

Interaction between nerve fiber formation and astrocytes

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Cover illustration: Photomicrograph of adult astrocytes and fetal ventral mesencephalon co-culture. Dopaminergic neurons (green), vimentin-positive astrocytes from fetal tissue (blue) and GFP-positive adult astrocytes (red).

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To my beloved Amir

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ABBREVIATIONS

AADC	L-aromatic amino acid decarboxylase
ANOVA	Analysis of variance
BBB	Blood brain barrier
β -tubulin	β -tubulin isotype III
β -xyloside	Methyl-umbelliferyl- β -D-xyloside
ChABC	Chondroitinase ABC
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CSPG	Chondroitin sulfate proteoglycan
DAPI	4',6-diamidino-2-phenylindole
DIV	Days <i>in vitro</i>
DMEM	Dulbecco's modified Eagle's medium
Dopa	Dihydroxyphenylalanine
E	Embryonic day
ECM	Extracellular matrix
FBS	Fetal bovine serum
GABA	Gamma-aminobutyric acid
GAG	Glycosaminoglycan
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GP	Globus pallidus
GPe	Globus pallidus externa
GPI	Globus pallidus interna
HBSS	Hanks' balanced salt solution
IAP	Integrin-associated protein
L-DOPA	3,4-dihydroxy-L-phenylalanine
MAO	Monoamine oxidase-B
MSN	Medium spiny neuron

P	Postnatal day
PBS	Phosphate buffered saline
PD	Parkinson's disease
PDL	Poly-D-lysine
PG	Proteoglycan
SC	Spinal cord
SHP-2	Src homology 2
SIRP α	Signal regulatory protein alpha
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TH	Tyrosine hydroxylase
TSP-1	Thrombospondin-1
VIM	Vimentin
VM	Ventral mesencephalon

ABSTRACT

Parkinson's disease, the second most common neurodegenerative disorder, is characterized by loss of nigrostriatal dopaminergic neurons. To date, there is no defined cause and cure for the disease. An ideal treatment strategy is to replace the lost neurons by transplanting fetal dopaminergic neurons to the brain of parkinsonian patients. Clinical trials have been performed and the outcome was variable where one significant obstacle was the limited graft reinnervation of the host brain. To study this issue, organotypic tissue culture can be utilized to monitor dopaminergic nerve fiber outgrowth *in vitro* and their association with astrocytes. Using this culture technique, dopaminergic nerve fibers appear in two morphologically and temporally different types. The early appearing nerve fibers are formed in the absence of astrocytes, reach long distances, and are called non-glial-associated tyrosine hydroxylase (TH) -positive nerve fibers. After a few days, the second sequence of nerve fibers, the glial-associated TH-positive nerve fibers, are formed, and their growth are limited to the presence of astrocytes, that migrate and form a monolayer surrounding the plated tissue. The aim of this thesis was to study the interaction between nerve fiber formation and astrocytes with a special focus on the long-distance growing nerve fibers. Ventral mesencephalic (VM) organotypic slice cultures from embryonic day (E) 12, E14, and E18 were incubated for 14, 21, 28, and 35 days *in vitro* (DIV). The results revealed that the two morphologically different processes were found in cultures from the younger stages, while no non-glial-associated growth was found in cultures of tissue from E18. Instead neurons had migrated onto the migrating astrocytes. Astrocytes migrated longer distances in tissue from older stages, and the migration reached a plateau at 21 DIV. Co-cultures of E14 VM tissue pieces and cell suspension of mature astrocytes promoted migration of neurons, as seen in E18 cultures. Thus,

the maturity of the astrocytes was an important factor for nerve fiber outgrowth. Hence, targeting molecules secreted by astrocytes might be beneficial for regeneration. Chondroitin sulfate proteoglycan (CSPG), a member of proteoglycan family, is produced by the astrocytes and has a dual role of being permissive during development and inhibitory after brain injury in adult brain. Cultures were treated with chondroitinase ABC (ChABC) or methyl-umbelliferyl- β -D-xyloside (β -xyloside) in two different protocols, early and late treatments. The results from the early treated cultures showed that both compounds inhibited the outgrowth of nerve fibers and astrocytic migration in cultures from E14 tissue, while β -xyloside but not ChABC promoted the non-glial-associated growth in cultures derived from E18 fetuses. In addition, β -xyloside but not ChABC inhibited neuronal migration in E18 cultures. Taken together, β -xyloside appeared more effective than ChABC in promoting nerve fiber growth. Another potential candidate, integrin-associated protein CD47, was studied because of its role in synaptogenesis, which is important for nerve fiber growth. Cultures from E14 CD47 knockout (CD47^{-/-}) mice were plated and compared to their wildtypes. CD47^{-/-} cultures displayed a massive and long non-glial-associated TH-positive nerve fiber outgrowth despite their normal astrocytic migration. Blocking either signal regulatory protein- α (SIRP α) or thrombospondin-1 (TSP-1), which bind to CD47, had no growth promoting effect. In conclusion, to promote nerve growth, younger tissue can grow for longer distances than older tissue, and inhibiting CSPG production promotes nerve growth in older tissue, while gene deletion of CD47 makes the astrocytes permissive for a robust nerve fiber growth.

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred in the text by their Roman numerals:

- I. Hashemian S, Phillips J, Af Bjerkén S, and Strömberg I.
 The age of the astrocytes affects neuronal growth.
 Manuscript

- II. Hashemian S, Marschinke F, Af Bjerkén S, Strömberg I.
 Degradation of proteoglycans affects astrocytes and neurite formation in organotypic cultures.
 Paper in press in Brain research journal, April 2014

- III. Marschinke F, Hashemian S, Matozaki T, Oldenborg P-A, Strömberg I.
 The absence of CD47 promotes nerve fiber growth from cultured ventral mesencephalic dopamine neurons.
 PLoS ONE 7: 0045218, 2012.

INTRODUCTION

Parkinson's disease

Parkinson's disease (PD) is a condition first described in ancient times by Indians under the name “Kampavata” (Kampa: shaking, vata: weakling). However it was not until 1817 that a detailed medical essay, “An Essay on the Shaking Palsy”, was published by doctor James Parkinson, based on his observation of six cases. The intention of the essay was to encourage others to study the disease. Around 60 years later, a French neurologist, Jean Martin Charcot, was the first to truly recognize the importance of Parkinson's work and named the disease after him “Parkinson's disease” (Goetz, 2011).



Figure 1: Typical appearance of a parkinsonian patient.

Parkinson's disease (PD), the second most common neurodegenerative disorder after Alzheimer's disease, has the prevalence of about 1 % among people over 60 years of age in industrialized countries (Nussbaum and Ellis, 2003). PD is clearly an age-related disorder, i.e. rare before the age of 50, and the prevalence increases up to 4 % among those over 80 (Claveria et al., 2002; de Rijk et al., 2000). A primary cause for PD is yet to be identified. However, a number of risk factors are clearly evident. Although most of the PD cases are apparently sporadic, there are several causative monogenetic mutations that likely explain a small proportion of all PD patients (de Lau and Breteler, 2006). Some of the genes known to be involved in familial PD are: *α -synuclein*, *Parkin*, *UCHL-1*, and *DJ-1* (Bonifati et al., 2003; Mizuno et al., 2006; Polymeropoulos et al., 1997). There are some non-genetic risk factors, which have been proposed to play role in the pathogenesis of PD, for

instance: environmental toxins such as pesticides, lack of dietary factors like vitamin D and B, inflammation and trauma (Betarbet et al., 2000; Factor and Weiner, 2004; Newmark and Newmark, 2007).

The cardinal features of PD are motor symptoms e.g. resting tremor, bradykinesia (slow movement), rigidity, and postural instability (Figure1) (Bernheimer et al., 1973; Selby, 1984). These are characteristics of the disease and are used for the clinical diagnosis of PD. They manifest when more than 50 % of the dopaminergic neurons in substantia nigra pars compacta (SNpc) are degenerated, and the dopamine levels are reduced in the striatum with 80 % (Agid, 1991; Bernheimer et al., 1973). However, there are non-dopaminergic and non-motor symptoms as well, such as olfactory deficits, mood disorders, constipation, sleep disturbances, and dementia, which contribute to impaired quality of life, shortened life expectancy, and severe disability (Chaudhuri et al., 2006; Meshulam et al., 1998). Non-motor symptoms could be explained by loss of serotonergic neurons of the raphe nuclei (Kish et al., 2008), noradrenergic neurons of the locus coeruleus (Zarow et al., 2003), and cholinergic neurons of the nucleus basalis (Nakano and Hirano, 1984). Besides the loss of dopaminergic neurons in the substantia nigra (SN), which is the most important pathological hallmark of the disease, there are other factors without established roles in the disease pathogenesis. Some examples are the presence of Lewy bodies, which are intracellular aggregated α -synuclein inclusions in the dopaminergic neurons, mitochondrial dysfunction, oxidative stress, and inflammatory changes that might be crucial in nigral cell death (Eriksen et al., 2005; Olanow and Tatton, 1999).

Parkinsonism is a common name for neurological syndromes that show at least two of the cardinal symptoms, either bradykinesia or tremor being one of them. Parkinsonism is divided into four subtypes: primary or idiopathic parkinsonism, secondary or acquired parkinsonism, the Parkinson plus

syndromes, and hereditary parkinsonism. Primary parkinsonism, being the most common form of PD, has a genetic or an unknown etiology, so the terms "familial Parkinson's disease" and "sporadic Parkinson's disease" can be used to truly differentiate idiopathic form of the disease from its genetic form. Secondary parkinsonism has a known origin, which is typically trauma or brain injuries such as stroke and toxins. Parkinson plus syndromes are extended neurodegeneration of several brain areas and include additional features to primary PD; and hereditary PD are inherited neurological conditions that give rise to symptoms resembling PD (Fahn, 2003).

An interesting theory, called the Braak theory, suggests that PD is a prion-like disease and that the enteric nervous system is a starting point of PD pathology, which progresses via vagus nerve to the brain stem and its nuclei (Braak et al., 2004). In support of this issue, Lewy pathology is found in a subset of patients transplanted with dopaminergic tissue (Kordower et al., 2008; Li et al., 2008). Degeneration of several brainstem nuclei during the disease progression and presence of α -synuclein-positive inclusions in the intestinal submucosa can be proofs of concept (Braak et al., 2006; Shannon et al., 2012).

The neurotransmitter dopamine

Dopamine is synthesized by hydroxylation of the amino acid tyrosine, by the rate-limiting enzyme tyrosine hydroxylase (TH), and further decarboxylation of dihydroxyphenylalanin (dopa), by L-aromatic amino acid decarboxylase (AADC). Discoveries by Arvid Carlsson and his colleagues demonstrated the presence of dopamine in the brain with similar concentrations as noradrenaline (Carlsson et al., 1958) but having a different distribution, and that dopamine is not merely an intermediate in the synthesis of adrenaline and noradrenaline. Carlsson showed that loss of motor control caused by the drug reserpine, through emptying dopamine terminals in the striatum, could

be counteracted by the dopamine precursor 3,4-dihydroxy-L-phenylalanine (L-DOPA) (Carlsson et al., 1957; Carlsson et al., 1958). These results were supported by demonstration of reduced dopamine levels in the striatum of PD patients (Ehringer and Hornykiewicz, 1960).

Dopamine exerts its effect via dopamine D1 (D1 and D5) and D2 (D2, D3, and D4) -like receptors (Kebabian and Calne, 1979; Missale et al., 1998). These receptors are found abundantly in the striatum on medium spiny neurons (MSNs), in the limbic, and cortical areas. Binding of dopamine to D1 receptors stimulates MSNs while dopamine inhibits neurons expressing the D2 receptors. It should be mentioned that there are some observations reporting that a subset of MSNs express both types of receptors (Lester et al., 1993; Surmeier et al., 1992), which intricates the issue.

Development of the dopaminergic system

During early development, neurons that will give rise to the SN are born in the proliferative zone in ventral mesencephalon (VM) between embryonic days (E) E11 and E16 with peak ontogeny at around E13-15 (Hanaway et al., 1971; Missale et al., 1998). However, according to a more recent study, the peak ontogeny occurs at E12 in rats (Gates et al., 2006). TH is expressed around the same time (Foster et al., 1988; Specht et al., 1981) and approximately one day later, dopamine can be visualized (Olson and Seiger, 1972; Voorn et al., 1988). The neurons migrate to their final location in the VM around E18 (Hanaway et al., 1971; Specht et al., 1981; Voorn et al., 1988). Dopaminergic neurons project their fibers to reach the striatal anlage, lateral ganglionic eminence, at E14 (Olson and Seiger, 1972; Voorn et al., 1988). The complete innervation and adult features of VM occurs at postnatal day (P) 21 (Kalsbeek et al., 1992; Voorn et al., 1988).

The regional distribution of dopamine was mapped by Dahlström and Fuxe using the Falck-Hillarp histochemical method (Falck et al., 1962). They

showed the dopaminergic nuclei in the VM as retrorubal area (A8), substantia nigra (A9), and ventral tegmental area (A10) (Dahlström and Fuxe, 1964). These areas give rise to the mesolimbic and mesocortical (A8 and A10), and nigrostriatal (A9) pathways. The nigrostriatal pathway innervates dorsal striatum (caudatus and putamen) that involves in the motor control (Björklund and Lindvall, 1984; Ungerstedt, 1971). To be able to distinguish between A9 and A10 neurons that are included in the pieces of VM when dissected, co-expression of TH with ALDH1 and GIRK2 are used to identify A9 dopamine neurons and co-expression of TH with calbindin or CCK are used to distinguish A10 neurons (Gerfen et al., 1987a; Gerfen et al., 1987b; German and Liang, 1993; McCaffery and Drager, 1994).

The basal ganglia

The basal ganglia are a subcortical group of interconnected nuclei consisting of four major nuclei: the striatum (caudate nucleus and putamen), the globus pallidus (GP) (interna: GPi and externa: GPe), the subthalamic nucleus (STN), and the substantia nigra (pars compacta: SNpc and pars reticulata: SNpr). There are two output pathways from the striatum that connect these components: the direct and the indirect pathways. The striatum is mostly composed of Gamma-aminobutyric acid (GABA) -ergic MSNs, but it also contains GABAergic and cholinergic interneurons (Graybiel, 1983; Kreitzer, 2009). Glutamatergic nerve fibers coming from cortex stimulate GABAergic MSNs in the striatum. In the direct pathway, GABAergic neurons expressing D₁ receptors and substance P project their fibers to the globus pallidus interna and substantia nigra pars reticulata, whereas in the indirect pathway, GABAergic neurons expressing D₂ receptors and enkephalin project their fibers to the globus pallidus interna through the globus pallidus externa and the subthalamic nucleus. The final GABAergic projections coming from the globus pallidus internal and substantia nigra pars reticulata are the main output nuclei of the basal ganglia, having the final effect on thalamocortical fibers (Groenewegen, 2003). According to the Albin-DeLong model, one of

the first modern models (Albin et al., 1989; Alexander et al., 1986; Crossman, 1989; DeLong, 1990), firing of MSNs in the direct pathway stimulates the thalamocortical fibers in order to initiate movement and activation of MSNs in the indirect pathways inhibit the thalamocortical fibers to suppress unwanted actions (Figure 2).

Controlling the activity of the GABAergic MSNs in the striatum are accomplished by the glutamatergic input coming from the cortex, and the dopaminergic input coming from the substantia nigra pars compacta. The net balance between both pathways accomplishes the motor control of the basal ganglia. The typical symptoms of Parkinson's disease, hypokinesia, tremor at rest, and rigidity could be explained by the loss of dopaminergic neurons in the SNpc. The reduced dopamine level results in a reduction in activity through the direct pathway and an increase in activity through the indirect pathway, causing reduced activity in the output of the basal ganglia. Therefore, the thalamic activation of the motor cortex is inhibited.

The symptoms of the disease are first seen when the dopamine concentration in the striatum is reduced with 80 %, and approximately 50 % of the nigral dopamine neurons are lost (Fearnley and Lees, 1991; Marsden, 1990). The loss of dopamine in PD patients is more prominent in the putamen compared to the caudate (Kish et al., 1988; Nyberg et al., 1983), which is a consequence of the more severe loss of dopamine neurons in the ventral tier of the SN (Fearnley and Lees, 1991).

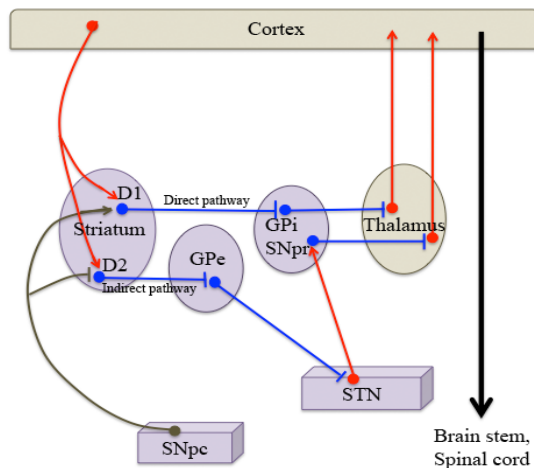


Figure 2: The basal ganglia. The striatum, globus pallidus (interna: GPi and externa: GPe), subthalamic nucleus (STN), and substantia nigra (pars compacta: SNpc, and pars reticulata: SNpr) regulate the net activation of thalamocortical fibers and motor control subsequently via the direct and the indirect pathways. Glutamatergic neurons (red arrows); GABAergic neurons (blue line); dopaminergic neurons (brown curve); stimulatory pathways (arrows); inhibitory pathways (blunt end lines).

Treatment strategies

At present, there is no cure for PD. However, therapeutic strategies can provide patients with temporary relief from the symptoms. The most commonly used treatment at the early stages of PD is administration of Levodopa (L-DOPA), the precursor of dopamine, which unlike dopamine can cross the blood brain barrier (BBB), be converted to dopamine in the brain, and lead to increased striatal dopamine content. The effects of L-DOPA was discovered when the motor deficiencies resulted from low levels of dopamine in the striatum, were counteracted by L-DOPA administration to animals (Birkmayer and Hornykiewicz, 1961; Carlsson et al., 1957; Ehringer and Hornykiewicz, 1960). Clinical trials with L-DOPA showed poor results due to low dosage and side effects of the drug (Birkmayer and Hornykiewicz, 1961), which was improved by stepwise introduction of higher dosage (Cotzias et al., 1967) in combination with peripheral carboxylase inhibitors (Bartholini and Pletscher, 1968; Dunner et al., 1971), and were efficiently yielding more L-DOPA to the brain. Although L-DOPA is efficient to treat PD during the initial period, there are limitations upon long-term use such as motor fluctuations and involuntary movements called L-DOPA-induced dyskinesia (Ahlskog and Muentner, 2001), and decreased

drug response known as “wearing-off” (Granerus, 1978). Metabolic inhibitors such as catechol-O-methyl transferase (COMT) and monoamine oxidase-B (MAO) (Inagaki et al., 2000) together with L-DOPA, which can extend the half-life of dopamine, are useful treatment strategies for the symptoms of PD. Dopamine agonists are other type of drugs that are used to enhance motor activity by stimulating postsynaptic dopamine receptors. Although, comparing to L-DOPA therapy, development of dyskinesia and the wearing-off effect are less prominent; side effects such as confusion and psychosis are more prone to occur (Rascol et al., 2000).

As the disease progresses, the motor features become harder to treat and do not respond to dopamine drug therapies. Deep brain stimulation, a surgical procedure utilizing electrical stimulation to adjust the activity in the basal ganglia, can be used for treatment of symptoms in PD. Depending on the motor symptoms electrode is inserted into the thalamus, the globus pallidus, or the subthalamic nucleus. Deep brain stimulation was preceded by a technique called pallidotomy, in which the GP was destroyed by heat (Narabayashi, 1990).

To restore the dopamine input in the dopamine-depleted striatum of parkinsonian patients, a novel approach was developed, which was grafting of fetal dopamine tissue. Preclinical transplantation studies (Bjorklund and Stenevi, 1979; Perlow et al., 1979) resulted in a compensatory effect of the motor deficits. Long-term graft survival (Freed et al., 1980; Stromberg and Bickford, 1996), functional synaptic connections (Bolam et al., 1987; Clarke et al., 1988; Mahalik et al., 1985; Stromberg et al., 1988), normalized striatal firing rate (Fisher et al., 1991; Stromberg et al., 1985; van Horne et al., 1990), and restored dopamine release in the reinnervated striatum (Rose et al., 1985; Stromberg et al., 1988; Zetterstrom et al., 1986) were reported. Following promising results of preclinical studies, clinical attempts using human fetal VM for grafting to parkinsonian patients were initiated

(Lindvall et al., 1988; Madrazo et al., 1988). Postmortem studies of transplanted patients revealed long-term graft survival, striatal reinnervation and functional effects due to integration of grafts in the neural circuits (Kordower et al., 1998; Piccini et al., 2000). However, the outcome was variable and some patients experienced no symptomatic relief (Freed et al., 2001; Olanow et al., 2003). The main problems with grafting of fetal dopaminergic neurons were poor survival of grafted dopamine neurons, ethical concerns about using fetal tissue, risk of infection, insufficient reinnervation of the striatum with the grafts (Barker et al., 1996) and the need for several implantation sites in order to cover the whole area of dopamine-depleted striatum, which in turn causes graft-induced dyskinesia (Agid, 1991; Freed et al., 2001; Hagell et al., 2002). The reason behind the halt in reinnervation is not known but lack of attractants, such as neurotrophic factors and extracellular matrix molecules, can be some possible causes. Due to the high response found in several patients, despite the negative outcomes, a new transplantation trial (TRANSEURO) funded by the European Union aims at achieving successful grafting in carefully selected patients in order to set up a template protocol for future use.

Another desirable approach is grafting of embryonic/adult stem cells, neuronal progenitor cells, neural stem cells generated in the subventricular zone, the hippocampus, the hindbrain and the forebrain (Conti et al., 2006) into the striatum of PD patients. The ideal situation would be that stem cells differentiate to functional dopaminergic neurons that integrate into the host brain (Riaz and Bradford, 2005). Animal transplant studies demonstrated long-term survival of the transplant and improved motor behavior (Bjorklund et al., 2002; Englund et al., 2002; Rodriguez-Gomez et al., 2007; Sanchez-Pernaute et al., 2008). Unfortunately there are problems with stem cell transplant such as poor survival, insufficient differentiation into dopaminergic neurons, poor innervation, and tumor development.

Nerve fiber formation in VM cultures

As mentioned above, grafting of fetal VM tissue to the striatum of PD patients might be one of the ideal treatment strategies. To improve graft function, it is important to control the graft outgrowth and improve survival of the graft for better reinnervation of the host brain. Hence, organotypic tissue culture technique is a useful tool to study nerve fiber formation from fetal VM tissue and monitor the interaction between nerve fibers and astrocytes while controlling the environmental conditions.

In organotypic tissue culture of fetal VM, two temporally different dopaminergic nerve fibers are observed; non-glial-associated TH-positive nerve fibers that are formed in the absence of glial cells, and glial-associated TH-positive nerve fibers, which are formed in close association with glial cells (Johansson and Stromberg, 2002). Non-glial-associated TH-positive nerve fibers can reach up to 4 mm, appear after some days but does not persist over time, and at later time points they display a dotted appearance as if they are degenerating. The non-glial-guided nerve fibers are followed by a secondary wave of nerve fiber outgrowth. Hence, these first nerve fibers might act as path finding fibers, in order to create a scaffold that the following nerve fibers can use for support (Jacobs and Goodman, 1989a; Jacobs and Goodman, 1989b). Glial-associated TH-positive nerve fibers that are formed after 5-7 days, create a network of fibers circulating around the VM tissue slice at a distance of approximately 1 mm, instead of radiating straight away from the tissue slice as the non-glial-guided fibers do. Notably, these glial-guided nerve fibers are persistent over time and innervate the lateral ganglionic eminence in co-culture studies (Johansson and Stromberg, 2003). Interestingly, in *in vivo* trials of intracranial graftings, 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 Stromberg, 2003). The reinnervation of the graft-derived nerve fibers in the dopamine-denervated striatum occurs often over areas

known as “permissive sites”, where glial processes radiate from the graft to the host (Stromberg et al., 1992). The elongation of the graft-derived nerve fibers terminates after about one week post-grafting, followed by weeks of branching (Barker et al., 1996). Hence, the glial-guided nerve fibers obtained in organotypic VM cultures are likely the nerve fiber outgrowth that corresponds to the graft-derived outgrowth when implanted into the brain. Figure 3 depicts a summary of the described nerve fiber outgrowth from organotypic tissue cultures.

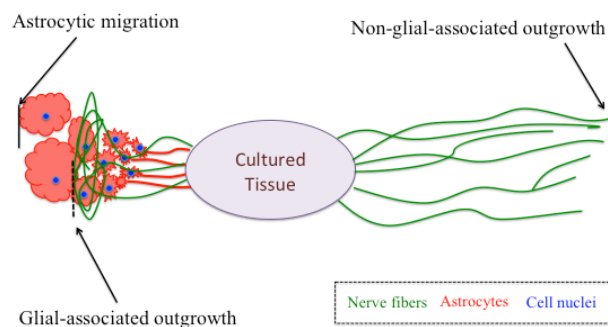


Figure 3: The schematic drawing displays the non-glial associated nerve fibers and the glial-associated outgrowth seen in ventral mesencephalon. Black arrows are pointing to the outermost border where astrocytic migration and nerve fiber outgrowth were measured.

Astrocytes

Neural precursor cells give rise to neurons followed by astrocytes, during mammalian nervous system development (Freeman, 2010). The generation and expansion of astrocytes is almost completed by early postnatal stages. Astrocytes express different types of ion channels, receptors and cell surface molecules to respond to neurotransmitters and environmental cues (Fiacco and McCarthy, 2006). Therefore, astrocytes have a crucial position in coordinating the neural circuits development. In transplantation studies of parkinsonian patients, nerve fibers and astrocytes derived from the fetal VM tissue interact with the adult astrocytes in the host brain. Hence, a need of having a model where these interactions can be monitored and controlled is important.

In adult brain, astrocytes form glial scar after for instance brain injury, which is one of the greatest obstacles in regenerative studies. Glial scar is an inevitable barrier that is made by reactive astrocytes with increased expression of Glial fibrillary acidic protein (GFAP) (Barrett et al., 1981; Bignami and Dahl, 1974), is inhibiting the regeneration of nerve fibers, and mainly constitutes of the proteoglycans (PGs) (McKeon et al., 1999). Hence their degradation is one of the highlights of regenerative studies (Bradbury et al., 2002). However, PGs have different cellular functions like neuronal survival, adhesion, migration, axonal growth, synapse formation and glial differentiation during the development of the nervous system (Margolis and Margolis, 1993; Oohira et al., 1994; Small et al., 1996).

During development, astrocytes participate in synaptogenesis. The first evidence that astrocytes instruct synapse formation, were studies related to cultures of the purified rodent retinal ganglion cells that were kept for several weeks in the absence of glia and other cell types (Barres et al., 1988). In the complete absence of glia, retinal ganglion cells form very few synapses (Pfrieger and Barres, 1997), while in the presence of cultured astrocytes or their conditioned medium, the number of excitatory synapses and their functionality were increased (Ullian et al., 2001). Examples of molecules, which were shown to play a role in the synaptogenesis, are thrombospondin-1 (TSP-1) (Christopherson et al., 2005) and CD47 (Ohnishi et al., 2005).

Proteoglycans (PGs)

Proteoglycans are a group of extracellular molecules and their structure consists of a core protein and glycosaminoglycan (GAG) side chains (Figure 4). PGs are divided into four major families based on the GAG side chains: keratan sulfate, hyaluronic acid, heparan sulfate/heparin, and chondroitin sulfate/dermatan sulfate (Ruoslahti, 1988). Degrading PGs is one of the

focuses of regenerative studies. In embryonic brain, chondroitin sulfate proteoglycans (CSPGs) are highly expressed and have a primary role in axonal guidance (Bandtlow and Zimmermann, 2000; Bovolenta and Fernaud-Espinosa, 2000). The main CSPGs in the brain during development are versican, brevican, neurocan, and aggrecan. Neurocan is expressed at the highest levels during embryogenesis, being present from E12 (Meyer-Puttlitz et al., 1995; Oohira et al., 1994). The distribution of neurocan and other CSPGs in the striatum during development suggests their involvement in guiding the nigral neurons for appropriate striatal innervation (Charvet et al., 1998a; Charvet et al., 1998b; Gates et al., 2006). During adulthood, CSPGs are the major components of glial scar, are increased after injury, and act like a barrier for axonal growth (Rhodes and Fawcett, 2004; Rhodes et al., 2006; Silver and Miller, 2004).

In glial scar, astrocytes secrete CSPGs at high levels, which inhibits regeneration (Jones et al., 2002; Matsui and Oohira, 2004; Properzi et al., 2003; Silver and Miller, 2004). However, functional regeneration and axonal growth can be observed in rodents e.g. after degrading the CSPGs in spinal cord (SC) injury (Barritt et al., 2006; Bradbury et al., 2002; Niederost et al., 1999; Yick et al., 2000), in the nigrostriatal pathway (Moon et al., 2001) or in neonatal organotypic co-cultures of cerebral cortex and SC (Nakamae et al., 2009). Thus, CSPGs appear to exert a general effect in the nervous system and are not devoted to control a specific circuit.

To unravel the effect of degraded CSPGs in tissue cultures, two different compounds with two different mechanisms of action, have been used. One of them is chondroitinase ABC enzyme (ChABC) that catalyzes hydrolysis of the GAG chains from the core protein without altering the core protein structure (Yamagata et al., 1968). ChABC treatment promotes axon regrowth both *in vivo* and *in vitro* (Bradbury et al., 2002; Fidler et al., 1999; Mckeon et al., 1995; Moon et al., 2001) and causes functional recovery of

injured spinal cord neurons (Bradbury et al., 2002). The other compound, methyl-umbelliferyl- β -D-xyloside (β -xyloside), can be used to inhibit the effects of the CSPGs by disruption of CSPG synthesis through blocking the activity of galactosyltransferase, which results in an insertion of xylosyl-serine instead of core protein (Schwartz et al., 1974). The altered GAG chains are unable to bind to the core protein, causing an increased amount of unbound core protein in the cytoplasm of the cell and released GAG chains (Niederost et al., 1999; Schwartz, 1977). Thus, β -xyloside blocks the production of CSPGs intracellularly by inhibiting the synthesis, while ChABC degrades CSPG extracellularly by enzymatic digestion. Hence, both compounds should exert similar effects on nerve growth, however, in tissue culture the results are contradicting i.e. while ChABC can promote axonal growth, β -xyloside has demonstrated to inhibit growth (Berglof et al., 2008; Nakamae et al., 2009).

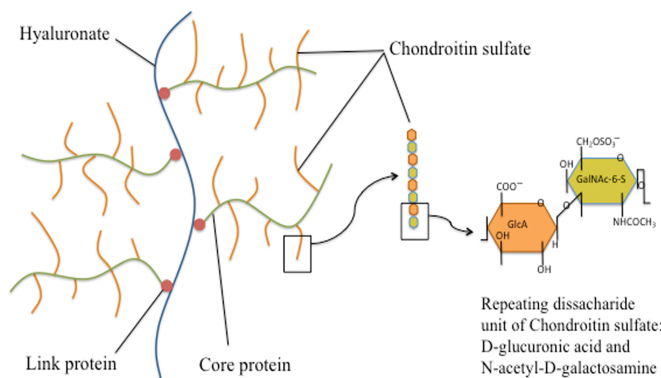


Figure 4: PGs consist of a core protein and GAG side chains. This is a schematic picture of CSPG structure.

CD47 and Sirp- α

CD47, also known as integrin-associated protein (IAP), is an extracellular matrix molecule, expressed by different cell types such as astrocytes, and is abundant in synapse-rich regions (Huang et al., 1998; Jiang et al., 1999; Ohnishi et al., 2005). The role of CD47 has been well studied in non-

neuronal cell populations for instance in activation and migration of leucocytes in response to bacterial infection (Verdrengh et al., 1999). It is also studied in the central nervous system (CNS) of CD47 deficient mouse model that showed a reduction of memory retention and long-term potentiation (Chang et al., 1999), and in overexpressing CD47 neurons with enhanced dendritic outgrowth and up-regulated synaptic proteins (Numakawa et al., 2004).

CD47 serves as a receptor for TSP-1. TSP-1 belongs to a family of extracellular matrix glycoproteins, is widely expressed in the developing and adult brain, is a multifunctional protein, and plays an important role in cell behavior such as migration, adhesion, and neurite outgrowth (O'Shea et al., 1990; Osterhout et al., 1992). Immature astrocytes produce TSP-1 and TSP-2 during brain development, and both promote the development of synapses (Christopherson et al., 2005).

CD47 acts as a ligand for signal regulatory protein alpha (SIRP α , also named SHPS-1), a cell adhesion molecule that was first localized to neurons (Jiang et al., 1999; Seiffert et al., 1999), and was later detected in immune cells like monocytes, granulocytes, and macrophages (Seiffert et al., 1999). Brain-derived neurotrophic factor (BDNF), a potent neurotrophic factor for dopamine neurons (Araki et al., 2000), exerts its effect through Src homology 2 (SHP-2) by affecting the phosphorylation of SIRP α (Araki et al., 2000; Ohnishi et al., 1999; Takai et al., 2002).

SPECIFIC AIMS OF THIS THESIS

- To assess the importance of the age of astrocytes on the interaction between dopaminergic nerve fibers and astrocytes (Paper I)
- To investigate the effects of degraded extracellular matrix (ECM) components produced by the astrocytes on nerve fiber outgrowth (Paper II)
- To study the effects of Integrins and integrin-associated proteins on the astrocytic migration and nerve fiber formation (Paper III)

MATERIALS AND METHODS

Animals

Fetal tissue from CD47 gene deleted (CD47^{-/-}) Balb/c mice (Jackson Laboratory, Bar Harbor, ME), SIRP α -mutant C57BL/6 mice (Inagaki et al., 2000), their wildtype (CD^{+/+}, SIRP α ^{+/+}) mice, and Sprague-Dawley rat was used for organotypic tissue cultures. Animals were housed under a 12:12 h light-dark cycle and provided with food and water *ad libitum*. Animals were kept and handled in accordance with internationally accepted guidelines. The experiments were approved by the local ethics committee. Efforts were made to minimize the number of animals used and to reduce any stress or discomfort.

Dissection of ventral mesencephalon and spinal cord

The ventral mesencephalic (VM) and spinal cord (SC) tissues from different developmental stages were dissected under sterile conditions using a microscope. The dissected pieces were chopped into 300 μ m coronal slices and transferred to Dulbecco's modified Eagle's medium (DMEM; Gibco, Stockholm, Sweden). The chopped VM and SC pieces were cut in the midline, and each piece was plated as one culture. The prepared tissue pieces were plated on previously washed, autoclaved and poly-D-lysine (PDL, 5 mg/100 ml dH₂O; Sigma-Aldrich, Stockholm, Sweden) -coated coverslips (12 x 24 mm) in a mixture of chicken plasma (Sigma-Aldrich, Stockholm, Sweden) and thrombin (1000 U/ml; Sigma-Aldrich, Stockholm, Sweden). Figure 5 is illustrating the procedure. The plasma/thrombin clot was allowed to dry for 15-20 min before the coverslip was placed in a 15 ml Falcon tube containing 0.9 ml medium. The tubes were inserted into a "roller-drum" placed in an incubator at 37°C and 5 % CO₂, rotating at a speed of 0.5 turns per min (Gähwiler et al., 1997).

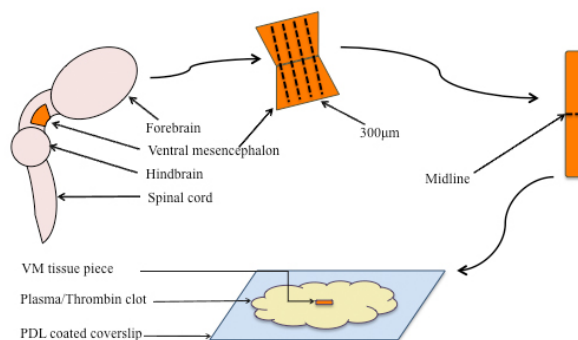


Figure 5: Schematic drawing for dissection procedure of VM piece from the embryonic brain. The tissue piece is chopped to 300 µm thick slices, which are cut in the midline. Each final piece is plated in plasma/thrombin clot on a PDL-coated cover slip and is representing one culture.

VM and GFP-positive astrocytes co-cultures

For co-culturing purpose, green fluorescent protein-positive (GFP⁺) adult astrocytes coming from cortex of postnatal day 2 (P2) Sprague-Dawley rat pups were mixed in medium containing 1 % antibiotics. The mixture was poured on a pre-coated PDL Petri dish and incubated for 96 h at 37°C. They were shaken at 300 rpm for 6 hours afterwards, to separate all other cells from the astrocytes and were discarded subsequently. For further proliferation, the medium was changed twice a week and the cells were incubated at 37°C until the day of co-culturing. At the time of co-culturing, the cells were trypsinized with 0.25% trypsin-EDTA and centrifuged at 180X g for 5 min. After aspiration of the supernatant, the cells were washed with fresh medium properly, and re-suspended in the medium to final concentration of 3.3×10^6 cells/ml. For plating the co-culture, 5 µl of the cell suspension was mixed with 20 µl of plasma together with one VM tissue piece, and was mixed with 10 µl thrombin to form a clot. The rest of the procedure was performed as described above.

Culture medium

The culture medium contained 55 % DMEM, 32.5 % Hanks' balanced salt solution (HBSS; Gibco, Stockholm, Sweden), 10 % fetal bovine serum (FBS; Gibco, Stockholm, Sweden), 1.5 % glucose (Gibco, Stockholm,

Sweden) and 1 % Hepes (Gibco, Stockholm, Sweden), and was sterilized through a filter with a pore size of 0.22 μm . The medium was stored at -20°C and thawed to 37°C before use. At plating, antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin, 25 $\mu\text{g/ml}$ amphotericin; Gibco, Stockholm, Sweden) of a final concentration of 1 % were added to the medium. Antibiotics were excluded from the first medium change. The medium was changed every 3-4 days.

Treatments of the cultures

In Paper II, cultures were treated either with 0.1 U/ml ChABC (Sigma-Aldrich, Stockholm, Sweden) or 1.5 mM β -xyloside (Sigma-Aldrich, Stockholm, Sweden) added to the medium from the plating day and for 14 days *in vitro* (DIV) named “early treatment”, or “late treatment” was performed when the cultures were established and treatment was initiated from day 14 and lasted through 21 DIV. For “n”s, see Table 1.

Treatment	Early treatment				Late treatment	
	VM E14	SC E14	VM E18	SC E18	VM E14	VM E18
Control medium	65	39	28	25	18	9
Medium+ β -xyl	36	13	15	17	21	9
Medium+ChABC	23	18	12	18	17	7

Table 1: Number of cultures used in paper II

In Paper III, antibodies against TSP-1 (2.5 $\mu\text{g/ml}$, A6.1 hybridoma, mouse IgG1, USA) were added to the medium of cultures derived from CD47^{+/+} tissue at the time of plating and throughout the experiment. Control cultures to the antibody treatment were treated with vehicle added to the medium of CD47^{+/+} tissue. For “n”s, see Table 2.

Genotype \ DIV	7 DIV	14 DIV
CD47 ^{+/+}	20	33
CD47 ^{-/-}	9	14
Sirpα-WT	—	19
Sirpα-mutant	—	6

Table 2: Number of cultures used in paper III

Immunohistochemistry

The organotypic tissue cultures were incubated for different DIV, in paper I for 14, 21, 28, and 35 DIV, in Paper II for 14 and 21DIV, and in Paper III for 7 and 14 DIV. Then the cultures were fixed in 2 % paraformaldehyde in 0.1 M phosphate buffer for 1 hour. After fixation, the cultures were rinsed in phosphate buffered saline (PBS; pH = 7.4) before primary antibody application. Primary antibodies were incubated for 48-72 h at 4°C. The cultures were incubated with goat serum (5 %; Sigma-Aldrich, Stockholm, Sweden) to block unspecific binding for 15 min at room temperature before incubation in secondary antibodies. Secondary antibodies were applied for 1h at room temperature. For staining the cell nuclei, 4',6-diamidino-2-phenylindole (DAPI) was used, and cultures were incubated for 10 min at room temperature. All antibodies and DAPI were diluted in 1 % Triton X-100 in 0.1 M PBS. After additional rinsing, the cultures were mounted in 90 % glycerin in PBS. All incubations were performed in a humidified atmosphere.

Antibodies used in experiments are listed in table 3 and 4.

Primary Antibodies	Type	Dilution	Source	Detection	Paper
β -tubulin isotype III (β -tubulin)	Mouse anti-human	1:200	Sigma-Aldrich	Microtubules ("pan neuronal" marker)	II
DAPI		1:50	Molecular Probes Inc.	Cell Nuclei	I & II & III
Neurocan	Mouse anti-rat	1:1000	Millipore AB	Core protein of CSPGs	II
Tyrosine hydroxylase (TH)	Mouse anti-rat	1:1500	ImmunoStar	Dopamine neurons	I & II & III
TH	Rabbit anti-rat	1:300	Millipore AB	Dopamine neurons	II & I
TH	Rabbit anti-rat	1:300	Pel-Freez	Dopamine neurons	III
Vimentin (VIM)	Chicken anti-rat	1:800	Abcam	Astrocytes	I & II & III
VIM	Mouse anti-pig	1:200	Sigma-Aldrich	Astrocytes	I & II

Table 3: Primary antibodies used for immunohistochemistry in papers I, II, and III

Secondary antibodies	Type	Dilution	Source	Paper
Alexa 594	Goat anti-mouse	1:500	Molecular Probes Inc.	I & II & III
Alexa 594	Goat anti-rabbit	1:500	Molecular Probes Inc.	I & II
Alexa 488	Goat anti-chicken	1:200	Molecular Probes Inc.	I & II & III
Alexa 488	Goat anti-mouse	1:500	Molecular Probes Inc.	I & II
Alexa 355	Goat anti-mouse	1:100	Molecular Probes Inc.	I

Table 4: Secondary antibodies used for immunohistochemistry in papers I, II, and III

Image analysis

The vimentin-positive astrocytic migration and TH/ β -tubulin-positive nerve fiber outgrowth from the organotypic fetal VM or SC slice cultures were measured using a microscale mounted in one ocular of the microscope. From each culture, astrocytic migration was measured from the periphery of the tissue slice in four perpendicularly placed directions and the distance to the

outermost migrated astrocytes was determined. Four measurements in areas with the longest nerve fiber outgrowth were measured. The mean values of astrocytic migration and nerve fiber outgrowth per culture were used for statistical analysis. Nerve fiber density measurements were performed using NIH image analysis program. The density measurements were performed over areas distal to the migration of astrocytes, near the astrocytic borderline. DAPI staining confirmed that no other cell types than vimentin-positive had migrated from the tissue slice. Image analysis using Openlab software (Improvision; Coventry, UK) was utilized to analyze the cultures.

Statistical analysis

Statistical analyses were performed using two-way analysis of variance (ANOVA) with genotype and DIV interactions in paper III, or fetal age and treatment as factors in Paper II, one-way ANOVA followed by Bonferroni *post hoc* test in papers I,II, and III, and Student's *t*-test in papers II and III. All data are expressed as means \pm SEM, and significant level was set at $p < .05$. All cultures were analyzed blind-coded during measurements.

RESULTS AND DISCUSSION

The focus of this thesis was to elucidate the two timely different nerve fiber outgrowths from organotypic cultures (Berglof et al., 2007; Johansson and Stromberg, 2002), and the effects of astrocytes on their formation to better understand how to improve graft-induced regeneration in the adult brain. The aims were to optimize the conditions for grafted dopaminergic neurons in dopamine-denervated striatum of parkinsonian patients, to improve their function by controlling and elongating the nerve fiber outgrowth. Transplantation of fetal VM is an effective treatment strategy in Parkinson's disease. However, the outcome of grafting experiments has been the limited reinnervation of the host striatum surrounding the graft. For this purpose organotypic VM tissue cultures were utilized and the two temporally and morphologically separated nerve fiber outgrowths, non-glial-associated- and glial-associated-TH-positive nerve fibers (Figure 3), and the effect of astrocytes on their formation were monitored.

The effect from the age of astrocytes (paper I)

The milieu that the fetal dopaminergic tissue piece will interact with, after being grafted to restore the lost dopamine function in PD, is the adult astrocytes of the striatum in parkinsonian patients. To control the prospective interaction between the fetal tissue and the adult brain in an optimized way, designing an *in vitro* system, in which fetal VM tissue and mature astrocytes can be monitored, is of great importance. Therefore a co-culture model has been developed where fetal VM tissue is co-cultured with mature astrocytes (paper I). Preceding the co-culture, finding the optimal age of the fetal tissue to use in the setup is important. For this purpose VM tissue from 3 developmental stages E12, E14, and E18 were cultured and followed up to 35 DIV (paper I). The astrocytes showed similar morphological pattern in all cultures, i.e. they formed a monolayer surrounding the tissue slice, and long

vimentin-positive processes were irradiating from the tissue piece, and at longer distances they were polygonal and large in their size (paper I; Fig. 1a-d, 2a-d, and 3a-d). In general, their migration reached a plateau after 21 DIV (paper I; Fig. 1e, 2e, and 3e). The outgrowth of two different types of nerve fibers, the non-glial- and glial-associated nerve fibers, which are independent or dependent on the presence of astrocytes, respectively, is a general phenomenon occurring in the E14 cultures from VM and SC (paper II and (Berglof et al., 2007)). Opposing to the earlier studies suggesting continuous growing of the non-glial-associated fibers in the absence of astrocytic migration (af Bjerken et al., 2008; Berglof et al., 2007; Johansson and Stromberg, 2003), the present study suggests that these nerve fibers reach a plateau at 21 DIV (paper I; Fig. 2g). Perhaps this contradiction is due to that the non-glial-associated growth has not been followed for such a long time in cultures before. Interestingly, their presence is pronounced within a timely narrow window during development that is in E14 cultures compared to E12 and E18 cultures. The glial-associated nerve fiber formation was observed in all embryonic ages, and their growth was also discontinued at 21 DIV. However, they had retracted at 35 DIV (paper I; Fig. 1f and 2f). These nerve fibers were not measurable in E18 cultures due to the specific characteristic of E18 cultures, which included neural migration that leaves no clear border for the measurements.

After monitoring the interaction between astrocytes and neurites at all embryonic stages, E14 was chosen for further studies due to that E14 cultures displayed long distance-growing nerve fibers, which could become a great hope for transplantation studies. Therefore, E14 VM tissue slices and GFP⁺ mature astrocytes were co-cultured for 21 DIV. The results revealed that both the glial-associate and non-glial-associated nerve fibers grew onto the GFP⁺ astrocytes (paper I; Fig. 4b and c). Surprisingly, TH-positive neurons migrated from the tissue slice on the monolayer of vimentin-positive/GFP-negative astrocytes (paper I; Fig. 4a). One possible explanation

for the neural migration is the presence of CSPGs produced by older astrocytes; since inhibition of CSPGs in E18 cultures hampered neuronal migration (paper II), and the co-culture experiment confirmed that neuronal migration was promoted in E14 cultures by the presence of the adult GFP⁺ astrocytes, which was not observed in the control E14 cultures. The monolayer of astrocytes, arose from the VM tissue piece, had changed their morphology, i.e. they formed a network and were disrupted by empty areas (paper I; Fig. 4a). Adult GFP⁺ cells formed a monolayer distal to the tissue piece and had large polygonal shapes, although some small single GFP⁺ astrocytes with thin processes were present closer to the VM tissue (paper I; Fig. 4d and e). Neural migration occurred nearby these small GFP⁺ astrocytes. Taken together, to optimize the presence of the long-distance growing nerve fibers, the age of the fetal tissue plays an important role. Paper I showed that the interaction between nerve fibers and immature astrocytes, arising from the tissue piece, has a limited time of outgrowth before they reach to a plateau, while the presence of mature astrocytes triggered neural migration independent of the age of the neurons. Interestingly, the adult astrocytes were not inhibitory for any of the nerve fiber outgrowths.

Effects of proteoglycans degradation (paper II)

Proteoglycans (PGs) play an important role in cellular function during neuronal development such as controlling the migration of fetal neurons (Snow et al., 1990). Degrading PGs has variable effects at different developmental stages in VM and SC cultures. Free GAG chains/core proteins may not support the migration of the neurons such as the intact PGs do. The effect of PGs can be inhibited by β -xyloside, a compound that inhibits the synthesis of PGs (Niederost et al., 1999), or ChABC, an enzyme that cleaves the GAG chains from the core protein extracellularly (Yamagata et al., 1968). β -xyloside and ChABC are used after SC injuries to promote

neuronal regeneration and might be helpful to increase neuronal outgrowth from VM grafts. In paper II, VM and SC “roller drum” cultures from E14 and E18 fetuses were treated with 1.5 mM β -xyloside or 0.1 U/ml ChABC for 14 DIV as the early treatment, or treated with normal medium for 14 DIV and thereafter for 7 DIV with the compounds, as the late treatment. Cultures were evaluated using TH- or β -tubulin- and vimentin-immunohistochemistry.

In general, morphological patterns of nerve fibers and astrocytes, and the distances that astrocytes migrated were similar in VM and SC control cultures. Thus, it seems that tissues from CNS, regardless of the area, follow the same pattern. However, embryonic age plays a role for instance, the distance for astrocytic migration was significantly enhanced in E18 cultures compared to E14 cultures (paper II, Fig. 1b). Additionally, the non-glial-associated nerve fiber outgrowth was present in both SC and VM E14 organotypic control cultures. However, their length and density was enhanced in β -tubulin-positive nerve fibers of SC cultures compared to TH-positive fibers in VM cultures (paper II, Fig. 1a). Based on previous studies, it was expected that their formation would be inhibited when astrocytic migration is promoted and vice versa, promoted when astrocytic migration is hampered (af Bjerken et al., 2008; Berglof et al., 2007; Marschinke and Stromberg, 2008). However, degrading PGs demonstrated novel results. Treatment of E14 VM and SC cultures with β -xyloside significantly hampered the outgrowth of these fibers and also astrocytic migration compared to control cultures (paper II, Fig. 2). ChABC had more vague but similar effects as β -xyloside on nerve fiber outgrowth in SC cultures, and on astrocytic migration in VM cultures (paper II, Fig. 2d and j). This effect could be explained by the fact that both ChABC and β -xyloside indirectly inhibit proliferation of glial cells (Berglof et al., 2008; Sirko et al., 2007), which consequently affects the astrocytic migration. To confirm the efficiency of the compounds, the control cultures were immunostained for

CS-56 and neurocan, as markers for GAG chains and core protein, respectively. Neurocan immunoreactivity revealed that ChABC-treated cultures had similar expression pattern as control cultures, i.e. neurocan was present in vesicles, near the nuclei of vimentin-positive astrocytes, while in β -xyloside treated cultures, it was diffuse in the cytoplasm of astrocytes including their processes (paper II, Fig. 4). To study the role of CSPGs at later stages during development, E18 VM and SC tissue pieces were cultured and treated with β -xyloside and ChABC and compared to control cultures. In E18 control cultures, the non-glial-associated nerve fiber outgrowth was not produced, instead neuronal migration occurred (paper II, Fig. 3a and e). Glial-associated nerve fiber outgrowth found in E18 cultures was not measured due to the lack of a precise border for estimation of nerve fiber growth since migrated neurons were present outside the tissue slice. Astrocytic migration was robust surrounding the tissue slices. Treatment with β -xyloside prevented the migration of neurons (paper II, Fig. 3c and g) and stimulated the outgrowth of β -tubulin-positive non-glial-associated fibers (paper II, Fig. 3d and g). This finding demonstrated that this type of nerve fiber outgrowth could be produced by other neurons than the dopaminergic. Besides, reduced distance for astrocytic migration was observed in β -xyloside treated cultures (paper II, Fig. 3h and i). ChABC exerted similar effects to β -xyloside treatment on astrocytic migration in VM but not SC cultures (paper II; Fig. 3h and i).

Overall, the results from the early treatment demonstrated opposing effects on E14 and E18 cultures, i.e. the absence of CSPGs generated nerve growth inhibition and promotion, respectively. The reason behind this difference is unclear, however, it is known that immature astrocytes that produce CSPGs promote nerve growth (Filous et al., 2010; Smith and Miller, 1991), and obviously this effect is dominant in E14 versus E18 cultures. Thus, the effects were most likely dependent on the presence of intact CSPGs rather than the astrocytes themselves.

To evaluate the effects of ChABC and β -xyloside on nerve fiber growth on already migrated and mature astrocytes, late treatment was performed on E14 and E18 VM cultures. Due to the lack of differences between VM and SC from previous experiments, studies were continued only on VM cultures. The results revealed no difference between the treatments in E14 cultures in the distance reached by the non-glial-associated nerve fibers and astrocytes (paper II, Fig. 5). In late treated E18 cultures, nerve fiber density was in general poor, and was restricted to areas with migrated astrocytes. Neural migration pattern was similar between ChABC-treated cultures and controls (paper II, Fig. 6a-b). A remarkable difference seen in the β -xyloside-treated cultures was that neurons did not migrate (paper II, Fig. 6c). After ChABC and β -xyloside treatments, nerve fibers within the tissue piece and in nerve fibers associated with the astrocytes had swellings in their nerve endings as if they were sprouting (paper II, Fig. 6i and j). The most distal astrocytes reached to similar distances independent of treatment (paper III, Fig. 6d). In both early and late treatment protocols, vimentin-immunoreactivity revealed similar morphology in ChABC-treated cultures and controls, whilst it was changed in β -xyloside treated cultures, from large polygonal-shaped to stellate-shaped cells with long processes (paper II, Fig. 7). The monolayer of migrating astrocytes had changed from confluent in controls to disrupted monolayer by empty areas (paper II, Fig. 2c and h, 3c and g, 5c, 6c, 7). This disruption of the astrocytic monolayer appeared to be stimulating the glial-associated nerve fibers rather than inhibiting, since nerve fibers reached to astrocytes located most distally to the tissue slice (paper II, Fig. 6). Taken together, this study suggests a switch of PGs function occurring already during developmental stages, between E14 and E18, and supports other studies showing this switch in fetal tissue compared to adult (Mark et al., 1989; Mark et al., 1990).

Effects of CD47 gene deletion on TH-positive nerve fiber outgrowth (paper III)

Controlling the non-glial-associated long-distance growing nerve fibers for VM graft regeneration in PD would be useful, besides graft survival and functionality. Achieving a proper functionality craves correct functional synapses. Hence, manipulating the level of the molecules that are important for synaptogenesis might lead to improved therapeutic effects. Previous studies have demonstrated that CD47 gene deletion improves regeneration in the spinal cord (Myers et al., 2011). On the other hand, overexpression of CD47 promotes dendritic outgrowth and up-regulates the synaptic proteins (Numakawa et al., 2004), thus making CD47 a potential candidate to improve graft function, and therefore CD47 was the focus of paper III.

To further study this issue one interesting strategy is to use CD47 knockout mouse model and monitor nerve fiber growth. Therefore, fetal VM tissue slices from E14 CD47^{+/+} and CD47^{-/-} mice were cultured for 7 and 14 DIV and examined for TH- and vimentin-immunohistochemistry (paper III). The results revealed no differences in astrocytic migration and presence of healthy TH-positive neurons between the different genotypes (paper III, Fig. 2c, 3a and b). However, the time in culture affected the distance reached by migrating astrocytes in both genotypes, i.e. it was significantly enhanced at 14 DIV compared to 7 DIV (paper III, Fig. 2c). Interestingly, the length of the non-glial-associated nerve fiber outgrowth as well as their density found in CD47^{-/-} cultures were significantly enhanced compared to CD47^{+/+} cultures (paper III, Fig. 1c and d, 2a, b, d and e). In addition, the nerve endings were enlarged in the cultures derived from CD47^{-/-} (paper III, Fig. 2d and e, inserts). The results suggested that astrocytes were permissive for nerve growth in the absence of CD47. From previous studies, it appears that in general when the astrocytic migration is inhibited, the non-glial-associated growth is stimulated (af Bjerken et al., 2008; Marschinke and Stromberg, 2008). This study demonstrated nerve fiber growth without any degenerative

signs when astrocytes had migrated, in the absence of CD47, which gives great hope for future grafting experiments.

To further investigate the observed effects on nerve fiber growth in CD47^{-/-} cultures, the two most important signaling pathways, in which CD47 plays an important role, were tested: CD47-SIRPα in which CD47 is the ligand, and CD47-TSP-1 in which CD47 is the receptor. Therefore, cultures of SIRPα mutant mice, that has normal extracellular but truncated intracellular domains of the receptor and can thus not mediate the signaling following CD47 binding (Inagaki et al., 2000), were studied and compared to their wildtypes for possible CD47-SIRPα interaction. To study CD47-TSP1 interaction, functional blocking antibodies against TSP-1 were added to the medium of CD47^{+/+} cultures. The overall results revealed healthy TH-positive neurons (paper III, Fig. 3a, c and d), similar distances of nerve fiber outgrowth (paper III, Fig. 3f, 4g), similar morphology (paper III, Fig. 4a-e) and density of TH-positive nerve fibers (paper III, Fig. 3e and 4f), and similar distances of migrated astrocytes (paper III, Fig. 3g and 4h) in SIRPα mutant and antibodies against TSP-1-treated cultures compared to their controls. Hence, the enhanced nerve fiber growth found in cultures derived from CD47 gene deleted tissue was not an effect mediated through blocking CD47-SIRPα or CD47-TSP1 interactions. Therefore it seems that other mechanisms are involved in CD47-dependent regulation of nerve fiber outgrowth that maybe make the astrocytes permissive. A likely explanation for similarities observed between SIRPα mutants compared to their wildtypes, could be that the interaction between cleaved and released extracellular domain of SIRPα is bidirectional and that the signal may be mediated through CD47 (Latour et al., 2001; Matozaki et al., 2009).

CONCLUDING REMARKS

- There is a narrow time limit for achieving long-distance growing nerve fiber outgrowth instead of neuronal migration and that is E14 in rats.
- In cultures derived from later stages, like E18, nerve fiber outgrowth is sparse and instead neurons are migrating onto the migrating astrocytes.
- The nerve fiber outgrowth and astrocytic migration reaches to a plateau at 21 DIV.
- The presence of adult astrocytes appears not to inhibit the outgrowth of nerve fibers, which might be important in future grafting studies. Their presence in the vicinity of the E14 tissue piece stimulates the neural migration, which might be due to the increased CSPGs production of the mature astrocytes.
- Formation of the non-glial-associated growth is probably a general phenomenon in different CNS tissues from early stages like E14.
- Early treatment with ChABC and β -xyloside gives similar results for E14 cultures, while β -xyloside but not ChABC promotes nerve fiber growth and inhibits neuronal migration in E18 cultures both after early treatment and late treatment. Thus, β -xyloside appears more effective than ChABC in promoting nerve fiber growth.
- The absence of CD47 may prevent drawback of the axons that form the non-glial-associated nerve fibers. The effect seems to be on the astrocytes rather than the nerve fibers. Furthermore, this effect is not mediated via SIRP α or TSP-1.

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