopamine agonists acting on the dopamine receptors are also given as an alternative to enhance the motor activity. Further, treatments to hamper the breakdown of dopamine by inhibiting the catechol-O-methyl transferase (COMT) and monoamine oxidase B are undertaken. Various combinations of the above-mentioned drugs are used as therapies for Parkinson's disease. However, the medications only improve motor activity deficiencies while do not cure the disease.

One way to counteract the symptoms of the disease is to use deep brain stimulation, where high frequently stimulating electrodes are implanted in the basal ganglia. Implanting the electrodes to the ventral intermediate nucleus of the thalamus can reduce tremor (Benabid et al., 1991; Putzke et al., 2003). Stimulation of either the subthalamic nucleus or the globus palludus interna diminishes bradykinesia, rigidity, as well as reduces L-dopa-induced dyskinesia (Benabid et al., 1998; Kumar et al., 1998a; Kumar et al., 1998b).

Yet another method to counteract the symptoms of Parkinsons's disease is to replace the lost dopaminergic neurons. Already in 1979, fetal dopaminergic transplants were evaluated as a putative method to counteract the symptoms of Parkinson's disease in a 6-hydroxydopamine (6-OHDA) animal model (Björklund and Stenevi, 1979; Perlow et al., 1979). The transplants were proven to be long-term surviving (Freed et al., 1980; Stromberg and Bickford, 1996), and the striatum of the host was reinnervated in a target specific manner (Strömberg et al., 1992; Wictorin et al., 1992). Further, new synapses were formed (Freund et al., 1985; Jaeger, 1985; Mahalik et al.,

1985; Stromberg et al., 1988), and the grafts were able to release dopamine in the reinnervated striatum (Rose et al., 1985; Stromberg et al., 1988; Zetterstrom et al., 1986). Ten years after the first animal transplantation, human fetal VM was grafted for the first time to patients. Postmortem analysis displayed graft survival and reinnervation of the striatum (Kordower et al., 1998; Lindvall et al., 1988; Madrazo et al., 1988). Unfortunately, the outcome of the clinical trials revealed poor cell survival and only portions of the host brain became reinnervated, which was also found in animal experiments together with the findings that reinnervation of grafted neurons terminated shortly after implantation, the striatum was not fully reinnervated, only subpopulations of the grafted neurons innervated the striatum, and recent findings show that some patients develop dyskinesia (Barker et al., 1996; Bjorklund et al., 2003; Brundin and Bjorklund, 1998; Freed et al., 2001; Hagell et al., 2002; Kordower et al., 1996; Olanow et al., 2003; Törnqvist et al., 2001). Thus, it is important to find various neuroprotective and neurotrophic factors that can enhance survival and neuritic formation.

A major problem with grafting fetal tissue is the limited availability of tissue. Stem cells, i.e. self renewal and multipotent cells, are alternative sources for neuronal transplantation. During the last couple of years, much effort has been performed regarding stem cells to be used in various neurodegenerative disorders. However, major concerns regarding scientific, clinical and ethical issues need to be resolved before stem cells can be used in Parkinson's disease as therapy, reviewed in (Newman and Bakay, 2008; Parish and Arenas, 2007; Wang et al., 2007).

Experimental analysis of Parkinson's disease

In order to investigate Parkinson's disease, various animal models have been developed where different neurotoxins are utilized. A common method used includes injections of the neurotoxin 6-OHDA, an analogue to dopamine. The toxin 6-ODHA can be taken up by the dopamine transporter in the dopamine neurons, and causes cell death of the neurons by formation of reactive oxygen species, as well as hamper the mitochondrial respiratory chain, for review see (Blum et al., 2001). 6-OHDA does not cross the blood brain barrier and therefore, needs to be administrated locally. Injecting the neurotoxin into the SN or the medial forebrain bundle, a complete lesion of the nigrostriatal pathway can be obtained, whereas injecting the 6-OHDA into the striatum results in a slower progression of the neurodegeneration due to a reterograde transport of the 6-OHDA compounds to the SN (Sauer and Oertel, 1994; Ungerstedt, 1968). Thus, a Parkinson's disease animal model is generated. Another neurotoxin commonly used in animal studies is MPTP, which is described above, for review see (Blum et al., 2001). Yet another animal model of Parkinson's disease is rotenone, a commonly used pesticide. Similar to MPTP, rotenone crosses the blood-brain-barrier and hampers the mitochondrial respiratory chain (Betarbet et al., 2000) (Perier et al., 2003) (Ferrante et al., 1997).

Another approach to study neurodegenerative disorders is to isolate brain regions of interest in order to evaluate interactions. The *in oculo* grafting method presents an unique approach to study interactions since tissues can be evaluated in an isolated *in vivo* co-culture system. The tissue pieces are implanted through the cornea to the anterior chamber of the eye and the grafts can survive as long as the host animal (Eriksdotter-Nilsson et al., 1988). The grafted tissue pieces maintain the cytoarchitecture and organization, hence resembling the normal conditions in the brain. Further, the *in oculo* grafts develop an intact blood-brain-barrier some weeks postgrafting, creating small brain circuits under *in vivo* condition (Granholm et al., 1996). The transplants are easily identifiable through the translucent cornea, and tissue pieces can be grafted in sequence allowing different ages of the implanted tissues. Therefore, this grafting method is very useful in order to evaluate factors involved in aging diseases, e.g. Parkinsons's disease, for review see (Strömberg, 2000). The intraocular grafting method has for example been undertaken to explore VM nerve fiber innervation to either immature or mature striatum. It was proven that the fetal dopaminergic nerve fiber innervation of the mature striatum was rather sparse and widespread in comparison to the innervation of the striatal anlage, which displayed a dense patchy pattern (Strömberg et al., 1997; Törnqvist et al.,

2001; Vidal et al., 1998). This correlates to the normal development of the brain, where this patchy pattern normally is found in the striatal anlage during development (Olson et al., 1972). Further, the role of different neurotrophic factors, e.g. glial cell line-derived neurotrophic factor (GDNF) on VM have been investigated (Johansson et al., 1995; Stromberg et al., 1993).

Different neurotrophic factors have been investigated in order to enhance the nerve fiber outgrowth from the VM graft to the target. Some factors implicated to promote VM dopamine neurons, besides the already mentioned GDNF, are fibroblast growth factors (FGFs), brain derived neurotrophic factor (BDNF), neurturin, and neurotrophin-4/5 (Date et al., 1993; Giacobini et al., 1993; Horger et al., 1998; Hyman et al., 1991; Hynes et al., 1994; Knusel et al., 1990; Lin et al., 1993; Mayer et al., 1993), for review see (Collier and Sortwell, 1999). When analyzing the border between the graft and the host, some areas display more nerve fibers innervating the striatum than other areas. Interestingly, glial processes radiating from the graft to the host are found within these areas (Strömberg et al., 1992; Strömberg et al., 1995). Astrocytes, with their multiple different functions, e.g. interactions with neuronal cells and production of various extra cellular matrix (ECM) molecules, are important in controlling and guiding neuronal growth (Gates et al., 1993; O'Brien et al., 1992; Powell and Geller, 1999). ECM molecules, e.g. proteoglycans (PGs), are suggested important for the formation of the nigrostriatal pathway by participating in the guidance of the dopaminergic growth during development (Charvet et al., 1998a; Charvet et al., 1998b; Gates et al., 1996; O'Brien et al., 1992). Hence, it is important to investigate how to guide the fetal dopaminergic neurons in order to achieve a better innervation of the target when grafting the fetal VM to the dopamine-denervated striatum.

Performing cell cultures, it has been demonstrated that immature fetal VM produce TH-positive nerve fibers that either are formed in the presence or absence of astrocytes (Johansson and Strömberg, 2002; Takeshima et al., 1994) (Figure 3). Notably, only the nerve fibers found in the presence of astrocytes (glial-guided nerve fibers) were persistant over time, and terminated their growth within the same time period as when intracranial VM grafts halt their reinnervation to the striatum (Barker et al., 1996; Johansson and Strömberg, 2002). Further, the glial-guided nerve fibers innervated, while the nerve fibers found in the absence of astrocytes (non-glial-guided nerve fibers) avoided the LGE, which revealed a presence of subpopulations of TH-positive neurons within the VM tissue pieces (Johansson and Strömberg, 2003). This correlates with data from intracranial grafting where

only a subpopulation of the grafted neurons innervated the striatum (Strömberg et al., 2001; Törnqvist et al., 2001).

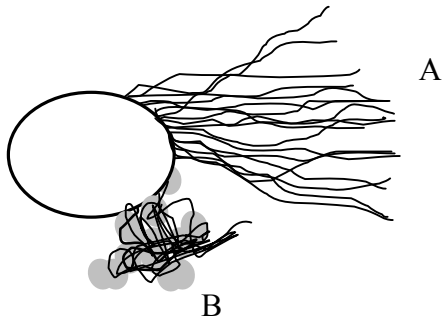

Figure 3. A schematic drawing of the two TH-positive nerve fiber outgrowths found when culturing fetal VM tissue pieces. The neurites are found either in the absence of glial cell (A), or in the presence of glial cells (visualized by grey circles) (B). Instead of radiating straight out from the tissue, the glial-guided nerve fibers change direction and start to arborize creating a network of nerve fibers surrounding the tissue, reaching a plateau in length of approximately 1 mm from the tissue slice after 10-14 DIV. Only the glial-guided nerve fiber outgrowth is persistent over time.

To improve survival of grafted dopamine neurons, antioxidants have shown to be beneficial (Björklund et al., 1997; Nakao et al., 1994). Antioxidants, often rich in fruits and vegetables, can easily be administrated through the diet, e.g. blueberry-enriched diet, and participate in scavenging oxygen free radicals in the circulation (Williams et al., 2004). Antioxidants from supplemented diets can cross the blood-brain-barrier and are found in for example the cortex, cerebellum, eye, and liver (Kalt et al., 2008; Matsumoto et al., 2006). Indeed, the addition of antioxidants to the diet has been proposed to be beneficial for neuronal maintenance in both aging and neurodegenerative disorders (Bickford et al., 1999; Bickford et al., 2000; Joseph et al., 1999; Joseph et al., 2005; Martin et al., 2000; Shukitt-Hale et al., 2008). Further, although many kinds of fruits and vegetables contain antioxidants, it has been shown that blueberries actually are one of the best sources for antioxidants (Prior and Cao, 2000). In animal experiments investigating Parkinson's disease, the addition of blueberries enhances the release of dopamine in the striatum as well as the reinnervation after injury, and facilitates motor improvement (Datla et al., 2004; Joseph et al., 1998; Joseph et al., 1999; Martin et al., 2000; Stromberg et al., 2005). Hence, it

appears as if the supplementation of antioxidants to the diet is beneficial for the outcome of various neurodegenerative disorders, including Parkinson's disease.

In this thesis the two nerve fiber formations found in cultured fetal VM will be investigated in more detail to elucidate how to guide the neurons, and thus achieve improved outcome in future grafting clinical trials. Antioxidants have been proven to be important in neurodegenerative disorders but the role of antioxidants during development is rather unexplored. Additionally, LC appears to protect the dopaminergic neurons while nothing is known about the role of LC during VM development.

AIMS

- To explore the role of glial cells for the nerve fiber formation, and the presence of dopamine subpopulations in organotypic VM cultures

- To examine the function of proteoglycans regarding nerve fiber formation in VM cultures

- To elucidate the role noradrenaline exerts on fetal VM *in oculo* transplants

- To study the effects a blueberry-enriched diet in combination with the presence of fetal LC would have on fetal VM *in oculo* transplants

MATERIAL AND METHODS

Animals

Female Sprague-Dawley rats (150 g) were used as recipients for *in oculo* grafting in paper III and IV. Fetal tissue for cultures (paper I, II) or grafting (paper III, IV) was obtained from pregnant rats of the same strain (Scanbur B&K, Sollentuna, Sweden). Animals were housed on a 12-hour day/night cycle under constant ambient temperature, given water and chow *ad libitum*. Blueberry-enriched diet (2%, NIH-31 diet) was given animals in paper IV. All experiments were approved by the local ethics committee.

Dissection procedure

Pregnant rats were anaesthetized with 4% isofluran (Baxter Medical AB, Sweden) using Univentor 400 anesthesia unit (AgnThos, Sweden), palpated to confirm pregnancy, and the age of the fetuses was determined before dislocation of the neck. The abdomen was washed with 70% ethanol, cut open, and fetuses of embryonic day (E) 13-14.5 (crown-rump-length 10-14 mm) were taken out and placed in a Petri dish. Thereafter, the dissection procedure was performed under sterile conditions in Dulbecco's modified Eagle medium (DMEM; Gibco, Sweden). Ventral mesencephalon (VM) (paper I-IV) and locus coeruleus (LC) (paper III-IV) were bilaterally dissected using a dissection microscope. The mesencephalic flexure was identified and the ventral part isolated to obtain VM tissue, and for LC the pontine flexure was used as an orientation marker. Isolated tissue pieces were kept in sterile DMEM for a maximum of two hours before grafting or cell culturing (Figure 4).

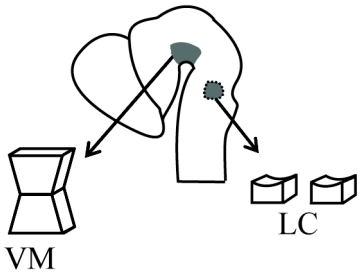

Figure 4. Bilateral dissection of fetal VM and LC utilized for cell cultures and intraocular grafting.

Roller drum cultures

The roller drum culture technique (Gähwiler et al., 1997; Stoppini et al., 1991) was used in paper I and II. Isolated butterfly-shaped VM pieces were chopped in 300 μm coronal sections (Mcllwain tissue chopper). The slices were transferred to fresh DMEM and divided at the midline, and tissue was plated as single pieces on coverslips. The coverslips (12x24 mm) had been washed (xylene 4 hr, acetone 4 hr, isopropanol 12 hr, distilled water 30 minutes, isopropanol 30 minutes), dried at 80ºC for 30 minutes, autoclaved, and coated with poly-D-lysine (5mg/100ml dH_2O; Sigma-Aldrich, Sweden), and left to air dry before being used. Two drops of chicken plasma (1.7mg/ml, Sigma-Aldrich, Sweden) were added to each coverslip, one organotypic VM tissue piece was placed on each glass, one drop of thrombin (1000 units/ml; Sigma-Aldrich, Sweden) was added, and the plasma and thrombin was stirred together. The plasma/thrombin clot was left to air dry for approximately 20 minutes. The coverslips were placed in 15-ml Falcon tubes containing 0.9 ml preheated (37ºC) medium. The tubes were placed in a roller drum, which rotated half a turn every minute in an incubator (Figure 5). The cultures were kept at 37ºC in 5% CO_2 for 1, 2, 3, 7, 14, 21, and 28 days *in vitro* (DIV) (paper I) and 9 DIV (paper II). Medium was changed every third day.

Insert cultures

The membrane insert culture technique was utilized in paper I. Inserts (pore size 0.4 μm, Millipore) were placed in six-well dishes containing 1.7 ml preheated (37°C) medium. One unilateral organotypic VM tissue piece (for dissection procedure, see roller drum cultures) was put on each insert using a spatula (Figure 5). Cultures were kept at 37°C in 5% CO_2 for 7 and 14 days *in vitro* and the medium was changed every third day.

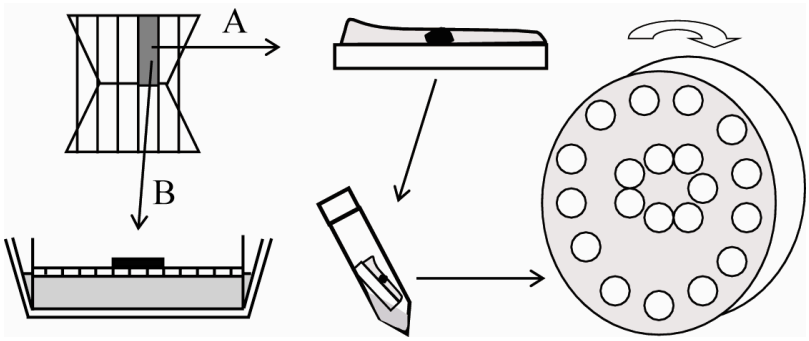

Figure 5. Fetal VM organotypic cultures were performed in paper I and II, using the roller drum (A) and insert culture (B) techniques. The picture is adapted from (Gähwiler et al., 1997).

Astrocytic cell suspension cultures

Astrocytic cell suspension cultures were undertaken in paper II. The butterfly-shaped VM tissue was dissected as described above and placed in a 15 ml Falcon tube containing 1 ml DMEM. Using a standard Pasteur pipette, the tissue was mechanically dissociated by trituration (10-15 strokes). A 1 ml syringe with a 18G needle and then a 23G needle further dissociated the tissue. The cell suspension was centrifuged for 5 minutes at 1000 rpm, the supernatant was removed and the cell pellet was resolved in 1 ml culture medium. Using a Bürcher chamber, the number of cells was counted and seeded with a density of 3.3 x 10^6 cells/ml in 50 ml culture flasks (Cellstar, Frickenhausen, Germany). Cells were allowed to settle for 48 hours, the flasks were shaken and medium was changed. Cells were kept in culture (37°C, 5% CO_2) for 28 DIV, and medium was changed every third day. Cells were detached from the flask surface by adding 5ml Hanks' buffered salt solution (HBSS, Ca^{2+}/Mg^{2+}-free, Gibco, Sweden) supplemented with 0.025% trypsin and 0.025% EDTA and incubated for approximately half an hour at 37°C. The cells were collected using a cell scraper, the cell solution was transferred to a 15 ml Falcon tube containing 5 ml of culture medium, centrifuged for 5 minutes at 1000 rpm, the supernatant was removed and cells were resuspended in 1 ml culture medium. Cells were once again counted and seeded into eight-well glass-slide culture dishes (Falcon) with the density of 25 000 cells/well, and kept in culture for another 14 DIV. Medium was changed every third day.

Culture medium

Culture medium was prepared of the following components: 55% DMEM, 32.5% HBSS, 10% fetal bovine serum (Gibco), 1.5% glucose and 1% hepes (Gibco). The medium was sterile filtered (0.22 µm pore size filters, Sterivex, Millipore) and aliquots were kept in -20°C, thawed and preheated (37°C) before use. At plating, the medium was supplemented with antibiotics-antimycotics (10,000 units/ml penicillin, 10 mg/ml streptomycin, and 25 µg/ml amphotericin; Gibco) to a final concentration of 1%. The antibiotics/antimycotics was excluded from the first medium change. Additionally, methyl-umbelliferyl-β-D-xyloside (β-xyloside; Sigma) was added to both roller drum cultures (0.3 mM, 1.5 mM) and cell suspension cultures (1.5 mM) in paper II to inhibit proteoglycan synthesis.

Western blot

In paper II, western blot analysis was undertaken. After 9 DIV, VM roller drum cultures were scraped off, 25-30 cultures were pooled into 100 µl 1% sodium dodecyl sulfate (SDS) and kept at -70°C until being processed. Samples were thawed on ice, sonicated (3 x 10 sec) and centrifuged. The BCA Protein Assay Kit (Pierce, Rockford; Illinois, USA) was used to determine the protein concentration according to the user's manual. From each sample, 40 µg protein was mixed with 2 x loading buffer containing 15% 2-mercaptoethanol, boiled (95 °C, 10 minutes), and cooled on ice. The samples were loaded on 7.5% SDS polyacrylamide gels using the Protean II XI vertical electrophoresis system to separate the proteins (Biorad, Hercules, Ca, USA) according to the user's manual. In order to determine the molecular weights of the proteins, the kaleidoscope molecular weight standard was used (Biorad). Proteins were transferred onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Uppsala, Sweden) using the Trans-Blot Semidry transfer (Biorad) according to the user's manual. Poncreau S stain (Sigma) was used to evaluate efficient transfer. Membranes blotted for TH and actin were preblocked in 5% milk in 1 x tris buffer saline-tween (TBS-T). Membranes blotted for neurocan were preblocked in 1% BSA in 1 x TBS-T. Preblocking the membranes was performed for 1 hour at room temperature while shaking. Incubation in primary antibodies was performed at room temperature using surface tension, and the incubation time for TH and actin was 1 hour, while neurocan was incubated for 2.5 hours. Secondary antibodies were incubated for one hour at room temperature while shaking. For information regarding antibodies, see table 1 and 3. Between and after incubations with antibodies, the membranes were washed 3 x 10 minutes in 1 x TBS-T. Chemiluminescence detection was utilized using ECL plus and Hyperfilm ECL (Amersham Biosciences) in a table top processor (CEAPRO), all according to manufacturers' instructions.

Intraocular grafting

Intraocular grafting was performed in paper III and IV. Two weeks prior the intraocular implantation, rats were bilaterally sympatectomized during deep isofluran anesthesia (Baxter Medical AB, Sweden) using Univentor 400 anesthesia unit (AgnThos, Sweden). The endogenous innervation of noradrenaline to the host iris was thus removed. The time interval between the removal of the ganglion and the intraocular implantation is preferred to normalize the levels of neurotrophic factors, which become elevated after the denervation (Ebendal et al., 1983; Ebendal et al., 1985).

Atropin was given each eye prior to grafting to dilate the pupil, thus preventing injuries to the iris when the cornea is opened. During deep anesthesia, a slit in the cornea was made using a razor blade and the rats received the transplants into the anterior chamber of the eye using a Pasteur pipette with a modified tip. In paper III, rats with intact superior cervical ganglia were also used as host animals when grafting. In paper IV, 28 days prior the sympatectomy animals received 2% blueberry-enriched diet. The animals were kept on this diet throughout the experiment. Unilateral tissue pieces from VM or LC were transplanted in paper III and IV (Figure 6 and 7).

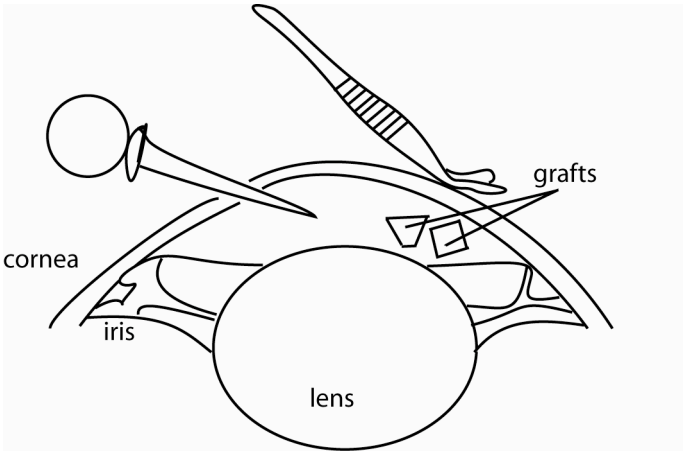

Figure 6. In paper III and IV, unilateral VM and LC were grafted using the intraocular method where tissue pieces were implanted into the anterior chamber of the eye using a Pasteur pipette and gently moved into place by pushing the cornea with a pair of forceps.

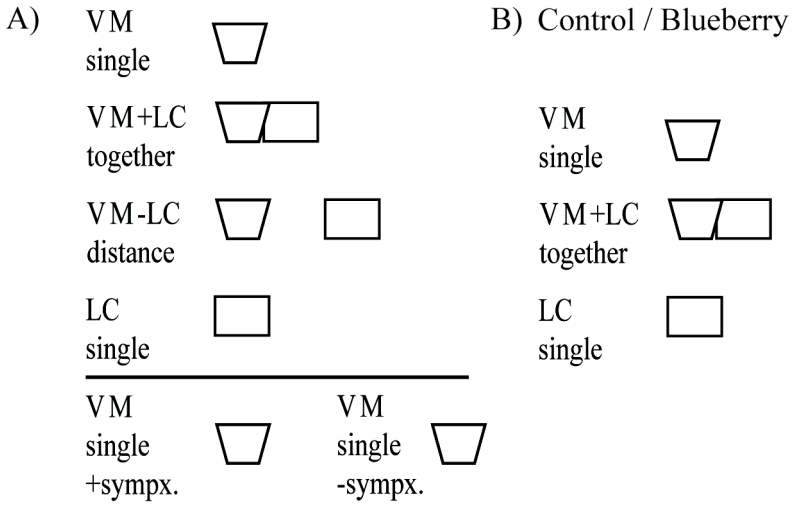

Figure 7. Experimental design for paper III (A) and IV (B). Unilateral VM and LC were implanted as single VM or LC grafts, VM+LC co-grafts attached to each other, or VM-LC with a distance between the tissue pieces. Additionally, single VM was grafted to animals with intact superior cervical ganglia. In paper IV, animal received 2% blueberry-enriched diet.

Immunohistochemistry

Grafted animals (paper III and IV) were anesthetized i.p. with pentobarbitalnatrium, followed by intracardial injections of 50 ml Tyrode solution (Ca^{2+} free), and 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). The intraocular transplants were dissected, postfixed for one hour, and rinsed in 10% sucrose in 0.1 M phosphate buffer. The intraocular grafts were stored in sucrose solution at 4ºC until processed for indirect immunohistochemistry. The transplants were rapidly frozen in gaseous CO_2, and cryostat sections (14 µm) were collected and thawed onto gelatine-coated glass slides. Cultures (paper I, II) were fixed in 2% paraformaldehyde in 0.1 M phoshate buffer for 1 hour before being processed for indirect immunohistochemistry.

Before and after incubations of antibodies, cultures and sections were rinsed 3 x 10 minutes in 0.1 M phosphate buffered saline (PBS). Cultures were incubated with antibodies dissolved in 1% triton-X-100 in 0.1 M PBS. Sections were incubated in antibodies diluted in 0.3% triton-X-100 in 0.1 M PBS. However, the primary antibodies ALDH1 and calbindin were diluted in 0.1 M PBS containing 2 and 10 % serum, respectively (Sigma-Aldrich, Sweden). When triton-X was excluded in incubations of primary antibodies, sections and cultures were preincubated in 0.3% triton X-100 in 0.1 M PBS containing 10% serum for one hour at room temperature and rapidly rinsed in 0.1 M PBS before the primary antibodies were applied. Incubations of primary antibodies were performed for 48 hours at 4°C. Blocking unspecific staining was performed using 5% goat serum (Sigma-Aldrich, Stockholm, Sweden) in 0.1 M PBS for 15 minutes at room temperature before incubations of secondary antibodies. Secondary antibodies were applied for one hour at room temperature. In paper I and II, cultures were stained with DAPI for 10 minutes at room temperature to visualize cell nuclei. Double or triple labeling was performed in sequence, with one antibody at a time. All incubations of antibodies were performed in a humified atmosphere. Antibodies used are listed in table 2 and 3. Cultures and sections were mounted in 90% glycerol. Insert cultures were processed for immunohistochemistry directly on the membranes, and mounted by cutting out a piece of the membrane, which was placed between an object glass and coverslip.

Table 1: Primary antibodies used for western blot in paper II.

Antibodies	**Dilution**	**Company**	**"Detection"**
Actin	1:10 000	Chemicon, mouse	actin, loading control
TH	1:10 000	Immunostar, mouse	dopamine neurons
Neurocan	1:500	Chemicon, mouse	proteoglycans

Table 2: Primary antibodies used for immunohistochemistry in paper I-IV.

Antibodies	**Dilution**	**Company, Species**	**Paper**	**"Detection"**
ALDH1	1:150	Abcam, rabbit	I, III	A9 dopamine neurons
βIII-tubulin	1:200	Sigma-Aldrich, mouse	I	microtubules ("pan-neuronal" marker)
$Calbindin_{D28k}$	1:2000	Swant, mouse	III	A10 dopamine neurons
DBH	1:150	Chemicon, mouse	III, IV	noradrenaline neurons
GAD 65/67	1:100	Sigma-Aldrich, rabbit	I	GABA neurons
Iba-1	1:1000	Wako Chemicals, rabbit	I, IV	pan-microglia marker
Neurocan	1:1000	Chemicon, mouse	II	proteoglycans
NG2	1:200	Chemicon, rabbit	I	oligodendrocyte precursors
Ox-6	1:200	Serotec, mouse	IV	MHC class II microglia
S100β	1:200	Dako. rabbit	II	astrocytes
TH	1:1500	Immunostar, mouse	I-IV	dopamine neurons
TH	1:300	Pel-Freez, rabbit	I-IV	dopamine neurons
Vimentin	1:200	Sigma-Aldrich, rabbit	I	astrocytes
DAPI	1:50	Molecular Probes	I, II	Cell nuclei

Table 3: Secondary antibodies used in article I-IV.

Antibodies (immuno-histochemistry)	**Species**	**Dilution**	**Company**	**Paper**
Alexa 488	Goat anti-mouse	1:200	Molecular Probes	I-IV
Alexa 488	Goat anti-rabbit	1:500	Molecular Probes	I-IV
Alexa 594	Goat anti-mouse	1:500	Molecular Probes	I-IV
Alexa 594	Goat anti-rabbit	1:500	Molecular Probes	I-IV
Antibodies (western blot)	**Species**	**Dilution**	**Company**	**Paper**
ImmunoPure IgG	Goat anti-mouse	1:70 000	Pierce	II

Graft volume, nerve fibers, and astrocytic migration

In paper I and II, nerve fiber outgrowth and astrocytic migration were measured from the peripheral borderline of the tissue slice to the distal end that fibers or astrocytes had reached. Four measurements were collected from each culture and parameter, and the mean values were used for statistical analysis.

In paper II, immunoblot density was evaluated using the NIH Image software (Gel plotting macro, version 1.63). Each grey value obtained for TH and neurocan 160 kDa was divided by the grey value of the loading control (actin) and expressed as percentage of the controls. Each sample was evaluated 2-3 times and mean values for each sample were calculated and used for the statistics.

In paper III and IV, the placement of the intraocular grafts was documented when implanted to the anterior chamber of the eye. Grafts were monitored through the cornea and graft sizes could be measured over time using a microscope equipped with a scale in one of the oculars. By measuring the longest diameter and the diameter perpendicular to it, the volume of the grafts was estimated by multiplying the two measurements (the thickness of the grafts had been estimated to 1 mm). This estimation of graft volume has been demonstrated to be valid (Björklund et al., 1980).

In paper III and IV, the density of DBH-positive nerve fibers were analyzed. Images using a CCD camera (ProgRes C14, Jenaoptic) were captured using the 20 x objective. The NIH Image software (Image macro, version 1.63) was used to measure the optical density of DBH-immunoreactive fibers,

where binary images were utilized to calculate grey values (Stromberg et al., 2005). In LC, areas containing DBH-positive neurons were avoided. Mean values were calculated and analyzed from 3-4 measurements of each transplant.

Cell counts

In paper II, astrocytic cell suspension cultures were analyzed using a standardized frame at 10 x magnification. Four measurements were performed on each suspension culture and mean values were calculated and statistically evaluated.

The number of Iba-1-positive microglia was counted in paper I and IV. At 20 x magnification, a standardized frame mounted in one of the oculars was used, and the number of microglia within the frame was counted. Approximately four measurements were performed on each culture or transplants, and mean values were calculated and statistics was performed.

In paper III and IV, neurons were counted on every fifth section throughout the grafts, and the Abercrombie formula was used to estimate the total numbers of neurons in the grafts (Abercrombie, 1946). In VM grafts, neurons being TH-positive/DBH-negative were counted, while in LC grafts TH-positive/DBH-positive neurons were counted. The border between the two grafts was determined using the DAPI filter. ALDH1/TH-positive neurons and calbindin/TH-positive neurons were counted and expressed as number of neurons displaying double labeling. Mean values were used for statistical analysis.

A digital camera (Hamamatsu) was used to capture images of cultures and transplants, and Open lab software (Improvision) was used to process the images. Identical areas were photographed at different wavelengths and merged to achieve pictures of double or triple labeling.

Statistics

Throughout the experiments, cultures and transplants were blind-coded when analyzed. Statistical comparison was performed using one-way analysis of variance (ANOVA), followed by Fisher's *post hoc* analysis (paper I, II) and Bonferroni *post hoc* analysis (paper III, IV). In paper II, the proportion of cultures demonstrating the different nerve fiber outgrowths was analyzed using the χ^2 –method. In paper III, Student's independent *t*-test was performed to analyse grafts ± sympatectomy. Values are expressed as means ± SEM. Differences were determined as significant when $p<0.05$*, $p<0.01$**, and $p<0.001$***.

RESULTS AND DISCUSSION

Attempts have been made to replace the degenerating dopaminergic neurons in Parkinson's disease by grafting fetal VM to the dopamine-denervated striatum. The outcome of the grafting experiments displays reinnervation of the host striatum in a narrow zone surrounding the graft. Thus, to explore factors that might participate when the grafted dopaminergic neurons innervate the dopamine denervated striatum, organotypic E14 VM tissue cultures have been performed. Two different TH-positive nerve fiber outgrowth patterns from the tissue slice were detected (Johansson and Strömberg, 2002). Soon after plating, TH-positive nerve fibers were seen projecting straight away from the tissue slice, reaching distances above 3 mm at 21 DIV. Although the length of these TH-positive nerve fibers was extremely long, the nerve fibers were not persistent over time, and appeared to degenerate or retract after some days *in vitro* (DIV). These first formed TH-positive nerve fibers were observed in the absence of glial cells (non-glial-guided). A second TH-positive nerve fiber outgrowth from the VM tissue slice was formed onto migrating astrocytes (glial-guided). This second wave of TH-positive nerve fibers was persistent over time, and reached the length of approximately 1 mm (Figure 3). Interestingly, when co-cultures of VM and LGE were undertaken, only the glial-guided TH-positive nerve fiber outgrowth projected to the LGE (Johansson and Strömberg, 2003). These results suggest the presence of subpopulations of TH-positive neurons within the VM tissue, which act differently when forming their nerve fibers. Therefore, paper I was conducted to characterize these two different nerve fiber formations from organotypic VM tissue cultures in more detail regarding the presence of different glial cells and subpopulations of dopaminergic neurons. These two nerve fiber projections will from now on be referred to as non-glial-guided-, and glial-guided nerve fibers.

The non-glial-guided nerve fibers in VM cultures

Already at one day after plating, β-tubulin-positive nerve fibers were observed to project from the organotypic VM tissue slice in the absence of cell bodies, i.e the first sequence of nerve fiber outgrowth (paper I). First one day later, TH-positive nerve fibers displaying the non-glial-guided outgrowth were observed. Both β-tubulin-positive/TH-negative and β-tubulin/TH-positive non-glial-guided nerve fibers displayed a dotted appearance at later time points as if they were degenerating. However, in the tissue cultures displaying healthy neurites, the length of the non-glial-guided nerve fibers reached up to 4 mm. Notably, thin vimentin-positive fibers were observed

among these first non-glial-guided nerve fibers, which originated from large polygonal vimentin-positive cells, located in the periphery of the tissue slice. At seven days after plating 69% of the cultures displayed these vimentin-positive fibers, while at 21 DIV only 23% of the cultures demonstrated these vimentin-positive fibers. At this time point the vimentin-positive processes were as long as the TH-positive nerve fibers, i.e. approximately 3 mm.

The results from paper I revealed that the non-glial-guided nerve fibers found in organotypic VM cultures rather is a general phenomenon than specific for dopaminergic neurons since β-tubulin-positive/TH-negative nerve fibers also demonstrated this pattern. Hence, the TH-positive non-glial-guided nerve fibers were preceded by other neurons. These first nerve fibers might act as guiding/pathfinding fibers, in order to create a scaffold that the following nerve fibers can use for support (Jacobs and Goodman, 1989a; Jacobs and Goodman, 1989b). The finding that vimentin-positive processes were detected along these non-glial-guided fibers supports this pathfinding theory. Vimentin is an early astrocytic marker also utilized to characterize radial glia, acting as scaffold for other nerve fibers and cells during development (Hartfuss et al., 2001; Hidalgo and Booth, 2000; Malatesta et al., 2000; Schnitzer et al., 1981). However, it has previously been suggested that radial glial might be neuronal progenitors, which can develop into either neuronal stem cells, neurons, or glial cells (Gotz et al., 2002; Malatesta et al., 2000). Unfortunately, no conclusions about the nature of these vimentin-positive fibers could be drawn from the present study. However, the fact that more cultures displayed these vimentin-positive processes early after plating supports the pathfinding theory. Taken together, the non-glial-guided nerve fibers are not formed in the total absence of glial cells.

The glial-guided nerve fibers in VM cultures

At three days after plating, vimentin-positive astrocytes were migrating from the VM tissue slice, forming a monolayer. This migration was rather sparse at 3 DIV. Over time, the migration of astrocytes proceded, and reached a plateau at approximately 14 DIV. The glial-guided nerve fibers were found on the migrating astrocytes. Instead of radiating straight away from the tissue slice without changing direction as the non-glial-guided nerve fibers did, both β-tubulin-positive/TH-negative and β-tubulin/TH-positive nerve fibers of the glial-guided nerve fibers changed the direction, creating a network of fibers circulating around the VM tissue slice at the distance of approximately 1 mm. Notably, these glial-guided nerve fibers were persistent over time.
As already mentioned, the glial-guided nerve fibers innervate the LGE, and the innervating nerve fibers could be correlated to areas displaying astrocytes (Johansson and Strömberg, 2003). Further, in intracranial grafting, the

reinnervation of the graft-derived nerve fibers in the dopamine denervated striatum often occurs over areas, i.e. “permissive sites”, where glial processes radiate from the graft to the host (Strömberg et al., 1992; Strömberg et al., 1995). The elongation of the graft-derived nerve fibers terminates after about one week postgrafting, followed by weeks of branching (Barker et al., 1996). The length of the graft-derived innervation is similar to the length of the glial-guided nerve fibers obtained from organotypic VM cultures (Barker et al., 1996; Johansson and Strömberg, 2003). Hence, the glial-guided nerve fibers obtained in organotypic VM cultures is likely the nerve fiber outgrowth that corresponds to the graft-derived outgrowth when implanted into the brain.

The roller drum culture technique vs. the membrane culture technique

To further investigate the two nerve fiber projections, the VM roller drum cultures were compared to VM cultures where the membrane insert culture technique was utilized (paper I) (Gähwiler et al., 1997; Stoppini et al., 1991). Surprisingly, the non-glial-guided nerve fibers were never observed. At 7 DIV, no astrocytes were observed outside the tissue slice. At 14 DIV, astrocytes had migrated from the tissue slice and the glial-guided nerve fibers were displayed. Hence, although the glial-guided nerve fiber ourgrowth was revealed in membrane cultures, a delay in time was observed. When performing roller drum cultures, the tissue slices were attached to the cover slips directly at plating, proven by the fact that β-tubulin-positive nerve fibers displayed the non-glial-guided outgrowth already at one day after plating. However, it seems like the attachment of tissue cultures to the membranes takes longer time. This delayed attachment might be the reason for why the non-glial-guided nerve fibers were absent. Notably, the absence of the non-glial-guided fibers did not result in the lack of glial-guided nerve fibers. This implies that the persistent glial-guided nerve fibers can be formed without being preceded by the non-glia-guided nerve fibers.

A9 vs. A10 dopaminergic neurons in VM cultures

When dissecting fetal VM, both A9 and A10 neurons are included. The two nerve fiber outgrowth patterns observed in organotypic VM cultures could perhaps be correlated to either A9 (SN) or A10 (VTA) neurons, thus immunohistochemistry for ALDH1 was performed. ALDH1 is preferably expressed in the A9 dopaminergic neurons, while A10 dopaminergic neurons are ALDH1-negative (Chung et al., 2005; McCaffery and Drager, 1994). Both the non-glial-guided, and the glial-guided nerve fiber outgrowths displayed TH/ALDH1-positive nerve fibers. Hence, these two nerve fiber patterns observed in VM cultures could not be correlated to either A9 or A10 dopaminergic neurons. This was, however, not expected since the glial-guided nerve fibers targets the LGE, i.e. should display an A9 dopaminergic feature (Johansson and Strömberg, 2003). Additionally, recent data in our group have shown that the non-glial-guided nerve fiber outgrowth targets corical tissue (af Bjerken et al., 2008). To conclude, these results indicate that the correlation between the non-glial-guided and the glial-guided nerve fibers is far more complex that initially thought.

Oligodendrocyte precursors and microglia in VM cultures

Nerve fiber formation in organotypic VM cultures appeared to be correlated to the presence of astrocytes, but the influence from other glial cells needed to be elucidated. Therefore, the presence of oligodendrocyte precursors (NG2-positive cells) was evaluated. Shortly after plating, small and round NG2-positive cells with short processes were found within the tissue slice. At later time points, the NG2-positive cells displayed more processes and formed a network inside the tissue piece, a pattern normal for cultured NG2-positive cells (Bouslama-Oueghlani et al., 2005). From 7 DIV, the NG2-positive cells migrated from the tissue slice, following the same route as the migrating astrocytes, although NG2-positive cells were still present in the tissue slice. The presence of oligodendrocyte precursors could not be correlated to either the dopaminergic neurons or their nerve fiber outgrowths.

The presence of microglia was monitored in the VM cultures using the pan-microglia marker Iba-1. The role of microglia can be both protective and toxic. For example, neuroinflammation has been implicated to participate in the degeneration of dopamine neurons in Parkinson's disease, and elevated levels of various proinflammatory cytokines, for example TNFα and IL-1β, are found in post mortem brains (Mogi et al., 1994). Microglia has also been shown to enhance survival of cultured dopaminergic neurons (Nagata et al., 1993; Zietlow et al., 1999). In our VM cultures, microglia were present in the tissue slice from seven days after plating as small, rounded cells without

processes. From 14 DIV, the microglia displayed short, arborized processes. Notably, at 14 and 21 DIV, microglia were localized in areas devoid of, rather than areas dense with, TH-positive neurons. It has previously been documented that microglia are more rounded immediately after plating, and are thought to be more prone to migrate, which correlates to the rounded cells found in paper I at 7 DIV. Additionally, the fact that the even distribution of microglia found at 7 DIV in the tissue slice changed to an uneven distribution in the cultures at later time points further supports the theory that the microglia migrated at the earlier time point (Grossmann et al., 2002; Stence et al., 2001). Additionally, at 14 DIV some microglia had migrated away from the tissue slice onto the monolayer of vimentin-positive astrocytes, however, no correlation to the presence of nerve fibers was made. Taken together, the fact that astrocytes appeared to guide the migration of both oligodendrocytes and microglia outside the tissue slice implies that astrocytes actually are the key players regarding guidance in the VM cultures.

The role of proteoglycans during dopaminergic development

Since astrocytes appeared important for nerve fiber formation, paper II focused on effects of astrocytic induction of proteoglycans (PGs). PGs are ECM molecules consisting of a core protein and glycosaminoglycan (GAG) chains, and participate in dopamine nerve fiber guidance during development (Charvet et al., 1998a; Charvet et al., 1998b; Gates et al., 1993), and are increased at the border of dopaminergic grafts (Barker et al., 1996; Gates et al., 1996). A major family of PGs is chondroitin sulfates, consisting of aggrecan, versican, brevican, and neurocan, where neurocan has been found in high levels during development (Meyer-Puttlitz et al., 1995). Therefore, neurocan was utilized as marker for PGs in paper II.

Methyl-umbelliferyl-β-D-xyloside (β-xyloside), a PG synthesis inhibitor, was added in the medium when culturing the VM tissue for 9 DIV. This time point was chosen because the astrocytic migration is intense and both the non-glial-guided and the glial-guided nerve fibers are normally present (Johansson and Strömberg, 2002). β-xyloside prevents the GAG chains to bind to the core protein, which results in no release of the proteoglycans (Lander et al., 1998; Niederost et al., 1999; Schwartz, 1977). Hence, β-xyloside blocks the effects of PGs and has been utilized to investigate the role of PGs regarding neuritic growth, proliferation, and migration (Carey et al., 1987; Fichard et al., 1991; Niederost et al., 1999; Ratner et al., 1985).

Effects of β-xyloside supplementation in VM cultures

In paper II, both the non-glial-guided, and the glial-guided nerve fiber formation, documented in paper I, were observed. In control cultures, the non-glial-guided nerve fibers were observed in 54% of the cultures, whereas 98% of the cultures demonstrated the glial-guided nerve fibers. β-xyloside treatment did not affect the length of the non-glial guided nerve fibers, while the length of the glial-guided nerve fibers decreased. Notably, 1.5 mM β-xyloside treatment resulted in that only 63% of the cultures displayed the glial-guided nerve fibers, while the numbers of cultures displaying the non-glial-guided nerve fibers increased to 80%. Interestingly, western blot analysis revealed that the β-xyloside treatment did not alter the amount of TH-protein, although the presence of the two different nerve fiber outgrowths was shifted. The presence of TH-positive nerve fibers located on the migrating astrocytes was not altered after the β-xyloside treatment

The supplementation of β-xyloside to the medium resulted in an impaired proliferation of astrocytes as well as a hampered astrocytic migration. The migrating astrocytes in control cultures displayed neurocan-immunoreactivity located to vesicles, whereas β-xyloside-treated astrocytes demonstrated a diffuse cytoplasmatic expression of neurocan located in the astrocytic processes. Further, the amount of neurocan core protein without any GAG chains was increased after the β-xyloside treatment compared to control cultures.

The finding in paper II that hampered astrocytic migration reduced the number of cultures displaying the glial-guided nerve fibers, while the number of cultures demonstrating the non-glial-guided nerve fibers increased, is rather intriguing. This suggests a relationship between these two nerve fiber formations, which has also been revealed previously where a promoted astrocytic migration resulted in fewer cultures displaying the non-glial-guided nerve fibers, and an inhibition of the astrocytic migration enhanced the presence of the non-glial-guided nerve fibers (af Bjerken et al., 2007; Johansson and Strömberg, 2002). This assumption of a constant relationship between the two nerve fiber outgrowths is further supported by the fact that in paper II, the amount of TH-protein was not altered. The length of the glial-guided nerve fibers was diminished after β-xyloside treatment. Whether this reduction in nerve fiber length was a direct effect due to loss of growth promoting molecules or an indirect effect due to hampered astrocytic migration is not clear from the present study. However, since TH-positive nerve fibers were located on the astrocytes despite the β-xyloside treatment, it is more likely that the reduction in nerve fiber growth was caused by a reduced migration. This is supported by findings demonstrating reduced

migration after β-xyloside treatment (Ratner et al., 1985). Controversially, in the adult brain PGs have for example been found to be upregulated after injury, and the degradation of PGs results in increased regeneration (Moon et al., 2002; Properzi et al., 2005; Steinmetz et al., 2005; Tropea et al., 2003). However, in normal adult brains, PGs are low in expression. Thus, inhibiting the PG expression during development results in reduced nerve fiber growth, while after injury of the adult brain the same inhibition results in increased nerve fiber regeneration.

When grafting dopaminergic neurons, unfortunately low cell survivalis obtained, which stresses the importance to evaluate factors that might promote survival (Brundin and Bjorklund, 1998). It has been postulated that both the LC and antioxidants are beneficial for neuronal maintenance (Heneka et al., 2003; Martin et al., 2000; Mavridis et al., 1991; Prior and Cao, 2000; Srinivasan and Schmidt, 2004; Stromberg et al., 2005), but little is known about the protective properties during development. Thus, papers III and IV were conducted to analyze the effects LC and antioxidant-enriched diet exert on fetal VM utilizing the *in oculo* grafting method, as described in the introduction. Unilateral fetal VM and LC were grafted either as single grafts, or as co-grafts. Additionally, 2% blueberry-enriched diet was given the recipients animals (Figure 7).

Survival of VM and LC intraocular grafts

Measurements of grafts size of VM single, LC single, and VM+LC cografts revealed enhanced volume of the VM grafts attached to the LC, independently of diet given, when compared to single VM grafts (paper III and IV). The volume of VM grafted together with LC but with a distance between the tissue pieces, was not increased. However, the addition of blueberries did not increase the volume of grafted VM. Neither the presence of VM nor the supplementation of blueberries did enhance the volume of LC grafts.

Dopaminergic neurons in the VM grafts were TH-positive/DBH-negative, while noradrenergic neurons in the LC grafts were TH/DBH-positive. In animals given the control diet, the attachment of the LC to the VM resulted in enhanced survival of TH-positive/DBH-negative neurons. When the tissue pieces were grafted with a distance, no increased number of surviving neurons in the VM graft was obtained. When the blueberry-enriched diet was given, single VM grafts displayed more TH-positive/DBH-negative neurons compared to single VM grafts on the control diet. However, no additive effect on the survival of TH-positive/DBH-negative neurons in the VM grafts was obtained in VM co-grafts given diet with supplementation of

blueberries. Further, the presence of the VM or the addition of blueberries did not improve the survival of TH/DBH-positive neurons in the LC grafts.

Interestingly, volume enlargement was observed in the VM grafts attached to the LC, while the addition of blueberries did not influence the size of the VM grafts, however, an increase of surviving dopaminergic neurons were revealed in VM grafts in both experimental setups. Thus, while increase in volume might be an indication for better survival of grafts, it is not necessary in order to obtain improved neuronal survival. What causes the increase in the VM volume when attached to the LC is unfortunately not known. A hypothesis might be that the innervation of noradrenergic nerve fibers found in the VM grafts attached to the LC (see discussion below) promotes growth-stimulating factors. It was surprising to find that the supplementation of blueberries did not yield enlargement in graft volume since *in oculo* hippocampal grafts demonstrated larger grafts after feeding the host with blueberries (Willis et al., 2005). A recent study, where intracranially transplanted VM cell suspension cultures displayed enhanced survival in animals given blueberry-enriched diet, supports our findings in paper IV that increased number of TH-positive neurons was observed after blueberry supplementation (McGuire et al., 2006). Taken together, the presence of LC or the addition of blueberries promoted survival of VM intraocular grafts, while no additive effect was obtained. Notably, neither the presence of VM, nor the addition of blueberries promoted survival of LC grafts.

A9 vs. A10 dopaminergic neurons in intraocular VM grafts

In paper III, we wanted to evaluate whether the presence of the LC would favor survival of subpopulations of VM dopamine neurons, i.e. A9 and A10. Thus, ALDH1/TH-positive as well as calbindin/TH-positive neurons were counted in the VM portion of single- and co-grafts. Dopamine neurons preferably located to the SN are ALDH1-positive, while dopamine neurons preferably located in the VTA are calbindin-immuoreactive (Chung et al., 2005; Gerfen et al., 1985; McRitchie et al., 1996; Rogers, 1992). Single VM grafts displayed ALDH1 immunoreactivity in 31.8 % of the grafts, while VM attached to LC displayed ALDH1-positive neurons in 81.8% of the grafts. Further, the number of neurons displaying ALDH1/TH double labeling in the VM grafts attached to the LC was higher compared to single VM grafts. When analyzing calbindin/TH-immunoreactive neurons, no difference in the number of neurons in the VM grafts was observed.

The results implies that the presence of the LC actually is more beneficial for the dopaminergic neurons preferably located in the SN than in the VTA, although the LC *in situ* innervate both the SN and the VTA (Collingridge et al., 1979; Jones and Moore, 1977; Jones and Yang, 1985). In Parkinson's disease, the dopaminergic neurons in the SN degenerate, which could be a secondary effect due to the loss of the protective input from the LC, and herein we show that the presence of LC increases the number of TH/ALDH1-positive neurons. Thus, the fact that the direct presence of LC could promote survival in the VM is worth considering in grafting trials for Parkinson's disease.

Noradrenergic innervation in VM intraocular grafts

Further attempts were undertaken in paper III to evaluate the role of LC for the survival of VM grafts. When LC was attached to VM, a dense innervation of DBH-positive nerve fibers was observed in the VM graft. When VM and LC were placed at a distance, some DBH-positive nerve fibers were found in the VM grafts, although not as dense as when attached to the LC. Notably, the density of DBH-positive nerve fibers in VM grafts when attached to the LC was similar to that found within the LC grafts. The noradrenergic input from the peripheral nerve fibers in the iris was also evaluated. However, although an intact iris demonstrated DBH-immunoreactivity, the VM grafts were DBH-negative.

It has been shown that noradrenaline improves survival of VM dopaminergic neurons in culture (Troadec et al., 2001). Thus, our finding that dense noradrenergic innervation was correlated to improved survival of the VM grafts, is supported by the data presented by Troadec. Notably, a distance between the LC and the VM grafts resulted in noradrenergic innervation of the VM, although not to such an extent where survival was promoted. Most cell loss occurs early after grafting (Mahalik et al., 1994; Zawada et al., 1996). Considering the fast onset of cell death upon grafting, and most likely a prolonged time for the noradrenergic nerve fibers to reach into the VM when grafted with a distance from the LC, might explain why only the direct attachment of LC promoted survival of dopamine neurons.

Microglia in intraocular grafts

Neuroinflammation participates in the degeneration of the dopamine neurons in Parkinson's disease, and activated microglia have been detected in the SN (McGeer et al., 1988). Further, antioxidant-enriched diet has been proven to affect neuroinflammation (Joseph et al., 2005; Shukitt-Hale et al., 2008; Stromberg et al., 2005). Thus, the presence of microglia was studied in paper IV, using antibodies against Iba-1 and Ox-6. The supplementation of blueberries decreased the number of Iba-1-positive microglia in VM single grafts. The attachment of LC resulted in decreased number of Iba-1-positive microglia in VM compared to single VM grafts from animals given control diet. Iba-1-positive microglia within the grafts from animals given the control diet were rather small and rounded with thin processes, while the Iba-1-positive microglia found in grafts from animals given the blueberry-enriched diet were more distended with thicker processes and appeared to be more activated. However, although the microglia in grafts supplemented with blueberries appeared as more active by their morphology, almost no double immunoreactivity for Iba-1/Ox-6 was observed.

Animals fed with antioxidants display reduced levels of TNFα (Cartford et al., 2002), and our results showing that the number of Iba-1-positive microglia was decreased in the VM grafts when attached to the LC, or in the VM single grafts from animals given blueberry diet, might mimic this effect of levels of micoglia. The Iba-1-positive microglia differed in their morphology, but Iba-1 is a pan-microglia marker, thus more characterization of the microglia was needed. Surprisingly, although the microglia appeared as more active after blueberry treatment, almost no double labeling of Iba-1/Ox-6 was observed. Ox-6 is a marker for MHC class II microglia, and is thought to be a marker for more activated microglia. Therefore, more studies are required in order to elucidate this effect of less microglia observed in our VM intraocular grafts. However, it is clear from this present study that the addition of blueberries or the attachment of LC affects the number of microglia in fetal grafted *in oculo* VM tissue.

CONCLUSIONS

- ✓ The two nerve fiber outgrowth patterns observed in fetal VM organotypic tissue cultures appear to be a general phenomenon during nerve fiber production and cannot be correlated to either A9 or A10 dopaminergic neurons. Further, astrocytes are the key players for guidance within these cultures, while microglia and oligodendrocytes most likely do no influence the nerve fiber formation.

- ✓ Inhibiting the proteoglycans synthesis results in hampered astrocytic migration and proliferation, and the presence of the persistent glial-guided nerve fibers is decreased. Further, the relationship between the two nerve fiber outgrowths from the VM tissue is probable constant, i.e. when the glial-guided nerve fibers are retracted, the non-glial guided nerve fibers are promoted, and vise versa.

- ✓ When transplanting fetal VM and LC tissue together, the attachment of LC yields a dense innervation of noradrenergic nerve fibers in the VM, which facilitates survival of dopaminergic neurons, derived preferably from the SN.

- ✓ Blueberry-enriched diet enhances the survival of dopamine neurons in VM *in oculo* graft. However, no additive effects on VM grafts are obtained when combining the presence of LC grafts with the supplementation of blueberries. Further, both the presence of LC or the addition of blueberries decrease the number of microglia in VM single grafts.

- ✓ Neither the presence of VM, nor the addition of blueberries improved survival of LC *in oculo* grafts.

To conclude, this thesis has revealed the importance of astrocytic guidance for nerve fiber formation in VM organotypic cultures, and the promoting role LC and the addition of blueberries exert on intraocular VM grafts. These findings are important since nerve fiber formation and/or survival of grafted neurons are crucial parameters to study to achieve improved grafting outcome in Parkinson's disease.

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REFERENCES

Abercrombie, M., 1946. Estimation of nuclear population from microtome sections. Anat. Rec. 94**,** 239-247.

af Bjerken, S., Boger, H.A., Nelson, M., Hoffer, B.J., Granholm, A.C., Stromberg, I., 2007. Effects of glial cell line-derived neurotrophic factor deletion on ventral mesencephalic organotypic tissue cultures. Brain Res. 1133**,** 10-9.

af Bjerken, S., Marschinke, F., Strömberg, I., 2008. Inhibition of astrocytes promotes long-distance growing nerve fibers in ventral mesencephalic cultures. *Submitted.*

Agid, Y., 1991. Parkinson's disease: pathophysiology. Lancet. 337**,** 1321-4.

Albin, R.L., Young, A.B., Penney, J.B., 1989. The functional anatomy of basal ganglia disorders. Trends Neurosci. 12**,** 366-75.

Altman, J., Bayer, S.A., 1981. Development of the brain stem in the rat. V. Thymidine-radiographic study of the time of origin of neurons in the midbrain tegmentum. J Comp Neurol. 198**,** 677-716.

Baldereschi, M., Di Carlo, A., Vanni, P., Ghetti, A., Carbonin, P., Amaducci, L., Inzitari, D., 2003. Lifestyle-related risk factors for Parkinson's disease: a population-based study. Acta Neurol Scand. 108**,** 239-44.

Barker, R.A., Dunnett, S.B., Faissner, A., Fawcett, J.W., 1996. The time course of loss of dopaminergic neurons and the gliotic reaction surrounding grafts of embryonic mesencephalon to the striatum. Exp Neurol. 141**,** 79-93.

Beach, T.G., Sue, L.I., Walker, D.G., Lue, L.F., Connor, D.J., Caviness, J.N., Sabbagh, M.N., Adler, C.H., 2007. Marked microglial reaction in normal aging human substantia nigra: correlation with extraneuronal neuromelanin pigment deposits. Acta Neuropathol. 114**,** 419-24.

Benabid, A.L., Pollak, P., Gervason, C., Hoffmann, D., Gao, D.M., Hommel, M., Perret, J.E., de Rougemont, J., 1991. Long-term suppression of tremor by chronic stimulation of the ventral intermediate thalamic nucleus. Lancet. 337**,** 403-6.

Benabid, A.L., Benazzouz, A., Hoffmann, D., Limousin, P., Krack, P., Pollak, P., 1998. Long-term electrical inhibition of deep brain targets in movement disorders. Mov Disord. 13 Suppl 3**,** 119-25.

Bernheimer, H., Birkmeyer, W., Hornykiewicz, O., Jellinger, K., Seitelberger, F., 1973. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. J. Neurol. Sci. 20**,** 415-455.

Bertler, A., Rosengren, E., 1959. Occurrence and distribution of dopamine in brain and other tissues. Experientia. 15**,** 10-1.

Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V., Greenamyre, J.T., 2000. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci. 3**,** 1301-6.

Bickford, P., Shukitt-Hale, B., Joseph, J., 1999. Effects of aging on cerebellar noradrenergic function and motor learning: nutritional interventions. Mech. Ageing Dev. 111**,** 141-154.

Bickford, P., Gould, T., Briederick, L., Chadman, K., Pollock, A., Young, S., Shukitt-Hale, B., Joseph, J., 2000. Antioxidant-rich diets improve cerebellar physiology and motor learning in aged rats. Brain Res. 866**,** 211-217.

Birkmayer, W., Hornykiewicz, O., 1962. [The L-dihydroxyphenylalanine (L-DOPA) effect in Parkinson's syndrome in man: On the pathogenesis and treatment of Parkinson akinesis.]. Arch Psychiatr Nervenkr Z Gesamte Neurol Psychiatr. 203**,** 560-74.

Bjarkam, C.R., Sorensen, J.C., 2004. Therapeutic strategies for neurodegenerative disorders: emerging clues from Parkinson's disease. Biol Psychiatry. 56**,** 213-6.

Bjorklund, A., Dunnett, S.B., Brundin, P., Stoessl, A.J., Freed, C.R., Breeze, R.E., Levivier, M., Peschanski, M., Studer, L., Barker, R., 2003. Neural transplantation for the treatment of Parkinson's disease. Lancet Neurol. 2**,** 437-45.

Björklund, A., Stenevi, U., 1979. Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. Brain Res. 177**,** 555-560.

Björklund, A., Lindvall, O., 1984. Dopamine-containing systems in the CNS. In *Classical transmitters in the CNS.* Elsevier.

Björklund, H., Olson, L., Seiger, Å., Hoffer, B., 1980. Chronic lead and brain development: intraocular brain grafts as a method to reveal regional and temporal effects in the central nervous system. Environ. Res. 22**,** 224-236.

Björklund, L., Spenger, C., Strömberg, I., 1997. Tirilazad mesylate increases dopaminergic neuronal survival in the *in oculo* grafting model. Exp. Neurol. 148**,** 324-333.

Blandini, F., Nappi, G., Tassorelli, C., Martignoni, E., 2000. Functional changes of the basal ganglia circuitry in Parkinson's disease. Prog Neurobiol. 62**,** 63-88.

Blum, D., Torch, S., Lambeng, N., Nissou, M., Benabid, A.L., Sadoul, R., Verna, J.M., 2001. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. Prog Neurobiol. 65**,** 135-72.

Bouslama-Oueghlani, L., Wehrle, R., Sotelo, C., Dusart, I., 2005. Heterogeneity of NG2-expressing cells in the newborn mouse cerebellum. Dev Biol. 285**,** 409-21.

Braak, H., Braak, E., 2000. Pathoanatomy of Parkinson's disease. J Neurol. 247 Suppl 2**,** II3-10.

Brown, T.P., Rumsby, P.C., Capleton, A.C., Rushton, L., Levy, L.S., 2006. Pesticides and Parkinson's disease--is there a link? Environ Health Perspect. 114**,** 156-64.

Brundin, P., Bjorklund, A., 1998. Survival of expanded dopaminergic precursors is critical for clinical trials. Nat Neurosci. 1**,** 537.

Carey, D.J., Rafferty, C.M., Todd, M.S., 1987. Effects of inhibition of proteoglycan synthesis on the differentiation of cultured rat Schwann cells. J Cell Biol. 105**,** 1013-21.

Carlsson, A., Lindqvist, M., Magnusson, T., 1957. 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. Nature. 180**,** 1200.

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

Cartford, M.C., Gemma, C., Bickford, P.C., 2002. Eighteen-month-old Fischer 344 rats fed a spinach-enriched diet show improved delay classical eyeblink conditioning and reduced expression of tumor necrosis factor alpha (TNFalpha) and TNFbeta in the cerebellum. J Neurosci. 22**,** 5813-6.

Charvet, I., Hemming, F.J., Feuerstein, C., Saxod, R., 1998a. Transient compartmental expression of neurocan in the developing striatum of the rat. J Neurosci Res. 51**,** 612-8.

Charvet, I., Hemming, F.J., Feuerstein, C., Saxod, R., 1998b. Mosaic distribution of chondroitin and keratan sulphate in the developing rat striatum: possible involvement of proteoglycans in the organization of the nigrostriatal system. Brain Res Dev Brain Res. 109**,** 229-44.

Chen, H., Zhang, S.M., Hernan, M.A., Schwarzschild, M.A., Willett, W.C., Colditz, G.A., Speizer, F.E., Ascherio, A., 2003. Nonsteroidal anti-inflammatory drugs and the risk of Parkinson disease. Arch Neurol. 60**,** 1059-64.

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

Collier, T.J., Sortwell, C.E., 1999. Therapeutic potential of nerve growth factors in Parkinson's disease. Drugs Aging. 14**,** 261-87.

Collingridge, G., James, T., MacLeod, N., 1979. Neurochemical and electrophysiological evidence for a projection from the locus coeruleaus to the substantia nigra. J. Physiol. 290**,** 44.

Cotzias, G.C., Van Woert, M.H., Schiffer, L.M., 1967. Aromatic amino acids and modification of parkinsonism. N Engl J Med. 276**,** 374-9.

Crossley, P.H., Minowada, G., MacArthur, C.A., Martin, G.R., 1996. Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. Cell. 84**,** 127-36.

Dahlström, A., Fuxe, K., 1964. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of the brain stem neurons. Acta Physiol Scand. Suppl. 232. 62**,** 1-55.

Date, I., Yoshimoto, Y., Imaoka, T., Miyoshi, Y., Gohda, Y., Furuta, T., Asari, S., Ohmoto, T., 1993. Enhanced recovery of the nigrostriatal dopaminergic system in MPTP-treated mice following intrastriatal injection of basic fibroblast growth factor in relation to aging. Brain Res. 621**,** 150-4.

Datla, K.P., Bennett, R.D., Zbarsky, V., Ke, B., Liang, Y.F., Higa, T., Bahorun, T., Aruoma, O.I., Dexter, D.T., 2004. The antioxidant drink effective microorganism-X (EM-X) pre-treatment attenuates the loss of nigrostriatal dopaminergic neurons in 6-hydroxydopamine-lesion rat model of Parkinson's disease. J Pharm Pharmacol. 56**,** 649-54.

Davis, C., Williams, A., Markey, S., Ebert, M., Caine, E., Reichert, C., Kopin, I., 1979. Chronic parkinsonism secondary to intravenous injection of merperidine analogues. Psychiatr. Res. 1**,** 249-254.

Dearry, A., Gingrich, J.A., Falardeau, P., Fremeau, R.T., Jr., Bates, M.D., Caron, M.G., 1990. Molecular cloning and expression of the gene for a human D1 dopamine receptor. Nature. 347**,** 72-6.

DeLong, M.R., 1990. Primate models of movement disorders of basal ganglia origin. Trends Neurosci. 13**,** 281-5.

Di Porzio, U., Zuddas, A., Cosenza-Murphy, D.B., Barker, J.L., 1990. Early appearance of tyrosine hydroxylase immunoreactive cells in the mesencephalon of mouse embryos. Int J Dev Neurosci. 8**,** 523-32.

Ebendal, T., Olson, L., Seiger, Å., 1983. The level of nerve growth factor (NGF) as a function of innervation. A correlative radioimmunoassay and bioassay study of the rat iris. Exp. Cell Res. 148**,** 311-317.

Ebendal, T., Strömberg, I., Olson, L., Seger, Å., 1985. Parasympathetic neurotrophic activity in the rat iris: determination after different denervation. Neurosci. 16**,** 181-185.

Echelard, Y., Epstein, D.J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J.A., McMahon, A.P., 1993. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell. 75**,** 1417-30.

Ehringer, H., Hornykiewicz, O., 1960. Verteilung von noradrenalin und dopamin (3-hydroxytyramin) im gehirn des menschen und ihr verhalten bei erkrankungen des extrapyramidalen systems. Klin. Wschr. 38**,** 1236-1239.

Eriksdotter-Nilsson, M., Gerhardt, G., Seiger, Å., Olson, L., Hoffer, B., Granholm, A.-C., 1988. Age-related alterations in noradrenergic input to the hippocampal formation: Structural and functional studies in intraocular trasnsplants. Brain Res. 478**,** 269-280.

Falck, B., Hillarp, N.-Å., Thieme, G., Torp, A., 1962. Fluorescence of catecholamines and related compounds condenced with formaldehyde. J. Histochem. Cytochem. 10**,** 348-354.

Ferrante, R.J., Schulz, J.B., Kowall, N.W., Beal, M.F., 1997. Systemic administration of rotenone produces selective damage in the striatum and globus pallidus, but not in the substantia nigra. Brain Res. 753**,** 157-62.

Fichard, A., Verna, J.M., Olivares, J., Saxod, R., 1991. Involvement of a chondroitin sulfate proteoglycan in the avoidance of chick epidermis by dorsal root ganglia fibers: a study using beta-D-xyloside. Dev Biol. 148**,** 1-9.

Forno, L.S., DeLanney, L.E., Irwin, I., Langston, J.W., 1996. Electron microscopy of Lewy bodies in the amygdala-parahippocampal region. Comparison with inclusion bodies in the MPTP-treated squirrel monkey. Adv Neurol. 69**,** 217-28.

Freed, C.R., Greene, P.E., Breeze, R.E., Tsai, W.Y., DuMouchel, W., Kao, R., Dillon, S., Winfield, H., Culver, S., Trojanowski, J.Q., Eidelberg, D., Fahn, S., 2001. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med. 344**,** 710-9.

Freed, W.J., Perlow, M.J., Karoum, F., Seiger, Å., Olson, L., Hoffer, B.J., Wyatt, R.J., 1980. Restoration of dopaminergic function by grafting fetal rat substantia nigra to the caudate nucleus: long-term behavioral, biochemical, and histochemical studies. Ann. Neurol. 8**,** 510-519.

Freeman, T.B., Spence, M.S., Boss, B.D., Spector, D.H., Strecker, R.E., Olanow, C.W., Kordower, J.H., 1991. Development of dopaminergic neurons in the human substantia nigra. Exp Neurol. 113**,** 344-53.

Freund, T.F., Bolam, J.P., Bjorklund, A., Stenevi, U., Dunnett, S.B., Powell, J.F., Smith, A.D., 1985. Efferent synaptic connections of grafted dopaminergic neurons reinnervating the host neostriatum: a tyrosine hydroxylase immunocytochemical study. J Neurosci. 5**,** 603-16.

Funayama, M., Hasegawa, K., Kowa, H., Saito, M., Tsuji, S., Obata, F., 2002. A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. Ann Neurol. 51**,** 296-301.

Galpern, W.R., Lang, A.E., 2006. Interface between tauopathies and synucleinopathies: a tale of two proteins. Ann Neurol. 59**,** 449-58.

Gasser, T., 2007. Update on the genetics of Parkinson's disease. Mov Disord. 22 Suppl 17**,** S343-50.

Gates, M.A., O'Brien, T.F., Faissner, A., Steindler, D.A., 1993. Neuron-glial interactions during the in vivo and in vitro development of the nigrostriatal circuit. J Chem Neuroanat. 6**,** 179-89.

Gates, M.A., Fillmore, H., Steindler, D.A., 1996. Chondroitin sulfate proteoglycan and tenascin in the wounded adult mouse neostriatum in vitro: dopamine neuron attachment and process outgrowth. J Neurosci. 16**,** 8005-18.

Gates, M.A., Torres, E.M., White, A., Fricker-Gates, R.A., Dunnett, S.B., 2006. Re-examining the ontogeny of substantia nigra dopamine neurons. Eur J Neurosci. 23**,** 1384-90.

Gerfen, C.R., Baimbridge, K.G., Miller, J.J., 1985. The neostriatal mosaic: Compartmental distribution of calcium-binding protein and parvalbumin in the basal ganglia of the rat and monkey. Proc. Natl. Acad. Sci. USA. 82**,** 8780-8784.

Gerfen, C.R., Baimbridge, K.G., Thibault, J., 1987a. The neostriatal mosaic: III. Biochemical and developmental dissociation of patch-matrix mesostriatal systems. J. Neurosci. 7**,** 3935-3944.

Gerfen, C.R., Herkenham, M., Thibault, J., 1987b. The neostriatal mosaic: II. Patch- and matrix-directed mesostriatal dopaminergic and non-dopaminergic systems. J. Neurosci. 7**,** 3915-3034.

Gerfen, C.R., Wilson, C.J., 1996. The Basal Ganglia. In: Handbook of chemical neuroanatomy: Integrated systems of the CNS, part III. Vol. 12, L.W. Swanson, A. Björklund, T. Hökfelt, ed.^eds. Elsevier Science B.V, Amsterdam, pp. 371-468.

German, D., Manaye, K., White III, C., Woodward, D., McIntire, D., Smith, W., Kalaria, R., Mann, D., 1992. Disease-specific patterns of locus coeruleus cell loss. Ann. Neurol. 32**,** 667-676.

German, D.C., Liang, C.L., 1993. Neuroactive peptides exist in the midbrain dopaminergic neurons that contain calbindin-D28k. Neuroreport. 4**,** 491-4.

Giacobini, M.M., Stromberg, I., Almstrom, S., Cao, Y., Olson, L., 1993. Fibroblast growth factors enhance dopamine fiber formation from nigral grafts. Brain Res Dev Brain Res. 75**,** 65-73.

Gibb, W.R., Lees, A.J., 1988. The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. J Neurol Neurosurg Psychiatry. 51**,** 745-52.

Gotz, M., Hartfuss, E., Malatesta, P., 2002. Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. Brain Res Bull. 57**,** 777-88.

Granerus, A.K., 1978. Factors influencing the occurrence of "on-off" symptoms during long-term treatment with L-dopa. Acta Med Scand. 203**,** 75-85.

Granholm, A.-C.E., Curtis, M., Diamond, D.M., Branch, B.J., Heman, K.L., Rose, G.M., 1996. Development of an intact blood-brain barrier in brain tissue transplants is dependent on the site of transplantation. Cell Transplant. 5**,** 305-314.

Graybiel, A.M., Ragsdale Jr, C.W., 1983. Biochemical anatomy of the striatum. In: Chem. Neuroanat., Vol., P.C. Emson, ed.^eds. Raven Press, New York, pp. 427-504.

Grossmann, R., Stence, N., Carr, J., Fuller, L., Waite, M., Dailey, M.E., 2002. Juxtavascular microglia migrate along brain microvessels following activation during early postnatal development. Glia. 37**,** 229-240.

Gähwiler, B.H., Capogna, M., Debanne, D., McKinney, R.A., Thompson, S.M., 1997. Organotypic slice cultures: A technique has come of age. Trends in Neurosci. 20**,** 471-477.

Hagell, P., Piccini, P., Bjorklund, A., Brundin, P., Rehncrona, S., Widner, H., Crabb, L., Pavese, N., Oertel, W.H., Quinn, N., Brooks, D.J., Lindvall, O., 2002. Dyskinesias following neural transplantation in Parkinson's disease. Nat Neurosci. 5**,** 627-8.

Haque, N.S., LeBlanc, C.J., Isacson, O., 1997. Differential dissection of the rat E16 ventral mesencephalon and survival and reinnervation of the 6-OHDA-lesioned striatum by a subset of aldehyde dehydrogenase-positive TH neurons. Cell Transplant. 6**,** 239-48.

Harrower, T.P., Michell, A.W., Barker, R.A., 2005. Lewy bodies in Parkinson's disease: protectors or perpetrators? Exp Neurol. 195**,** 1-6.

Hartfuss, E., Galli, R., Heins, N., Gotz, M., 2001. Characterization of CNS precursor subtypes and radial glia. Dev Biol. 229**,** 15-30.

Heikkila, R.E., Hess, A., Duvoisin, R.C., 1984. Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. Science. 224**,** 1451-3.

Heneka, M., Gavriluyk, V., Landreth, G., O'Banion, M., Weinberg, G., Feinstein, D., 2003. Noradrenergic depletion increases inflammatory responses in brain: Effects on kappaB and HSP70 expresssion. J. Neurochem. 85**,** 387-398.

Hidalgo, A., Booth, G.E., 2000. Glia dictate pioneer axon trajectories in the Drosophila embryonic CNS. Development. 127**,** 393-402.

Horger, B.A., Nishimura, M.C., Armanini, M.P., Wang, L.C., Poulsen, K.T., Rosenblad, C., Kirik, D., Moffat, B., Simmons, L., Johnson, E., Jr., Milbrandt, J., Rosenthal, A., Bjorklund, A., Vandlen, R.A., Hynes, M.A., Phillips, H.S., 1998. Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurons. J Neurosci. 18**,** 4929-37.

Hyman, C., Hofer, M., Barde, Y.A., Juhasz, M., Yancopoulos, G.D., Squinto, S.P., Lindsay, R.M., 1991. BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature. 350**,** 230-2.

Hynes, M.A., Poulsen, K., Armanini, M., Berkemeier, L., Phillips, H., Rosenthal, A., 1994. Neurotrophin-4/5 is a survival factor for embryonic midbrain dopaminergic neurons in enriched cultures. J Neurosci Res. 37**,** 144-54.

Jacobs, J.R., Goodman, C.S., 1989a. Embryonic development of axon pathways in the Drosophila CNS.I. A glial scaffold appears before the first growth cones. J Neurosci. 9**,** 2402-2411.

Jacobs, J.R., Goodman, C.S., 1989b. Embryonic development of axon pathways in the Drosophila CNS.II. Behavior of pioneer growth cones. J. Neurosci. 9**,** 2412-2422.

Jaeger, C.B., 1985. Cytoarchitectonics of substantia nigra grafts: A light- and electronmicroscopic study of immunocytochemically identified dopaminergic neurons and fibrous astrocytes. J. Comp. Neurol. 231**,** 121-135.

Javitch, J.A., D'Amato, R.J., Strittmatter, S.M., Snyder, S.H., 1985. Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6 - tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. Proc Natl Acad Sci U S A. 82**,** 2173-7.

Jellinger, K.A., 1991. Pathology of Parkinson's disease. Changes other than the nigrostriatal pathway. Mol Chem Neuropathol. 14**,** 153-97.

Johansson, M., Friedemann, M., Hoffer, B., Strömberg, I., 1995. Effects of glial cell line-derived neurotrophic factor on developing and mature ventral mesencephalic grafts *in oculo*. Exp. Neurol. 134**,** 25-34.

Johansson, S., Strömberg, I., 2002. Guidance of dopaminergic neuritic growth by immature astrocytes in organotypic cultures of rat fetal ventral mesencephalon. J. Comp. Neurol. 443**,** 237-249.

Johansson, S., Strömberg, I., 2003. Fetal lateral ganglionic eminence attracts one of two morphologically different types of tyrosine hydroxylase-positive nerve fibers formed by cultured ventral mesencephalon. Cell Transplant. 12**,** 243-255.

Jones, B., Moore, R., 1977. Ascending projections of the locus coeruleus in the rat II: Autoradiographic study. Brain Res. 127**,** 25-53.

Jones, B., Yang, T.-Z., 1985. The efferent projections from the reticular formation and the locus coeruleus studied by anteriograde and retrograde axonal transport in the rat. J. Comp. Neurol. 242**,** 56-92.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Prior, R.L., Cao, G., Martin, A., Taglialatela, G., Bickford, P.C., 1998. Long-term dietary strawberry, spinach, or vitamin E supplementation retards the onset of age-related neuronal signal-transduction and cognitive behavioral deficits. J Neurosci. 18**,** 8047-55.

Joseph, J.A., Shukitt-Hale, B., Denisova, N.A., Bielinski, D., Martin, A., McEwen, J.J., Bickford, P.C., 1999. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J Neurosci. 19**,** 8114-21.

Joseph, J.A., Shukitt-Hale, B., Casadesus, G., 2005. Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds. Am J Clin Nutr. 81**,** 313S-316S.

Kalt, W., Blumberg, J.B., McDonald, J.E., Vinqvist-Tymchuk, M.R., Fillmore, S.A., Graf, B.A., O'Leary, J.M., Milbury, P.E., 2008. Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. J Agric Food Chem. 56, 705-12.

Kebabian, J.W., Calne, D.B., 1979. Multiple receptors for dopamine. Nature. 277, 93-6.

Kish, S.J., Shannak, K., Hornykiewicz, O., 1988. Uneven pattern of dopamine loss in the striatum of patients with idiopathic Parkinson's disease. Pathophysiologic and clinical implications. New Engl. J. Med. 318, 876-880.

Knusel, B., Michel, P.P., Schwaber, J.S., Hefti, F., 1990. Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. J. Neurosci. 10, 558-570.

Kordower, J.H., Rosenstein, J.M., Collier, T.J., Burke, M.A., Chen, E.Y., Li, J.M., Martel, L., Levey, A.E., Mufson, E.J., Freeman, T.B., Olanow, C.W., 1996. Functional fetal nigral grafts in a patient with Parkinson's disease: chemoanatomic, ultrastructural, and metabolic studies. J Comp Neurol. 370, 203-30.

Kordower, J.H., Freeman, T.B., Chen, E.Y., Mufson, E.J., Sanberg, P.R., Hauser, R.A., Snow, B., Olanow, C.W., 1998. Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. Mov Disord. 13, 383-93.

Kumar, R., Lozano, A.M., Kim, Y.J., Hutchison, W.D., Sime, E., Halket, E., Lang, A.E., 1998a. Double-blind evaluation of subthalamic nucleus deep brain stimulation in advanced Parkinson's disease. Neurology. 51, 850-5.

Kumar, R., Lozano, A.M., Montgomery, E., Lang, A.E., 1998b. Pallidotomy and deep brain stimulation of the pallidum and subthalamic nucleus in advanced Parkinson's disease. Mov Disord. 13 Suppl 1, 73-82.

Lander, C., Zhang, H., Hockfield, S., 1998. Neurons produce a neuronal cell surface-associated chondroitin sulfate proteoglycan. J Neurosci. 18, 174-83.

Lang, A.E., Lozano, A.M., 1998a. Parkinson's disease. Second of two parts. N Engl J Med. 339, 1130-43.

Lang, A.E., Lozano, A.M., 1998b. Parkinson's disease. First of two parts. N Engl J Med. 339, 1044-53.

Lang, A.E., 2007. The progression of Parkinson disease: a hypothesis. Neurology. 68, 948-52.

Langston, J.W., Ballard, P., Tetrud, J.W., Irwin, I., 1983. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science. 219, 979-80.

Langston, J.W., Forno, L.S., Tetrud, J., Reeves, A.G., Kaplan, J.A., Karluk, D., 1999. Evidence of active nerve cell degeneration in the substantia

nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann. Neurol. 46, 598-605.
Le Moal, M., Simon, H., 1991. Mesocorticolimbic dopaminergic network: functional and regulatory roles. Physiol Rev. 71, 155-234.
Lewy, F., 1912. Paralysis agitans: I. pathologische anatomie.
Lin, L.F., Doherty, D.H., Lile, J.D., Bektesh, S., Collins, F., 1993. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science. 260, 1130-2.
Lindvall, O., Rehncrona, S., Gustavii, B., Brundin, P., Astedt, B., Widner, H., Lindholm, T., Bjorklund, A., Leenders, K.L., Rothwell, J.C., et al., 1988. Fetal dopamine-rich mesencephalic grafts in Parkinson's disease. Lancet. 2, 1483-4.
Liu, A., Joyner, A.L., 2001. Early anterior/posterior patterning of the midbrain and cerebellum. Annu Rev Neurosci. 24, 869-96.
Madrazo, I., Leon, V., Torres, C., Aguilera, M.C., Varela, G., Alvarez, F., Fraga, A., Drucker-Colin, R., Ostrosky, F., Skurovich, M., et al., 1988. Transplantation of fetal substantia nigra and adrenal medulla to the caudate nucleus in two patients with Parkinson's disease. N Engl J Med. 318, 51.
Mahalik, T.J., Finger, T.E., Strömberg, I., Olson, L., 1985. Substantia nigra transplants into denervated striatum of the rat: Ultrastructure of graft and host interconnections. J. Comp. Neurol. 260, 60-70.
Mahalik, T.J., Hahn, W.E., Clayton, G.H., Owens, G.P., 1994. Programmed cell death in developing grafts of fetal substantia nigra. Exp. Neurol. 129, 27-36.
Malatesta, P., Hartfuss, E., Gotz, M., 2000. Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development. 127, 5253-63.
Mann, D., 1983. The locus coeruleus and its possible role in ageing and degenerative disease of the human central nervous system. Mech. Ageing Dev. 23, 73-94.
Maroteaux, L., Campanelli, J.T., Scheller, R.H., 1988. Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. J Neurosci. 8, 2804-15.
Marsden, C.D., 1983. Neuromelanin and Parkinson's disease. J Neural Transm Suppl. 19, 121-41.
Martin, A., Prior, R., Shukitt-Hale, B., Cao, G., Joseph, J.A., 2000. Effect of fruits, vegetables, or vitamin E--rich diet on vitamins E and C distribution in peripheral and brain tissues: implications for brain function. J Gerontol A Biol Sci Med Sci. 55, B144-51.
Matsumoto, H., Nakamura, Y., Iida, H., Ito, K., Ohguro, H., 2006. Comparative assessment of distribution of blackcurrant anthocyanins in rabbit and rat ocular tissues. Exp Eye Res. 83, 348-56.

Mavridis, M., Degryse, A.-D., Lategan, A., Marien, M., Colpaert, F., 1991. Effects of locus coeruleus lesion on parkinsonian signs, striatal dopamine and substantia nigra cell loss after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in monkeys: a possibel role for the locus coeruleus in the progression of Parkinson's disease. Neurosci. 41**,** 507-523.

Mayer, E., Dunnett, S.B., Pellitteri, R., Fawcett, J.W., 1993. Basic fibroblast growth factor promotes the survival of embryonic ventral mesencephalic dopaminergic neurons-I. Effects in vitro. Neuroscience. 56**,** 379-88.

McCaffery, P., Drager, U.C., 1994. High levels of a retinoic acid-generating dehydrogenase in the meso-telencephalic dopamine system. Proc Natl Acad Sci U S A. 91**,** 7772-6.

McGeer, P.L., Itagaki, S., Boyes, B.E., McGeer, E.G., 1988. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology. 38**,** 1285-91.

McGuire, S.O., Sortwell, C.E., Shukitt-Hale, B., Joseph, J.A., Hejna, M.J., Collier, T.J., 2006. Dietary supplementation with blueberry extract improves survival of transplanted dopamine neurons. Nutr Neurosci. 9**,** 251-8.

McRitchie, D., Hardman, C., Halliday, G., 1996. Cytoarchitectural distribution of calcium binding proteins in midbrain dopaminergic regions of rats and humans. J. Comp. Neurol. 364**,** 121-150.

Meyer-Puttlitz, B., Milev, P., Junker, E., Zimmer, I., Margolis, R.U., Margolis, R.K., 1995. Chondroitin sulfate and chondroitin/keratan sulfate proteoglycans of nervous tissue: developmental changes of neurocan and phosphacan. J Neurochem. 65**,** 2327-37.

Missale, C., Nash, S.R., Robinson, S.W., Jaber, M., Caron, M.G., 1998. Dopamine receptors: from structure to function. Physiol Rev. 78**,** 189-225.

Mogi, M., Harada, M., Riederer, P., Narabayashi, H., Fujita, K., Nagatsu, T., 1994. Tumor necrosis factor alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from Parkinsonian patients. Neurosci. Lett. 165**,** 208-210.

Mogi, M., Harada, M., Kondo, T., Narabayashi, H., Riederer, P., Nagatsu, T., 1995. Transforming growth factor b-1 levels are elevated in the striatum and in ventricular cerebrospinal fluid in Parkinson's disease. Neurosci. Lett. 193**,** 129-132.

Mogi, M., Togari, A., Kondo, T., Mizuno, Y., Komure, O., Kuno, S., Ichinose, H., Nagatsu, T., 1999. Brain-derived growth factor and nerve growth factor concentrations are decreased in substantia nigra in Parkinson's disease. Neurosci. Lett. 270**,** 45-48.

Moon, L.D., Asher, R.A., Rhodes, K.E., Fawcett, J.W., 2002. Relationship between sprouting axons, proteoglycans and glial cells following unilateral nigrostriatal axotomy in the adult rat. Neuroscience. 109**,** 101-17.

Nagakubo, D., Taira, T., Kitaura, H., Ikeda, M., Tamai, K., Iguchi-Ariga, S.M., Ariga, H., 1997. DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. Biochem Biophys Res Commun. 231, 509-13.

Nagata, K., Takei, N., Nakajiama, K., Saito, H., Kohsaka, S., 1993. Microglial conditioned medium promotes survival and development of cultured mesencephalic neurons from embryonic rat brain. J. Neurosci. Res. 34, 357-363.

Nakao, N., Frodl, E.M., Duan, W.-M., Widner, H., Brundin, P., 1994. Lazaroids improve the survival of grafted rat embryonic dopamine neurons. Proc. Natl. Acad. Sci. USA. 91, 12408-12412.

Newman, M.B., Bakay, R.A., 2008. Therapeutic potentials of human embryonic stem cells in Parkinson's disease. Neurotherapeutics. 5, 237-51.

Nicklas, W.J., Vyas, I., Heikkila, R.E., 1985. Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sci. 36, 2503-8.

Niederost, B.P., Zimmermann, D.R., Schwab, M.E., Bandtlow, C.E., 1999. Bovine CNS myelin contains neurite growth-inhibitory activity associated with chondroitin sulfate proteoglycans. J Neurosci. 19, 8979-89.

O'Brien, T.F., Faissner, A., Schachner, M., Steindler, D.A., 1992. Afferent-boundary interactions in the developing neostriatal mosaic. Brain Res Dev Brain Res. 65, 259-67.

Olanow, C.W., Tatton, W.G., 1999. Etiology and pathogenesis of Parkinson's disease. Annu Rev Neurosci. 22, 123-44.

Olanow, C.W., Goetz, C.G., Kordower, J.H., Stoessl, A.J., Sossi, V., Brin, M.F., Shannon, K.M., Nauert, G.M., Perl, D.P., Godbold, J., Freeman, T.B., 2003. A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. Ann Neurol. 54, 403-14.

Olson, L., Seiger, Å., Fuxe, K., 1972. Heterogeneity of striatal and limbic dopamine innervation: Highly fluorescent islands in developing and adult rats. Brain Res. 44, 283-288.

Parish, C.L., Arenas, E., 2007. Stem-cell-based strategies for the treatment of Parkinson's disease. Neurodegener Dis. 4, 339-47.

Perier, C., Bove, J., Vila, M., Przedborski, S., 2003. The rotenone model of Parkinson's disease. Trends Neurosci. 26, 345-6.

Perlow, M.J., Freed, W.J., Hoffer, B.J., Seiger, Å., Olson, L., Wyatt, R.J., 1979. Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. Science. 204, 643-647.

Powell, E.M., Geller, H.M., 1999. Dissection of astrocyte-mediated cues in neuronal guidance and process extension. Glia. 26, 73-83.

Prior, R.L., Cao, G., 2000. Analysis of botanicals and dietary supplements for antioxidant capacity: a review. J AOAC Int. 83, 950-6.

Properzi, F., Carulli, D., Asher, R.A., Muir, E., Camargo, L.M., van Kuppevelt, T.H., ten Dam, G.B., Furukawa, Y., Mikami, T., Sugahara, K., Toida, T., Geller, H.M., Fawcett, J.W., 2005. Chondoitin 6-sulphate synthesis is up-regulated in injured CNS, induced by injury-related cytokines and enhanced in axon-growth inhibitory glia. Eur. J. Neurosci. 21, 378-390.

Putzke, J.D., Wharen, R.E., Jr., Wszolek, Z.K., Turk, M.F., Strongosky, A.J., Uitti, R.J., 2003. Thalamic deep brain stimulation for tremor-predominant Parkinson's disease. Parkinsonism Relat Disord. 10, 81-8.

Ratner, N., Bunge, R.P., Glaser, L., 1985. A neuronal cell surface heparan sulfate proteoglycan is required for dorsal root ganglion neuron stimulation of Schwann cell proliferation. J Cell Biol. 101, 744-54.

Rinne, U.K., 1981. Treatment of Parkinson's disease: problems with a progressing disease. J Neural Transm. 51, 161-74.

Rogers, J.H., 1992. Immunohistochemical markers in rat brain: Colocalisation of calretinin and calbindin-D28k with tyrosine hydroxylase. Brain Res. 587, 203-210.

Rose, G., Gerhardt, G., Stromberg, I., Olson, L., Hoffer, B., 1985. Monoamine release from dopamine-depleted rat caudate nucleus reinnervated by substantia nigra transplants: an in vivo electrochemical study. Brain Res. 341, 92-100.

Sasaki, M., Shibata, E., Tohyama, K., Takahashi, J., Otsuka, K., Tsuchiya, K., Takahashi, S., Ehara, S., Terayama, Y., Sakai, A., 2006. Neuromelanin magnetic resonance imaging of locus ceruleus and substantia nigra in Parkinson's disease. Neuroreport. 17, 1215-8.

Saucedo-Cardenas, O., Quintana-Hau, J.D., Le, W.D., Smidt, M.P., Cox, J.J., De Mayo, F., Burbach, J.P., Conneely, O.M., 1998. Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc Natl Acad Sci U S A. 95, 4013-8.

Sauer, H., Oertel, W.H., 1994. Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. Neurosci. 59, 401-415.

Schein, J.C., Hunter, D.D., Roffler-Tarlov, S., 1998. Girk2 expression in the ventral midbrain, cerebellum, and olfactory bulb and its relationship to the murine mutation weaver. Dev Biol. 204, 432-50.

Schnitzer, J., Franke, W., Schachner, M., 1981. Immunocytochemical demonstration of vimentin in astrocytes and ependymal cells of developing and adult mouse nervous system. J. Cell Biol. 90, 435-447.

Schwartz, N., 1977. Regulation of chondroitin sulfate synthesis. J. Biol. Chem. 252, 6316-6321.

Seiger, Å., Olson, L., 1973. Late prenatal ontogeny of central monoamine neurons in the rat: Fluorescence histochemical observations. Z. Anat. Entwickl.-Gesch. 140**,** 281-318.

Selby, G., 1984. The Graeme Robertson memorial lecture, 1983. The long-term prognosis of Parkinson's disease. Clin Exp Neurol. 20**,** 1-25.

Semchuk, K.M., Love, E.J., Lee, R.G., 1993. Parkinson's disease: a test of the multifactorial etiologic hypothesis. Neurology. 43**,** 1173-80.

Shastry, B.S., 2001. Parkinson disease: etiology, pathogenesis and future of gene therapy. Neurosci Res. 41**,** 5-12.

Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., Suzuki, T., 2000. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat Genet. 25**,** 302-5.

Shukitt-Hale, B., Lau, F.C., Joseph, J.A., 2008. Berry fruit supplementation and the aging brain. J Agric Food Chem. 56**,** 636-41.

Shults, C.W., Hashimoto, R., Brady, R.M., Gage, F.H., 1990. Dopaminergic cells align along radial glia in the developing mesencephalon of the rat. Neuroscience. 38**,** 427-36.

Sillitoe, R.V., Vogel, M.W., 2008. Desire, disease, and the origins of the dopaminergic system. Schizophr Bull. 34**,** 212-9.

Smidt, M.P., Cox, J.J., van Schaick, H.S., Coolen, M., Schepers, J., van der Kleij, A.M., Burbach, J.P., 2000. Analysis of three Ptx2 splice variants on transcriptional activity and differential expression pattern in the brain. J Neurochem. 75**,** 1818-25.

Specht, L.A., Pickel, V.M., Joh, T.H., Reis, D.J., 1981. Light microscopic immunocytochemical localization of tyrosine hydroxylase in prenatal rat brain. I. Early ontogeny. J. Comp. Neurol. 199**,** 233-253.

Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M., Goedert, M., 1998. alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc Natl Acad Sci U S A. 95**,** 6469-73.

Srinivasan, J., Schmidt, W., 2004. Behavioral and neurochemical effects of noradrenergic depletions with *N*-(2-cloroethyl)-*N*-ethyl-2-bromobenzylamine in 6-hydroxydopamine-induced rat model of Parkinson's disease. Behav. Brain Res. 151**,** 191-199.

Steinmetz, M.P., Horn, K.P., Tom, V.J., Miller, J.H., Busch, S.A., Nair, D., Silver, D.J., Silver, J., 2005. Chronic enhancement of the intrinsic growth capacity of sensory neurons combined with degradation of inhibitory proteoglycans allows functional regeneration of sensory axons through the dorsal root entry zone in the mammalian spinal cord. J. Neurosci. 25**,** 8066-8076.

Stence, N., Waite, M., Dailey, M.E., 2001. Dynamics of microglial activation: A confocal time-lapse analysis in hippocampal slices. Glia. 33**,** 256-266.

Stoppini, L., Buchs, P.-A., Muller, D., 1991. A simple method for organotypic cultures of nervous tissue. J. Neursci. Meth. 37**,** 173-182.

Stromberg, I., Almqvist, P., Bygdeman, M., Finger, T.E., Gerhardt, G., Granholm, A.C., Mahalik, T.J., Seiger, A., Hoffer, B., Olson, L., 1988. Intracerebral xenografts of human mesencephalic tissue into athymic rats: immunochemical and in vivo electrochemical studies. Proc Natl Acad Sci U S A. 85**,** 8331-4.

Stromberg, I., Bjorklund, L., Johansson, M., Tomac, A., Collins, F., Olson, L., Hoffer, B., Humpel, C., 1993. Glial cell line-derived neurotrophic factor is expressed in the developing but not adult striatum and stimulates developing dopamine neurons in vivo. Exp Neurol. 124**,** 401-12.

Stromberg, I., Bickford, P., 1996. Reduced ageing effects of striatal neuronal discharge rate by aged ventral mesencephalic grafts. Neuroreport. 7**,** 693-6.

Stromberg, I., Gemma, C., Vila, J., Bickford, P.C., 2005. Blueberry- and spirulina-enriched diets enhance striatal dopamine recovery and induce a rapid, transient microglia activation after injury of the rat nigrostriatal dopamine system. Exp Neurol. 196**,** 298-307.

Strömberg, I., Bygdeman, M., Almqvist, P., 1992. Target-specific outgrowth from human mesencephalic tissue grafted to cortex or ventricle of immunosuppressed rats. J. Comp. Neurol. 315**,** 445-456.

Strömberg, I., Adams, C., Bygdeman, M., Hoffer, B., Boyson, S., Humpel, C., 1995. Long-term effects of human-to-rat mesencephalic xenografts on rotational behavior, striatal dopamine receptor binding, and mRNA levels. Brain Res. Bull. 38**,** 221-233.

Strömberg, I., Björklund, L., Förander, P., 1997. The age of striatum determines the pattern and extent of dopaminergic innervation: A nigrostriatal double graft study. Cell Transplant. 6**,** 287-296.

Strömberg, I., 2000. Intraocular grafting. In: NeuroMethods. Neural Transplantation Methods. Vol. 36, S.B. Dunnett, A.A. Boulton, G.B. Baker, ed.^eds. Humana Press, Totowa, New Jersey, pp. 149-169.

Strömberg, I., Törnqvist, N., Johansson, S., Bygdeman, M., Almqvist, P., 2001. Evidence for target-specific outgrowth from subpopulations of grafted human dopamine neurons. Microscopy Res. Tech. 54**,** 287-297.

Takeshima, T., Shimoda, K., Suaves, Y., Commissiong, J.W., 1994. Astrocyte-dependent and -independent phases of the development and survival of rat embryonic day 14 mesencephalic, dopaminergic neurons in culture. Neurosci. 60**,** 809-823.

Tanner, C.M., Chen, B., Wang, W., Peng, M., Liu, Z., Liang, X., Kao, L.C., Gilley, D.W., Goetz, C.G., Schoenberg, B.S., 1989. Environmental factors and Parkinson's disease: a case-control study in China. Neurology. 39**,** 660-4.

Tanner, C.M., Langston, J.W., 1990. Do environmental toxins cause Parkinson's disease? A critical review. Neurology. 40**,** suppl 17-30; discussion 30-1.

Tanner, C.M., Ottman, R., Goldman, S.M., Ellenberg, J., Chan, P., Mayeux, R., Langston, J.W., 1999. Parkinson disease in twins: an etiologic study. JAMA. 281**,** 341-6.

Trétiakoff, C., 1919. Contribution à l'étude de l'anatomie pathologique du locus niger de Soemmering avec quelques déductions relatives à la pathogénie des troubles du tonus musculaire et de la maladie de Parkinson. Thése Médicine, Paris.

Troadec, J.-D., Marien, M., Darios, F., Hartmann, A., Ruberg, M., Colpaert, F.C., Michel, P.P., 2001. Noradrenaline provides long-term protection to dopaminergic neurons by reducing oxidative stress. J. Neurochem. 79**,** 200-210.

Tropea, D., Caleo, M., Maffei, L., 2003. Synergistic effects of brain-derived neurotrophic factor and chondroitinase ABC on retinal fiber sprouting after denervation of the superior colliculus in adult rats. J. Neurosci. 23**,** 7034-7044.

Törnqvist, N., Björklund, L., Strömberg, I., 2001. Evidence for target-specific outgrowth from subpopulations of grafted dopaminergic neurons. A retrograde study using *in oculo* and intracranial grafting. Exp. Neurol. 169**,** 329-339.

Ungerstedt, U., 1968. 6-Hydroxydopamine induced degeneration of central monoamine neurons. Eur. J. Pharmacol. 5**,** 107-110.

Ungerstedt, U., 1971. Stereotaxic mapping of the monoamine pathways in the rat brain. Acta Physiol. Scand. 197**,** 1-48.

Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., Albanese, A., Nussbaum, R., Gonzalez-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W.P., Latchman, D.S., Harvey, R.J., Dallapiccola, B., Auburger, G., Wood, N.W., 2004. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science. 304**,** 1158-60.

Wallen, A., Zetterstrom, R.H., Solomin, L., Arvidsson, M., Olson, L., Perlmann, T., 1999. Fate of mesencephalic AHD2-expressing dopamine progenitor cells in NURR1 mutant mice. Exp Cell Res. 253**,** 737-46.

Wang, Y., Chen, S., Yang, D., Le, W.D., 2007. Stem cell transplantation: a promising therapy for Parkinson's disease. J Neuroimmune Pharmacol. 2**,** 243-50.

Wictorin, K., Brundin, P., Sauer, H., Lindvall, O., Bjorklund, A., 1992. Long distance directed axonal growth from human dopaminergic mesencephalic neuroblasts implanted along the nigrostriatal pathway in 6-hydroxydopamine lesioned adult rats. J Comp Neurol. 323**,** 475-94.

Vidal, N., Björklund, L., Strömberg, I., 1998. Morphological and functional evidence for enhanced growth and potassium-evoked dopamine release in

striatal grafts innervated with a patchy growth pattern. An *in oculo* nigrostriatal cograft study. Cell Transplant. 7**,** 1-12.
Williams, R.J., Spencer, J.P., Rice-Evans, C., 2004. Flavonoids: antioxidants or signalling molecules? Free Radic Biol Med. 36**,** 838-49.
Willis, L., Bickford, P., Zaman, V., Moore, A., Granholm, A.C., 2005. Blueberry extract enhances survival of intraocular hippocampal transplants. Cell Transplant. 14**,** 213-23.
Wilms, H., Rosenstiel, P., Sievers, J., Deuschl, G., Zecca, L., Lucius, R., 2003. Activation of microglia by human neuromelanin is NF-kappaB dependent and involves p38 mitogen-activated protein kinase: implications for Parkinson's disease. FASEB J. 17**,** 500-2.
Wirdefeldt, K., Gatz, M., Schalling, M., Pedersen, N.L., 2004. No evidence for heritability of Parkinson disease in Swedish twins. Neurology. 63**,** 305-11.
Zarow, C., Lyness, S.A., Mortimer, J.A., Chui, H.C., 2003. Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. Arch Neurol. 60**,** 337-41.
Zawada, W.M., Kirschman, D.L., Cohen, J.J., Heidenreich, K.A., Freed, C.R., 1996. Growth factors rescue embryonic dopamine neurons from programmed cell death. Exp. Neurol. 146**,** 60-67.
Zecca, L., Fariello, R., Riederer, P., Sulzer, D., Gatti, A., Tampellini, D., 2002. The absolute concentration of nigral neuromelanin, assayed by a new sensitive method, increases throughout the life and is dramatically decreased in Parkinson's disease. FEBS Lett. 510**,** 216-20.
Zecca, L., Zucca, F.A., Albertini, A., Rizzio, E., Fariello, R.G., 2006. A proposed dual role of neuromelanin in the pathogenesis of Parkinson's disease. Neurology. 67**,** S8-11.
Zetterstrom, R.H., Solomin, L., Jansson, L., Hoffer, B.J., Olson, L., Perlmann, T., 1997. Dopamine neuron agenesis in Nurr1-deficient mice. Science. 276**,** 248-50.
Zetterstrom, T., Brundin, P., Gage, F.H., Sharp, T., Isacson, O., Dunnett, S.B., Ungerstedt, U., Bjorklund, A., 1986. In vivo measurement of spontaneous release and metabolism of dopamine from intrastriatal nigral grafts using intracerebral dialysis. Brain Res. 362**,** 344-9.
Zietlow, R., Dunnett, S.B., Fawcett, J.W., 1999. The effect of microglia on embryonic dopamine neuronal survival *in vitro*: diffusible signals from neurons and glia change microglia from neurotoxic to neuroprotective. Eur. J. Neurosci. 11**,** 1657-1667.
Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., Kachergus, J., Hulihan, M., Uitti, R.J., Calne, D.B., Stoessl, A.J., Pfeiffer, R.F., Patenge, N., Carbajal, I.C., Vieregge, P., Asmus, F., Muller-Myhsok, B., Dickson, D.W., Meitinger, T., Strom, T.M., Wszolek, Z.K., Gasser, T., 2004. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron. 44**,** 601-7.

Zweig, R.M., Cardillo, J.E., Cohen, M., Giere, S., Hedreen, J.C., 1993. The locus ceruleus and dementia in Parkinson's disease. Neurology. 43**,** 986-91.