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Differentiation of Human Mesenchymal Stem Cells into Dopaminergic Neurons on Brain Electrode Materials

Master's Thesis
Espoo, May 13th, 2016

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School of Electrical Engineering
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ABSTRACT OF
MASTER'S THESIS

Title: Differentiation of Human Mesenchymal Stem Cells into Dopaminergic Neurons on Brain Electrode Materials			
Date:	May 13th, 2016	**Pages:**	9 + 83
Major:	Biotronics	**Code:**	S3037
Supervisor:	Prof. Tomi Laurila		
Advisor:	D.Sc. (Tech.) Emilia Peltola		

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into various cell types depending on their environment, but cannot undergo neurogenesis in normal conditions. In contrast, with the appropriate chemical and mechanical stimuli, these cells can be guided towards the neuronal lineage *in vitro*. Moreover, the generated neurons can be further directed into a dopaminergic (DA) subgroup. This process, however, requires optimal growth conditions, as well as suitable substrates for controlling the cell fate.

In this thesis work, human MSCs (hMSCs) were differentiated into DA neurons on four different carbon-based materials and the differentiation process with and without differentiation factors was assessed by following markers related to neurogenesis. The substrate materials were tetrahedral amorphous carbon (ta-C), ta-C coated with poly-D-lysine (PDL), ta-C coated with carbon nanodiamonds (vox) and vox functionalized with brain-derived neurotrophic factor (BDNF). The differentiation medium was a cocktail of BDNF, sonic hedgehog (Shh) and fibroblast growth factors (FGF2 and FGF8). The expressions of glial fibrillary acidic protein (GFAP), nestin, neuron-specific enolase (NSE) and thyrosine hydroxylase (TH) were tracked by immunofluorescence staining and quantitative real-time polymerase chain reaction (RT-qPCR).

The results showed the ability of the differentiation medium to induce neuron-like morphology in the cells cultured for 12 days on all material types. In addition, the marker profiles revealed a positive effect of the nanostructures on the MSC differentiation, while PDL coating was found unfavorable for MSCs. Furthermore, the results also indicate that the differentiation process had not been fully completed by the day 12, implying a need for a longer period in culture.

These experiments demonstrate various challenges related to developing an efficient protocol for DA differentiation from hMSCs, the most important being the optimization of the combined mechanical and chemical stimuli. Nevertheless, the use of MSCs holds great promise for therapeutic approaches in several medical conditions including spinal cord injuries and neurodegenerative disorders such as Parkinson's disease.

Keywords:	Human mesenchymal stem cell, adult neurogenesis, dopaminergic neuron, tetrahedral amorphous carbon, carbon nanodiamond, brain electrode
Language:	English

Aalto-yliopisto
Sähkötekniikan korkeakoulu
Bioinformaatioteknologian koulutusohjelma

DIPLOMITYÖN
TIIVISTELMÄ

Työn nimi: Ihmisen mesenkymaalisten kantasolujen erilaistaminen dopaminergisiksi neuroneiksi aivoelektrodimateriaaleilla			
Päiväys:	13. toukokuuta 2016	**Sivumäärä:**	9 + 83
Pääaine:	Biotroniikka	**Koodi:**	S3037
Valvoja:	Prof. Tomi Laurila		
Ohjaaja:	TkT Emilia Peltola		

Mesenkymaaliset kantasolut (MSC) ovat multipotentteja soluja, jotka voivat erilaistua useiksi eri solutyypeiksi ympäristöstään riippuen, mutta eivät kuitenkaan normaalisti tuota hermosoluja. Näitä kantasoluja voidaan kuitenkin ohjata kohti hermosolulinjaa *in vitro* sopivien kemiallisten ja mekaanisten ärsykkeiden avulla. Tuotetut solut voidaan vielä erilaistaa tietyksi alaryhmäksi, kuten dopaminergisiksi neuroneiksi. Tämä prosessi vaatii kuitenkin optimaaliset kasvuolosuhteet, sekä sopivan materiaalialustan erilaistumisen säätelemiseksi.

Tässä työssä ihmisen mesenkymaalisia kantasoluja erilaistettiin dopaminergisiksi neuroneiksi neljän erilaisen hiilipohjaisen materiaalin päällä ja tätä prosessia seurattiin neurogeneesiin liittyvien merkkiaineiden avulla sekä erilaistumismediassa että ilman erilaistumistekijöitä. Testatut materiaalit olivat tetrahedraalinen amorfinen hiili (ta-C), ta-C pinnotettuna poly-D-lysiinillä (PDL), ta-C hiilinanotimanttipinnoituksella (vox) ja vox funktionalisoituna BDNF:llä (brain-derived neurotrophic factor). Merkkiaineiden ilmentymistä seurattiin immunofluoresenssivärjäyksillä sekä kvantitatiivisella real-time polymeraasiketjureaktio -menetelmällä.

Tuloksista nähtiin, että materiaalista riippumatta erilaistumismedia sai aikaan neuronien kaltaista morfologiaa soluissa, joita oli erilaistettu 12 päivän ajan. Lisäksi havaittiin nanopinnoitteen positiivinen vaikutus kantasolujen erikoistumiseen, kun taas PDL-pinnoitus vaikutti epäsuotuisalta MSC-solujen viljelyyn. Tuloksista pääteltiin myös, että erilaistumisprosessi saattoi vielä olla käynnissä 12 päivän kohdalla. Soluja voisi siksi olla tarpeen erilaistaa pidemmän ajanjakson ajan.

Nämä kokeet osoittivat myös useita haasteita liittyen toimivan protokollan kehittämiseen MSC-solujen erilaistamiseksi dopaminergisiksi neuroneiksi. Tärkein näistä on sopivien kemiallisten ja mekaanisten stimulusten yhdistäminen optimaaliseksi erilaistumisympäristöksi. Tulevaisuudessa näitä kantasoluja voitaisiin käyttää hoitomuotona useissa hermoston sairauksissa, esimerkiksi selkärangan vaurioissa sekä hermorappeumasairauksissa kuten Parkinsonin taudissa.

Asiasanat:	Ihmisen mesenkymaalinen kantasolu, neurogeneesi, dopaminerginen neuroni, tetrahedraalinen amorfinen hiili, hiilinanotimantti, aivoelektrodi
Kieli:	Englanti

Acknowledgements

I would like to thank my supervisor professor Tomi Laurila for the opportunity to engage in such an interesting thesis topic. I also want to thank my instructor Emilia Peltola for teaching me everything I know about cell culture and giving me such great advice, even from another country.

I am also grateful to all my other workmates that have contributed to this work. To Niklas, Sami and Tommi, who prepared the carbon samples for my material experiments and to Noora, who kindly helped me with the enzyme linking system. In addition, I want to thank Katariina for taking care of my cells when they needed it and keeping me company when I needed it. Moreover, these two years in this group would not have been as fun without the lunch breaks with Noora, Katariina, Elli and Donna.

A special thanks goes to my dear family for all the encouragement and patience during this journey. To all my friends, I am extremely thankful for your loving support and understanding. And finally to my flatmate Aino, for putting up with me every day and for supporting me in everything I do.

Thank you, and keep up the good work!

Espoo, May 13th, 2016

Elsi Mynttinen

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Symbols and abbreviations

Abbreavation	Explanation
Ascl1	Achaete-scute homolog 1
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CNT	Carbon nanotube
DA	Dopamine
DAPI	4',6-diamidino-2-phenylindole
DG	Dentate gyrus
EGF	Epidermal growth factor
En1/En2	Homeobox protein engrailed 1/2
ESC	Embryonic stem cell
FGF2	Fibroblast growth factor 2
FGF8	Fibroblast growth factor 8
GABA	Gamma aminobutyric acid
GFAP	Glial fibrillary acidic protein
HD	Huntington's disease
hESC	Human embryonic stem cell
Hes1	Hairy enhancer of split
hMSC	Human mesenchymal stem cell
IGF	Insulin-like growth factor
iPS cell	Induced pluripotent stem cell
Lmx1	LIM homeobox transcription factor 1
LV	Lateral ventricles

MS	Multiple sclerosis
MSC	Mesenchymal stem cell
Msx1	Msh homeobox 1
MWCNT	Multi-walled carbon nanotube
NGF	Nerve growth factor
Ngn2	neurogenin 2
NSC	Neural stem cell
NSE	Neuron-specific enolase
Nurr1	Nuclear receptor related 1 protein
Otx2	Orthodenticle homeobox 2
PANi	Polyaniline
PBS	Phosphate-buffered saline
PCL	Polycaprolactone
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDL	Poly-D-lysine
PDMS	Polydimethylsiloxane
PEDOT	Poly(3,4-dioxyethylenethiophene)
PES	Polyethersulfone
PFA	Paraformaldehyde
Pitx3	Pituitary homeobox 3
PLLA	Poly-L-lactic acid
PPy	Polypyrrole
Ptc	Patched
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RBPj	Recombination signal binding protein for immunoglobulin kappa J region
RT	Room temperature
RT-qPCR	Real-time quantitative polymerase chain reaction
Shh	Sonic hedgehog
Smo	Smoothened
SVZ	Subventricular zone
SWCNT	Single-walled carbon nanotube

Ta-C	Tetrahedral amorphous carbon
TGFβ	Transforming growth factor β
TH	Tyrosine hydroxylase
VEGF	Vascular endothelial growth factor
VM	Ventral midbrain
WMC	Whole Molecule Control

1 Introduction

For decades, the prevailing belief throughout the scientific world was that no new neurons are born in the adult mammalian brain. It was not until the early 1960s that the discovery of neurogenesis in adult brain was first reported [1]. This finding soon led to an avalanche of studies confirming the presence of neural stem cells also in adult human brain as well as their capability to create new neurons throughout life. As culturing neural stem cells *in vitro* became a routine protocol in cell laboratories, it started a whole new era in the field of neuroregenerative medicine.

The growing understanding of the mechanisms involved in neurogenesis enabled the development of various methods to differentiate neuronal stem cells and embryonic stem cells into mature neurons, and later into specific neuronal cell types. In the year 2000, the next step forward was the successful stimulation of neurogenesis in mesenchymal stem cells, a subgroup of adult stem cells that are not capable of neurogenesis in normal conditions [2]. Several years later, human mesenchymal stem cells were further stimulated into dopaminergic neurons [3]. These milestones have led the path in the development of stem cell therapies in regenerating the central nervous system.

In several major neurological diseases such as Parkinson's disease, Huntington's disease, multiple sclerosis, spinal cord injury and stroke, the main pathological course is the destruction of neurons or their structures. The field of regenerative medicine aims to find solutions to replace the damaged tissue or assist the tissue in regenerating itself. Stem cells, particularly mesenchymal stem cells (MSCs), have shown strong potential in neuroregeneration as autologous, non-immunoreactive transplants. The use of stem cells in these applications has also been combined with stimulative factors [4] and biomaterial scaffolds with guiding topographies [5] to provide mechanical support and promote neural growth and neurogenesis at the injured areas.

Another topic of intense research for the past twenty years has been the use of electrical stimulation in treating various neurological conditions, especially Parkinson's disease [6]. The development of deep brain stimulation has required designing biocompatible materials with conductive properties

and the ability to support the growth of neural tissue at the implantation site. In this application, autologous stem cells could be used to further improve the functionality and long-term stability of the electrodes by tailoring the material-tissue interface. The current challenges include optimizing the stimulation method of MSCs to generate an active, implantable MSC-coated brain electrode and guide the stem cells into the neuronal lineage *in vivo*. The research areas around this future perspective include gathering more information of the signaling pathways governing natural neurogenesis, improving the efficiency and specificity of the guided MSC differentiation and designing suitable materials and differentiation factors for medical purposes.

This thesis is composed of a literature study and a following experimental part. In the literature review, section 2 gives an overview of the current level of knowledge on neurogenesis in the adult brain, covering the major cell signaling pathways in neural and dopaminergic differentiation. Section 3 introduces the different methods used in guiding stem cells into neurons *in vitro*, including transfection and chemical and mechanical stimulation. Finally, the last section of the literature work presents some existing medical applications of neuroregeneration as well as discusses the possible future directions of the field.

In the experimental part, the first objective is to assess the feasibility of a differentiation protocol [7] in stimulating human mesenchymal stem cells into dopaminergic neurons on electrode materials. Therefore, the cells are cultured and differentiated on four different carbon-based materials that have potential to be applied in brain electrode applications: tetrahedral amorphous carbon (ta-C), ta-C coated with poly-D-lysine (PDL), ta-C coated with carbon nanodiamonds (vox) and vox functionalized with brain-derived neurotrophic factor (BDNF).

The ta-C -based substrates have been proven suitable materials for bioelectrodes and more sensitive towards dopamine than pure platinum [8]. Furthermore, the sensitivity of the electrodes has been further improved by modifying the layer thickness and by inducing topography to the ta-C surface [9]. In particular, nanoscale structures have been seen to enhance the electrical properties of the brain electrodes. For example carbon nanotubes lower the electrode impedance and increase the observed current due to the increased surface area [10].

Here, the effect of nanotopography is evaluated by coating the ta-C samples with carbon nanodiamonds. This vox-coating contains multiple carboxyl groups enabling immobilization of various molecules to the surface. In these experiments, a growth factor molecule BDNF is functionalized onto the vox surface to assess the applicability of the carboxyl-linking as well as to inspect the effects of the immobilized BDNF on the cell behavior. In addition, PDL-

coating is used as a reference for its known ability to promote cell attachment, and the suitability of this coating for hMSCs is evaluated.

The purpose of the material experiments is to compare the extent of the differentiation on all these material types to determine potential conductive or even inducive effects of the coatings and nanostructures on the dopaminergic differentiation of the cells. In order to do this, the differentiation process is assessed with and without chemical factors by following neuronal markers with immunofluorescence staining and quantitative real-time polymerase chain reaction (RT-qPCR). The experimental part is comprised of two parts: section 5 documents the materials and methods used in the work and section 6 presents the results. In the end, section 7 concludes the highlights of the literature review and the main results of the experimental work.

2 Neural differentiation

Since the finding of the regeneration of neurons in mammalian brain in 1962 [1], the research in this area of neuroscience has been growing dramatically. A specifically interesting subject has been neuronal differentiation in human brain and its applications in regenerative medicine. To be able to fully take advantage of the stem cells and their potential in medical applications, it is essential to first understand the mechanisms and conditions through which the growth and development of the cells is guided in their natural environment. Hence, a major part of the research has been focusing on the signaling mechanisms and factors related to stem cell survival and neurogenesis in the mammalian and human brain. In this chapter, the main focus is on the neural stem cells and the cell signaling governing neurogenesis in the adult brain.

2.1 Stem cells

Stem cells are undifferentiated cells that have the ability to divide and produce other cell types with specific functions. A stem cell is also able to renew itself through mitotic cell division. Each division of a stem cell can lead to either two new stem cells (symmetric division) or one stem cell and one differentiated cell (asymmetric division). In mammals, there are two types of stem cells: embryonic and adult stem cells. Embryonic stem cells (ESCs) can be found in the inner cell mass of blastocysts, the early stage of the embryo development, whereas stem cells in adults are located in several tissues, e.g. blood, bone marrow and adipose tissue.

The differentiation potential, i.e. the potency of a stem cell, can be used to describe the functionality of different types of stem cells. Totipotent stem cells are capable of differentiating into any type of cells and a single cell can divide and differentiate to create a whole organism. The only fully totipotent cells in humans are the ones created during the first few days after fertilization, including the fertilized egg, until they start to specialize and

become pluripotent. These pluripotent stem cells, which include embryonic stem cells, can differentiate into all cell types, but cannot produce the entire organism by itself.

Multipotent stem cells, sometimes also called progenitor cells, have the potential to develop into multiple but limited cell types. A specific line of multipotent cells is called mesenchymal stem cells (MSCs), stromal cells that are found in most human tissues. These cells can differentiate into a variety of cell types *in vivo*, including bone, adipose and cartilage cells [11], but are not able to produce neurons in normal conditions. However, with appropriate stimuli, MSCs can also be guided into neuronal cells [2].

2.2 Neural stem and progenitor cells

The first evidence of neurogenesis, the regeneration of neurons, in mammalian brain was observed in 1962 by Altman *et al.* [1]. Two decades later, this finding led to the discovery of neural stem cells (NSCs). NSCs were first found in mammal embryonic central nervous tissue in 1989 [12] and were later confirmed to also exist in adult human brain [13]. NSCs are multipotent cells that are capable of differentiating into both neural and glial cells via intermediary progenitors and thus serve as the source of self-renewal in adult mammalian brain [14].

The NSCs in the human brain are also called slow proliferating cells or type B cells [15], and they give rise to type C cells, fast-proliferating transit-amplifying progenitor cells [15, 16]. These neural progenitors then generate either proliferating neuroblasts (type A cells), or glioblasts. Neuroblasts are dividing progenitor cells committed to the neuronal fate and further differentiate into neurons, whereas glioblasts generate non-neuronal glial cells, including astrocytes and oligodendrocytes [17] (see Figure 2.1).

NSCs are constantly generated in two parts of the brains of adult humans: the dentate gyrus (DG) of hippocampus and the subventricular zone (SVZ) of the lateral ventricles (LV) [13] (see Figure 2.2). SVZ is a remnant of the embryonic germinal neuroepithelium, and is the part of the forebrain, in which the NSCs are most abundant [19]. In human hippocampus, the neurogenesis takes place in the subgranular zone of the dentate gyrus, from which the generated neurons migrate into the granule cell layer [13].

In contrast to other mammals, there seems to be no detectable migration to or active neurogenesis in the olfactory bulb of the adult human brain [20]. The NSCs in the SVZ differentiate into neuroblasts, which then in the non-human mammalian brain migrate to the olfactory bulb through a structure called the rostral migratory stream [21]. On the contrary, in the human brain

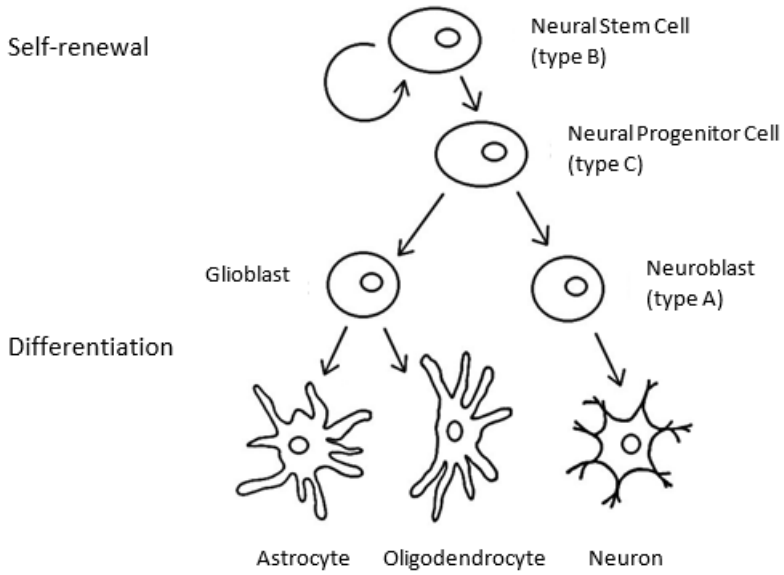

Figure 2.1: Differentiation of neural stem cells. Neural stem cells (type B) have the ability of both self-renewal and differentiation into all neuronal subtypes, e.g. astrocytes, oligodendrocytes and neurons, through intermediary progenitor cells (type A and type C).

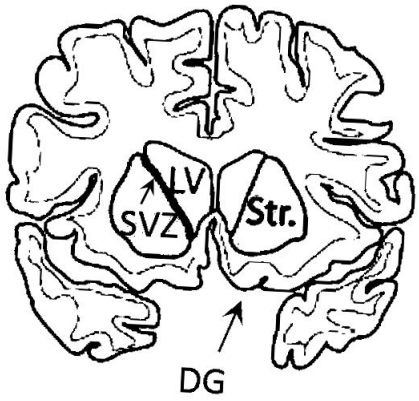

Figure 2.2: The adult human neurogenic system. The subventricular zone (SVZ; indicated by the arrow) is located between the lateral ventricle (LV) and the striatum (Str.). The hippocampal dentate gyrus (DG) lies near the center of the brain. (Modified from [18])

the destination of the migrating neuroblasts seems to be mainly the striatum of the brain, in which they differentiate into interneurons [22] (see Figure 2.2). However, the debate over whether a rostral migratory stream exists in human brain is still ongoing [22, 23].

2.3 Cell signaling during adult neural differentiation

The process of neurogenesis in the adult brain is tightly regulated and guided by a wide variety of cellular signaling pathways. Understanding these mechanisms underlying the behavior of the neural stem and progenitor cells during maturation and differentiation is the key to recreating the optimal growth environment for these cells outside the body. The knowledge of the intrinsic signaling is also a useful tool for tracking the progress of differentiation in *in vitro* conditions. The major pathways activated during adult neurogenesis are Wnt - beta-catenin, sonic hedgehog and notch, accompanied by an array of growth factors and neurotrophic factors (see Figure 2.3).

2.3.1 Notch pathway

The maintenance of adult NSCs is regulated through Notch pathway. Activation of a Notch receptor by ligand binding promotes cell cycle exit and progenitor differentiation, thus decreasing the pool of neural progenitors [24]. The Notch signaling is mediated through an intracellular signal mediator, RBPj (Recombination signal binding protein for immunoglobulin kappa J region) [25]. RBPj in turn acts as a transcriptional activator inducing the expression of transcription factors, such as a neurogenesis associated gene Hes1 (Hairy enhancer of split) [25, 26]. RBPj has been shown to exist both in adult SVZ [27] as well as the hippocampus [28].

Two of the receptors involved in the adult brain are Notch1 and Notch3. In the adult hippocampus, Notch1 is found to be required for NSC differentiation into progenitor cells both *in vivo* and *in vitro* [29], while activation of Notch3 seems to result in maintaining quiescence of the stem cells [30]. Furthermore, inactivation of RBPj induces an initial increase in neurogenesis by promoting neural differentiation, resulting in a subsequent depletion of the stem cell pool and a substantial decrease in neurogenesis [27, 28]. In the light of these findings, it seems clear that the Notch pathway indeed has an important role in regulating the maintenance of adult NSCs.

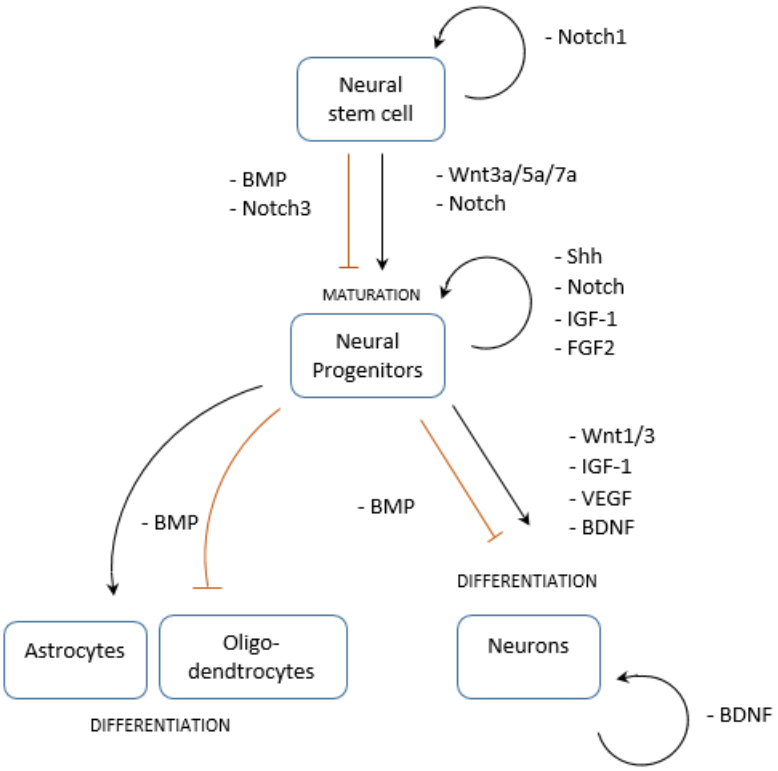

Figure 2.3: Neural differentiation and proliferation signaling in adult brain. The promoting factors are indicated with black arrows and the inhibitors with orange ones with end caps. The curved arrows represent the induction of self-renewal and maintenance.

2.3.2 Wnt signaling pathway

Wnt pathway has been shown to be involved in various different processes in the adult brain, including neuroblast proliferation, neuronal differentiation and development of dopaminergic (DA) neurons. Wnt signaling in the so called canonical Wnt pathway operates through a signal transducer protein, beta-catenin. In the absence of Wnt, beta-catenin is phosphorylated and degradated, resulting in a low intracellular beta-catenin level [31]. The degradation of beta-catenin prevents subsequent transcription of Wnt target genes, whereas a high level of beta-catenin allows initiation of Wnt-target gene transcription.

In adult hippocampus, Wnt signaling has been shown to regulate neuroblast proliferation and neuronal differentiation via beta-catenin [32]. A member of the canonical Wnt pathway, Wnt1, is especially associated with

the differentiation of neurons, and it is an important promotor of DA differentiation [33, 34]. In addition to Wnt1, Wnt3 also induces neurogenesis both *in vivo* and *in vitro*, and its inhibition results in reduced neurogenesis in adult hippocampus *in vivo* [32]. Two other signaling proteins, Wnt5a and Wnt7a together with Wnt3a, participate in the proliferation, self-renewal and differentiation of NSCs. Wnt7a is mostly studied in the adult hippocampus [35], while Wnt3a and Wnt5a have been found active in the SVZ neural progenitor cells *in vitro* [36].

2.3.3 Sonic hedgehog pathway

Sonic hedgehog (Shh) is an extracellular signaling protein, which regulates the proliferation of the neural progenitor cells in both adult hippocampus [37] and SVZ [38]. The Shh pathway includes two receptors, a transmembrane receptor protein Patched (Ptc), and its G protein-coupled co-receptor Smoothened (Smo) [39]. In the absence of an Shh ligand, Ptc receptor inhibits signal transduction from Smo, thus preventing the transcription of Shh target genes. In contrast, binding of an Shh ligand to Ptc enables the activation of Smo-regulated signaling cascade, which leads to transcription of Gli-dependent and other Shh target genes.

The receptors Ptc and Smo have been found to be expressed in both adult hippocampus [40] and the subventricular zone [38]. Overexpression of Shh increases the proliferation and the number of progenitor cells *in vitro* [41] and *in vivo* [37, 42], whereas blocking the pathway has been shown to have the opposite effect. Similarly, direct inhibition or dysfunction of Smo receptor has been found to result in significant reduction in progenitor cell proliferation in the postnatal hippocampus and SVZ [38] as well as in adult hippocampal dentate gyrus *in vivo* [43].

2.3.4 Growth factors and neurotrophic factors

Growth factors, a large family of extracellular signaling proteins, constitute to the neuronal development by promoting cell proliferation and supporting maintenance [31]. Important growth factors involved in adult neurogenesis include bone morphogenetic proteins (BMPs), insulin-like growth factor (IGF), fibroblast growth factor 2 (FGF2, also called basic fibroblast growth factor bFGF) and vascular endothelial growth factor (VEGF). These growth factors bind to receptors belonging to the tyrosine kinase family, which induces the activation of their specific downstream signaling pathways.

Bone morphogenetic proteins

Bone morphogenetic proteins are a group of extracellular signaling molecules that form the widest subgroup of the transforming growth factor-beta (TGF-β) superfamily [31]. BMPs have a multifunctional role in both embryonic and adult nervous systems affecting a variety of cellular processes including cell survival, proliferation and fate specification. In the embryonic brain, BMPs seem to be required for initializing stem cell proliferation and neurogenesis, while in the adult brain they function to promote quiescence of NSCs to prevent stem cell exhaustion [44].

The activity of BMPs is mediated by BMP receptors I and II. Upon BMP ligand binding, these receptors cooperate to set in motion an intracellular signaling cascade involving inhibitory Smad proteins, which in turn initiates the transcription of target proteins [45]. In the adult SVZ, BMPs serve as inhibitors for neuronal differentiation, while in the striatum it is suggested to direct differentiation towards the astroglial fate [46, 47]. BMPs are expressed in the subventricular zone by neural stem and progenitor cells, in which they prevent the differentiation of these type B and C cells into neurons.

One of the most important BMP inhibitors is Noggin, which is a protein produced by ependymal cells in the central nervous system [46]. Noggin antagonizes BMP signaling, thus promoting NSC proliferation and increasing neurogenesis in the adult hippocampus both *in vivo* and *in vitro* [46, 48]. Blocking BMP signaling by Noggin seems to result in initial increase in neurogenesis in adult DG, but a subsequent loss of precursors and neurogenesis, suggesting that BMPs are indispensable for maintaining neural stem cells and regulating neurogenesis [44].

Insulin-like growth factor

Insulin-like growth factor type 1 (IGF-1), a growth-promoting peptide hormone, is endogenously produced in adult neural progenitors in SVZ and is required for spontaneous neurogenesis [49]. In the adult hippocampus, IGF-1 has also been seen to directly stimulate neural progenitor cell proliferation *in vitro* together with FGF2 [50] as well as increase the rate of neurogenesis *in vivo* [51]. In addition to cooperating with FGF2, IGF-1 also seems to function as an instructive signaling molecule for the BMP pathway [52]. The inhibition of BMP signaling by IGF-1 seems to induce the differentiation of adult hippocampal progenitor cells and direct them into oligodendrocytes and neurons at the expense of the astroglial fate.

Fibroblast growth factor 2
FGF2 is another growth factor connected to adult neurogenesis and it has been found to affect especially hippocampal neurons. In the aging adult brain, the diminishing proliferation rate and dendtritic growth of neurons has been connected to a decrease in the levels of FGF2, alongside with IGF and VEGF [53]. The same effect has also been observed in FGF-receptor deficient mice, which show significant decrease in neural progenitor proliferation accompanied with reduced production of new neurons in the adult DG [54]. Inversely, the infusion of FGF2 into the adult brain results in addition of new neurons in the hippocampal DG, which indicates an important role of FGF2 in maintaining the self-renewal of neurons [55].

Vascular endothelial growth factor
VEGF has originally been known for its function as a promoter for vascular endothelial cell proliferation, but is also suggested to be involved in various processes in both developing and adult nervous system. This multifunctional growth factor seems to affect several different neuronal activities including neuronal growth and maturation during development [56] as well as hippocampal lesion-induced reorganization [57]. Furthermore, VEGF seems to act as both neurotrophic and neuroprotective factor in adult neurogenesis. The receptors for VEGF signaling are expressed on both endothelial and neuronal progenitor cells in adult hippocampus and SVZ [58]. VEGF acts directly via these receptors as a mitogenic factor for neuronal progenitor cells [59]. It has also been shown that direct infusion of VEGF into the lateral ventricle of adult rat brain promotes neurogenesis in both SVZ and SGZ [59].

Brain derived neurotrophic factor
Neurotrophins comprise a small group of proteins with various functions acting primarily on cells of the nervous system [60]. In mammals, four neurotrophic factors have been identified: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 and neurotrophin 4/5. Among these four, the brain-derived neurotrophic factor (BDNF) is the most extensively studied. The multiple roles of BDNF in the central nervous system include involvement in progenitor maturation and survival, neuronal development, differentiation and synapse formation and axonal regeneration [61].

BDNF acts through binding to a receptor tyrosine kinase abbreviated as TrkB receptor. This receptor is expressed on dividing progenitor cells both in the adult SVZ [62] as well as in the neurogenic zone of the hippocampal DG in mouse [63]. In contrast, while BDNF has also been reported to be expressed in the adult hippocampus, there is no evidence of BDNF activity

in the SVZ. In fact, it has recently been suggested that BDNF would not have a stimulative role in SVZ neurogenesis [64].

Despite the substantial amount of research done on BDNF function, its regulation and connections to other pathways has long remained unclear. Only recently have some studies started to link the activity of BDNF signaling to that of the Wnt/β-catenin pathway. In glial cells, BDNF seems to be a direct target of Wnt signaling [65], whereas in human neurons the roles of these two appear to interchange [66]. These studies imply a complex relationship between the BDNF and Wnt pathways - a crosstalk regulating the growth and development of human neurons.

2.3.5 Neurotransmitters

Neurotransmitters predominantly operate as the basis of chemical signaling between neuronal synapses. However, recent studies have also implicated a coexisting role for neurotransmitters in the regulation of adult neural progenitor cell proliferation, differentiation and adult neurogenesis [31]. The main effective neurotransmitters are glutamate, dopamine and GABA (gamma aminobutyric acid).

The evidence of glutamate intervention in neurogenesis is only an emerging subject for research, but it has been addressed by studying the expression of glutamate receptors on neural cells. In the adult hippocampus SVZ, the glutamate receptor NMDA (N-methyl-D-aspartate) seems to govern a regulatory role on neurogenesis [67].

GABA functions as the main inhibitory neurotransmitter in the adult brain. The most essential GABA receptor involved in neurogenesis is the $GABA_A$ receptor, which is activated during neurogenesis in the adult SVZ [68]. GABA is secreted by differentiating neuroblasts and bound to $GABA_A$ receptors on neural stem cells. The receptor activation then results in inhibition of NSC proliferation, thus providing a feedback mechanism regulating NSC proliferation. Furthermore, GABA has also been found to stimulate hippocampal newborn neurons in the adult brain, prominently improving their dendritic development and synaptic integration into the existing neuronal network [69].

Dopamine is a catecholamine regulating a vast range of functions in the adult brain. Similarly to glutamate, also the studies on dopamine as a neurotrophin have focused on dopamine receptor expression. The two types of dopamine receptors are D1-like (D1 and D5) and D2-like (D2, D3 and D4), divided by their structural and functional similarities [70]. In the adult SVZ, the D2-like receptors are especially expressed on transient amplifying cells

(type C), which are targets for hippocampal DA signaling. Loss of this signaling for example due to DA denervation leads to a significant reduction in the proliferation rate of the type C precursor cells in the SVZ [71, 72].

2.4 Signaling pathways in dopaminergic differentiation

Dopamine is a neurotransmitter secreted mostly by the DA neurons of the midbrain [73]. The loss of DA neurons in the midbrain structure called substantia nigra is the main cause of Parkinson's disease, a neurodegenerative disease affecting several neurological functions. The differentiation process of these neurons involves, for the major part, the same pathways that are active in neurogenesis. However, the later stages of the differentiation require specific signaling that guides the cells towards mature DA cells. These signaling factors, including Shh, Wnt, FGF8, notch and BDNF, act in a time dependent manner to regulate the development of the cells. The major signaling pathways are summarized in Figure 2.4. Studying the generation of DA neurons *in vivo* has enabled the first attempts to create these specific neurons *in vitro*, giving hope to future applications in regenerative medicine for example for Parkinson patients.

2.4.1 Sonic hedgehog

In addition to its role in the regular neurogenesis, Shh is also involved in the process of DA neuron development (see Fig. 2.5). In mammalian ventral midbrain (VM), Shh induces the expression of the homeodomain factor Lmx1a (LIM homeobox transcription factor 1 alpha), which subsequently induces the expression of its downstream effector, transcription factor Msx1 (Msh homeobox 1) [74]. Msx1 in turn initiates the expression of a proneuronal gene Ngn2 (neurogenin 2), thus supporting neuronal differentiation in DA neurogenesis.

The overexpression of Lmx1a in the VM results in generation of DA neurons, while reduced expression results in a loss of neurons with DA function [74]. A related factor, Lmx1b, has been shown to cooperate with Lmx1a in mediating the DA fate of neural precursors [75]. Similar to Lmx1a, overexpression of Lmx1b can also increase the production of DA neurons in VM [76] and conversely, reduce the number of DA neurons when depleted in VM [75].

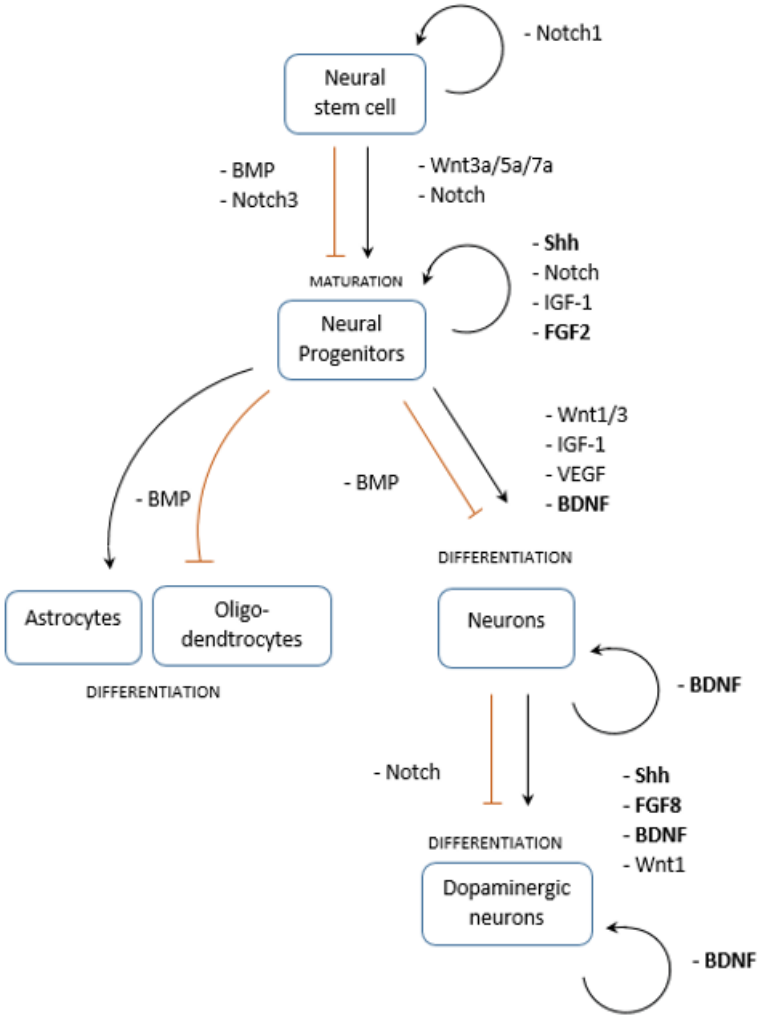

Figure 2.4: A summary of the major signaling factors and pathways regulating neuronal growth, differentiation and proliferation in the adult brain. The promoting factors are indicated with black arrows and the inhibitors with orange ones. The curved arrows represent the induction of self-renewal and maintenance. The differentiation stimulants used in the experiments of this work are highlighted in bold.

In addition to Shh, another transcription factor, Otx2 (Orthodenticle homeobox 2), is required for the expression of Lmx1a. Otx2 has been shown to regulate Ngn2 expression [77] by inducing the expression of Lmx1a [78]. In the absence of Otx2, neural precursors in VM fail to activate the expression

of Lmx1a, Msx1 and Ngn2, and consequently fail to differentiate into DA neurons [79].

Lmx1a expression is also modulated by FoxA2, a transcription factor part of a feedback loop with Shh [80]. Shh signaling is known to regulate Gli2 activator molecule, which in turn upregulates the expression of Gli1, an effector of Shh pathway [81]. Gli1 then induces the expression of FoxA2 [82], subsequently modulating Lmx1a expression. On the other hand, FoxA2 has also been suggested to regulate Shh expression [83], which implicates a possible feedback loop between Shh and FoxA2. The loss of either Gli1 or Gli2 *in vivo* has shown to result in reduction of DA neurogenesis [84].

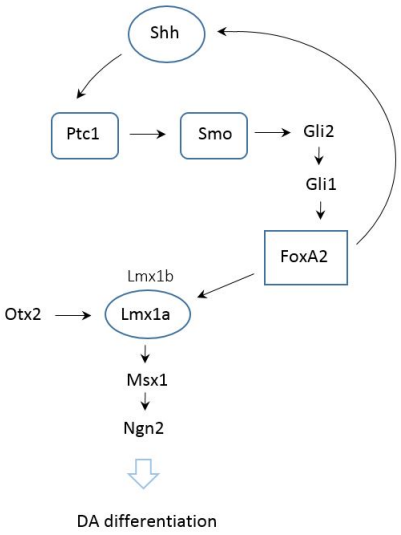

Figure 2.5: Shh signaling in adult DA neurogenesis. Shh promotes neuronal differentiation via various downstream effectors. The inducive pathways are indicated with arrows.

2.4.2 Wnt in dopaminergic neurogenesis

There seems to be a connection between the Wnt pathway and the development of DA neurons. The inactivation of Wnt1 and Wnt5a proteins have been associated with a loss of DA neurons in the adult midbrain [85]. In addition, it has been shown *in vitro*, that Wnt3a and Wnt5a might cooperate to induce neurogenesis by Wnt3a mediated progenitor proliferation and Wnt5a induced differentiation of NSCs.

Wnt1 seems to act as a key regulator of the proliferation and differentiation of VM DA progenitors into DA neurons both *in vitro* [33] and *in*

vivo [34]. Another Wnt protein, Wnt2, has also been implicated as a regulator of DA differentiation, since the absence of Wnt2 results in suppressed DA neurogenesis [86]. Furthermore, Wnt3a has been shown to induce DA progenitor proliferation but to inhibit their differentiation, whereas Wnt5a specifically enhances differentiation of progenitors into DA neurons [33, 87]. Conclusively, several signaling molecules of the Wnt family seem to have an essential role in regulating both maintenance and differentiation of the DA progenitors.

2.4.3 Sonic hedgehog and Wnt cooperation

Recent results have suggested a possible link between Shh and Wnt signaling in the regulation of DA neurogenesis (see Fig.2.6). It has been proposed, that Wnt is required to antagonise Shh, and the suppressed Shh levels allow the differentiation of VM progenitors into DA neurons [88]. This finding is not in an agreement with the previously stated results, which have shown the important role of Shh in inducing DA neurogenesis. It is possible, however, that Shh is initially required to create the VM DA progenitor pool by inducing neurogenesis, but is later involved in inhibiting their proliferation and differentiation. This theory suggests that once the Shh-induced progenitor pool has been established during development, Wnt signaling reduces Shh levels in VM, enabling DA neurogenesis.

Wnt has also been shown to promote Otx2 and Lmx1 expression [34, 88], both of which are known to participate in inducing neurogenesis. An autoregulatory loop between Wnt1 and Lmx1a has been proposed to control Otx2 expression via β-catenin during VM DA neurogenesis [89]. More recent data also shows that Lmx1a and Lmx1b might function cooperatively to regulate the proliferation of DA progenitors through modulating Wnt1 expression [90]. In addition, Otx2 has also been found to regulate Wnt1 expression therefore affecting DA neurogenesis [79], suggesting a Otx2-Wnt1 regulatory feedback loop involving both Lmx1a and Lmx1b.

It is still being investigated, whether the Wnt1-Lmx1a and the Shh-FoxA2 autoregulatory loops control VM DA neurogenesis synergistically [89], or whether the interaction between Wnt and Shh pathways is indeed antagonistic [88, 91]. However, taking these studies together it is evident that Wnt signaling is required for DA neurogenesis, involving a complicated cooperation with Shh as well as other signaling factors.

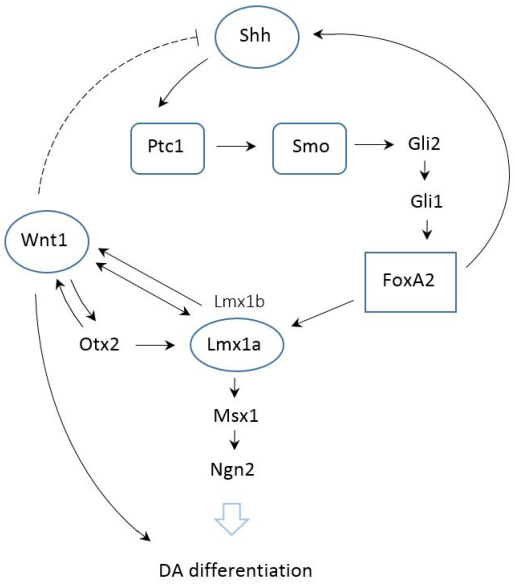

Figure 2.6: The interaction between Wnt and Shh signaling in adult DA neurogenesis. Wnt1 is linked to the Shh pathway via Lmx1 expression, and establishes a Wnt1-Otx2 regulatory loop that controls DA differentiation. The inducive pathways are indicated with arrows, while the dotted one with an end cap represents a suggested inhibitory regulation of Shh by Wnt1.

2.4.4 Notch in dopaminergic neurogenesis

Since Notch has been found to be the key regulator of the maintenance of NSCs, it is not surprising that it has also seen to affect DA differentiation. Studied in fruit flies, DA neurons were observed to differentiate only from cells without active Notch signaling [92]. This indicates that Notch signaling would function as a suppressor of DA neurogenesis. The Notch pathway is included in Figure 2.7.

One mediator in the Notch-induced repression of neurogenesis has been shown to be the downstream effector Hes1, a transcription factor inhibiting DA differentiation [93]. The involvement of Hes1 has been confirmed by studying its activator, Nato3. The transcription factor Nato3 was found to promote neurogenesis by repressing Hes1 expression. Nato3, on the other hand, acts downstream of FoxA2, thus suggesting a link between the Shh, Wnt and Notch pathways.

2.4.5 Neurotrophins and growth factors

In addition to their substantial role in neurogenesis, growth factors and neurotrophic factors also participate in the regulation of DA differentiation. The most important factors are FGF2, fibroblast growth factor 8 (FGF8) and BDNF. The contribution of these factors to DA neurogenesis is presented in Figure 2.7.

Fibroblast growth factors

FGF8 is another member of the fibroblast growth factor family, belonging to the same group of proteins with FGF2. FGF8 is required to induce the correct patterning of VM DA progenitors [94]. The loss of FGF receptors has been seen to result in altered patterning of the VM and failure of VM DA neuron maturation. FGF2, on the other hand has been shown to regulate DA progenitor proliferation, as well as the developmental cell death of mature DA neurons [95].

FGF8 has been suggested to have a connection with both Wnt and Shh pathways. Wnt1 is an essential regulator of the expression of two engrailed genes, En1 and En2 (Homeobox protein engrailed-1 and -2) [96]. The absence of these genes in mice generate a DA neuron depleted ventral midbrain similar to Wnt1-deficient mice [97] and thus are believed to be required to prevent apoptosis in DA neurons [98]. Apart from Wnt1, FGF8 has also been shown to regulate En1 expression in the developing VM [94], presumably through Wnt1. Since Wnt-β-catenin signaling has already been shown to upregulate FGF8 expression [99], an autoregulation loop similar to that of Shh and FoxA2, might also exist between Wnt and FGF8.

While Gli1 and Gli2 are shown to connect Shh to FoxA2, another transcription factor of the Gli family, Gli3, is involved in the Shh signaling. Gli3 functions as a repressor, and is suppressed by Shh to allow the de-repression of FGF8 expression [81]. In other words, by antagonising Gli3, Shh induces FGF8 expression and therefore facilitates DA neurogenesis. It has also been demonstrated *in vitro*, that the combination of Shh and FGF8 signaling molecules on NSCs results in the most effective induction of DA neurons [100].

Brain-derived neurotrophic factor

BDNF has been seen to support the survival of neurons in the adult forebrain [101] as well as the survival of DA neurons in the substantia nigra *in vitro* [102]. Subsequently, BDNF has also been demonstrated to be able to induce maturation of hMSCs into DA neurons and increase the DA release upon stimulation [103]. In addition, BDNF seems to have an inducive role in

neurogenesis *in vivo*, in neurons originating from both adult dentate gyrus [102] and SVZ [104, 105]. It might be even probable that BDNF is required for the survival of DA neurons in specific parts of the brain [106].

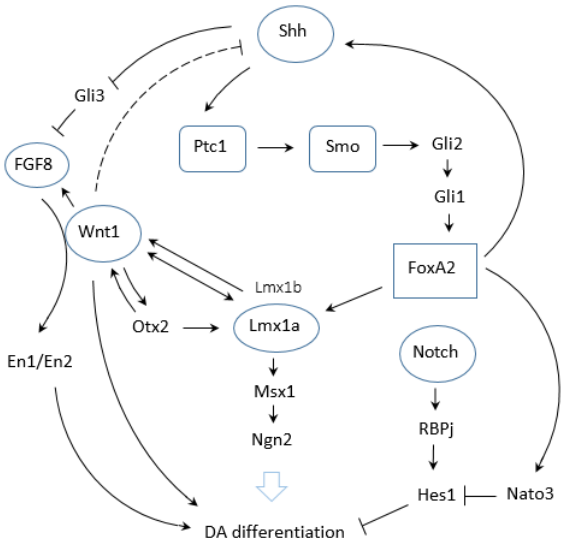

Figure 2.7: The major pathways and signaling factors affecting DA differentiation in adult brain - Wnt, Shh, FGF8 and Notch. Notch functions as an antagonist for DA neurogenesis, while FGF8 cooperates with both Wnt and Shh pathways to induce neurogenesis. The inducive pathways are indicated with arrows, while the ones with end caps represent inhibitory effects.

There is still a lot of research to be done in order to completely map the factors and pathways related to neurogenesis and DA differentiation. The signaling mechanisms directing stem and progenitor cells to differentiate into neurons also partly overlap with those of generating glial cells. This is especially seen during the embryonic development [107], but is also evident in the adult brain, as can be deduced from the multiple studies reviewed above. Another challenge regarding the research on cell signaling pathways is the fact that a major part of the experiments are conducted using rodent cells or other non-human models. Therefore, attention has to paid to the possibility that these results obtained from other organisms might not be analogous to the human nervous system [108].

In summary, the cellular signaling in the central nervous system comprises of a highly complex network of growth factors and morphogens, which are secreted in a time-dependent manner and controlled by intracellular, extracellular and environmental factors. This section presented the main chemical factors governing neurogenesis and DA differentiation in the adult brain according to the current literature (summarized in Fig. 2.4). While all of these factors have been demonstrated to have a role in the process of adult neurogenesis *in vivo*, it has been shown that the fate of stem cells in *in vitro* conditions can be affected already by a few of them, if applied into the cell culture at correct dosages. Currently, various mixtures of differentiation factors can be used to produce neural-like cells, and in this work, four different stimulants (highlighted in bold in Fig. 2.4) were chosen to induce DA differentiation in MSCs.

3 Guided neurogenesis

Guiding the differentiation of stem cells, especially MSCs, into neurons is a relatively new area of research in the field of neuroscience. The first demonstrations of guided neurogenesis from MSCs have been published in the beginning of the 21st century [2]. Starting with rodent stem cells and followed by numerous studies on human MSCs, these studies have introduced MSCs as a possible tool for regenerative medicine in neurological diseases. In guiding neuronal differentiation, the methods used today include chemical stimulation, transfection of pivotal genes and culturing on substrates designed to enhance neurogenesis.

3.1 Chemical stimulation

In chemical stimulation, a range of growth factors and neurotrophic factors have been used in differentiating stem cells into neural cells. Currently, BDNF, retinoic acid (RA) and FGF2 are among the most prevalent factors used in guiding MSC differentiation into neurons. For further inducing the cell fate towards DA neurons, the frequently used growth factors include Shh, FGF2 and FGF8, BDNF and ascorbic acid. The role of these molecules in the differentiation process is discussed in the previous section (see Section 2) and are summarized in Figure 2.4. Inducing neurogenesis in MSCs has been studied using several different media cocktails with varying combinations of growth factors.

In generating neurons from MSCs, BDNF, a member of the nerve growth factor (NGF) family, has been identified as a trophic factor for neuronal cells in the midbrain *in vitro* [102]. For its known ability to promote neurogenesis, BDNF has been used as a differentiation factor in several protocols aiming to increase neuronal and/or DA differentiation in stem cells [103, 109–111].

In addition to BDNF, RA is often used as a part of the induction medium for its known role in neural development [112]. In the early studies with adult stem cells, RA was seen to promote neural differentiation in both mouse and

human bone marrow MSCs [113]. Together with epidermal growth factor (EGF) in the proliferation medium, BDNF and RA were found successfully differentiate these cells into a neuronal lineage.

Although providing initial evidence of the capacity of stromal cells to differentiate into neurons, the first experiments resulted in significantly low yields. As the research on guided neurogenesis has evolved, the efficiency of the differentiation protocols has increased. Some of the differentiation factors contributing to the higher yields were FGF2 and FGF8, which together with EGF and BDNF were seen to be able to stimulate approximately 70% of hMSCs into neuron-like cells [112].

FGF2 and FGF8 are frequently used in guiding DA differentiation, along with Shh and ascorbic acid. The important role of Shh as a possible inducer of DA differentiation *in vitro* was first established by Hynes et al. [114]. Later on, the combination of Shh and FGF8 was demonstrated to increase the differentiation of both mouse and human embryonic stem cells into DA neurons *in vitro* [115], in which the role of FGF8 is though to be to promote midbrain specification [116]. Additionally, the co-effect of Shh and FGF8 together with FGF2 also results in a significant increase in DA differentiation in human ESCs [116].

Recently, studies with human bone marrow derived MSCs have further confirmed the important roles of Shh, FGF2 and FGF8 in DA neurogenesis [117]. These signaling molecules efficiently promote differentiation of MSCs into DA neurons both separately as well as in different combinations. Adding ascorbic acid to a mixture of Shh and FGF8 has also been used to induce DA differentiation in stem cells *in vitro* [118]. Furthermore, the addition of EGF and FGF2 into a differentiation protocol including FGF8, Shh and ascorbic acid, has been proven to further facilitate neuronal fate and differentiation of ESCs into DA neurons [119].

3.2 Transfection

Transfection is a technique that modifies the genetic expression of a cell by altering the nucleic acid sequence. The change in the genomic code results for example in overexpression of the corresponding protein. While traditionally used mainly in examining and studying neurogenesis, transfection has now been employed as an active tool in differentiating stem cells into neurons. The transcription factors studied as stimulative agents include Nurr1, Pitx3, Lmx1, En1/En2 and Ascl1.

Nurr1 (nuclear receptor related 1 protein) is a transcription factor that was shown to be related to neurogenesis in midbrain DA neurons already in

1997 [120]. A year later, the role of Nurr1 was specified to be involved in the later states of the differentiation process into DA neurons, the lack of Nurr1 leading to impaired DA specification and subsequent degeneration of the precursor cells [121].

When overexpressed in mouse NSCs, Nurr1 was seen to induce the majority of the cells to adapt a neuron-like morphology [100]. Moreover, the addition of Shh and FGF8 into the Nurr1-transfected cell culture induced DA differentiation, while this was not observed in control cells without transfection. These results were significant, since the cell line used in the experiments is not normally able to give rise to DA neurons *in vitro*.

The Nurr1-mediated guiding of differentiation towards DA neurons has been suggested to function through cooperation with another transcription factor, Pitx3 (pituitary homeobox 3) [122]. Pitx3 alone seems to be required, but not sufficient, for the development of DA neurons in the substantia nigra, since mice lacking this protein failed to develop these specified neurons [123]. In contrast, the co-transduction of Pitx3 with Nurr1 successfully promotes the maturation of DA neurons from both mouse and human ESCs [122]. This combination resulted in significantly increased levels of a terminal maturation DA marker DAT (dopamine transporter) as well as reduced the expression of non-neuronal markers. While this Nurr1/Pitx3 co-stimulation profile showed promising results *in vitro*, it was proven unsuitable for *in vivo*-implementation as such. The co-transduced ESCs were grafted into lesioned adult mice in the progenitor cell stage, but they showed only low TH expression, indicating a demand for additional inductive factors for *in vivo* applications.

A recent study assessed the transfection potential of another homeodomain protein, Lmx1 [74]. Lmx1a and Lmx1b are known targets for neurogenic Shh signaling (see section 2.4), and hence raised interest in direct use as stimulative factors. It was indeed shown that Lmx1a was both required and sufficient to trigger the process of DA differentiation in mouse ESCs. The cells were transfected with Lmx1a and cultured in a differentiation medium consisting of FGF2, FGF8 and Shh. The protein Lmx1b, on the other hand, introduces an independent pathway regulating midbrain DA differentiation [75]. The lack of Lmx1b production inhibited the induction and expression of Pitx3 leading to the death of specified midbrain DA neurons, whereas the expression of Nurr1 was not compromised.

In addition to the above mentioned homeodomain proteins Pitx3 and Lmx1, the fate of midbrain DA neurons is also controlled by two homeodomain transcription factors En1 and En2 [97]. These two engrailed genes (see section 2.4) are expressed by midbrain DA neurons throughout their life, controlling the survival of these neurons rather than affecting their differen-

tiation. In mice lacking these genes, the neurons are generated normally and differentiated into DA phenotypes, but then disappear in short time. While the absence of En2 alone results in nearly normal DA neuron distribution in the midbrain, the lack of En1 has a substantial degenerative effect leading to apoptosis of the majority of the DA neurons. For their role in maintenance of the generated DA neurons, En1 and En2 could have potential as transfection factors, but are yet to be implemented in this area.

Another type of transcription factor, Ascl1 (Achaete-scute homolog 1), has been linked to the cellular commitment of NSCs in both embryonic and adult brain [31]. In contrast to the genetic factors contributing to promoting neurogenesis, Ascl1 has been shown to induce adult hippocampal progenitor cells into oligodendrocytes instead of neurons [124]. In the adult brain, Ascl1 is shown to be transiently expressed by progenitor cells that subsequently differentiate into either oligodendrocytes or neurons [125]. However, the overexpression of this transcription factor instructed the cells to take up another pathway at the expense of their usual commitment towards glutamatergic neurons [124]. As this experiment demonstrates, it is not always particularly straightforward to predict the outcome of experiments when modifying the intricate machinery of the cells.

The challenges related to transfection-based stimulating strategies are mostly related to the complex nature of all cellular processes. The generation of new neurons and the specification into a certain subtype involves tightly controlled extracellular signaling as well as stage-specific gene expression programs. Hence, it is not likely that a single transcription factor could govern the complex process of neuronal and DA differentiation. On the contrary, it seems evident that a rather complicated combination of transcriptional factors have to be activated to complete the task.

3.3 Mechanical stimulation

Despite the positive results obtained from transfecting specific genes into cell cultures and animal models, this method might not be the most optimal one for clinical practice. As discussed above, it is not a simple matter to predict all the cumulative effects of the engineered genes on other interrelated pathways. In addition, the challenge of targeting the right cell population in a patient sets another obstacle for implementing this method in practice. Consequently, more effort has been put on taking advantage of inherent stimulants to correct neural dysfunction. While using soluble proteins and molecules to induce neuronal growth and differentiation has been a subject for intense research for the last decades, the success in therapeutic

applications has yet to be accomplished. This is mainly due to various problems related to solubility, stability, concentration and spatial and temporal positioning of the factors [126].

A potential strategy to overcome these limitations could be the use of suitable biomaterials to support the process. A growing interest has emerged towards taking advantage of mechanical factors and the properties of the substrate materials in order to affect cell behavior. Accumulating evidence shows the effects of both static and dynamic mechanical cues in guiding neuronal growth and neurogenesis. The possibilities in material modulation include chemical modification, topographic micropatterning and nanoscale surface featuring.

3.3.1 Material properties and chemical modification

Several material properties and modifications contribute to the cell behavior and can be utilized to promote neurogenesis. Favorable properties of the material itself include low stiffness, good biocompatibility and applicability of modifications, the most frequently used materials being different types of polymers and carbon substrates. The surface of the substrate can be modified for example by lithographic methods to create micropatterns or chemical modifications to promote neural growth and differentiation.

One of the underlying substrate properties contributing to the cell behavior is the stiffness of the material. Since the natural environment of the neural cells consists of the relatively soft extracellular matrix, it seems only logical that stem cells would prefer differentiating into neuronal lineages on softer surfaces rather than stiffer ones. Indeed, a variety of studies have shown that when MSCs are cultured on deformable substrates (E $\sim$ 0.1-1 kPa), such as soft gels, they tend to differentiate toward neuronal phenotype [127, 128], while stiffer matrices induce myogenesis ($\sim$ 11 kPa) and osteogenesis ($\sim$ 34 kPa) [127]. Compliant materials also specifically favor neuronal growth over that of glial cells [129].

Static micropatterning can also be combined with dynamic mechanical stimulation, such as stretching of the substrate, to create mechanical tension to the cells. To study the effects of both parallel and vertical tension, guiding microchannels were generated on elastic polydimethylsiloxane (PDMS) substrate and rat neuronal stem cells were cultured on the material [130]. The substrate was stretched repeatedly over several days and the effects were assessed by following neurite growth, axon elongation and differentiation behavior. The cells experiencing parallel tension were observed to express enhanced neurite and axonal outgrowth and orientation as well as promoted differentiation and maturation into neuronal cells.

Chemical modification is still another approach established for enhancing biomaterial properties. Graphene has been proposed as a biomaterial for neural interfacing, and its properties for stimulating neurogenesis have been enhanced by chemical processing. Graphene itself has excellent biocompatibility properties and it stimulates neurite growth of hippocampal neurons *in vitro* [131]. In addition, fluorinated graphene has been shown to facilitate hMSC fate toward neuronal lineage, making it a promising substrate for stimulating stem cells [132]. Furthermore, this carbon-based material has unusual electrical conductivity, and it can be fabricated into thin, flexible and highly transparent films, which could have great potential as a material for implantable nervous system applications.

3.3.2 Micropatterning

In the micrometer scale, stem cells can be guided by different patterns and pattern sizes. The width as well as the depth of the micropatterns has been seen to affect both the differentiation rate [133] and selective differentiation of stem cells [134]. With patterns too narrow and deep, the cells are highly constrained and cell-cell connections impaired, leading to smaller differentiation rates and decreased neurite development [133]. On the contrary, for wider micropatterns with a width larger than that of the soma (usually ∼13 μm), the differentiation rate and neurite growth of the cultured adult human NCSs increased and were comparable with flat, unpatterned surfaces. Similarly, no difference was observed in the differentiation rate of adult rat neural progenitor cells into neurons when cultured on micropatterns with a width of 16 μm [134].

These observations were also consistent with results from human MSC differentiation on both micro- and nanogratings [135]. Gratings with widths 10 μm, 1 μm and 350 nm were generated on a polymer substrate, and the results showed that while hMSCs were able to differentiate into neuronal lineages on all feature sizes, the effect was bigger on the nanoscale patterns. This is suggested to be due to the unconstrained environment, in which the neurites are free to spread and grow in multiple directions. The differentiation processes is hindered by a decrease in the number of neurites in the cell, and the neurites in turn seem to require a certain amount of physical space to protrude from the soma.

In addition to the size of the patterns, also the shape of the micropattern contributes to the stimulating effect of the substrate. This was addressed in a study comparing culturing of adult NSCs on linear, circular and dotted micropatterns, with feature sizes 2 μm and 10 μm [136]. The linear and circular patterns with both feature sizes were observed to promote NSC differentia-

tion towards neuronal lineage mainly by depressing astrocytic differentiation. Furthermore, the neuronal differentiation was allowed to a greater extent on the smaller patterns compared to the larger ones.

These studies suggest that the pattern itself would determine the differentiation fate, while the feature size affects the extent of the differentiation [136]. In the current literature, a vast array of different micro- and nanostructures, such as channels, grafts, grooves and dots have been examined in inducing differentiation of stem cells. Despite of the great amount of research, the detailed mechanisms governing the effect of the substrate topography on the cell behavior remain unclear. However, it is assumed that these mechanisms include the initial cell adhesion and spreading, the resulting geometry and orientation of the cells and consequent changes in gene expression leading to altered proliferation and differentiation.

3.3.3 Nanotopography

While both micro and nanometer features and topographies have been shown to promote cellular growth and differentiation of neurons, the effect of nanomaterials has proven even more substantial. This is likely due to the biomimicry of the nanostructures, which resemble the nanoscale features of the natural growth environment of the cells [137]. The contacts between the neurons and their environment rely on adhesion sites, so called focal adhesions, that are established via integrins [138]. These interactions occur in the 5-200 nm range and thus are greatly affected by nanotopographic features [138, 139].

Since nanomaterials are able to both favour neuronal adhesion as well as offer a natural-like microenvironment, they are a promising group of materials for promoting neuronal growth, directing neurogenesis and designing neural tissue scaffolds and transplants for medical applications. The frequently used materials to generate nanofeatures are polymers and carbon nanotubes and -fibers. By electrospinning, several polymer materials have been fabricated to create nanoscale surface features. These polymers include poly-L-lactic acid (PLLA), polycaprolactone (PCL) polyurethane and polyethersulfone (PES). To increase the biocompatibility of the materials, the polymers have also been coated with natural polymers such as collagen and laminin. For applications requiring electric conductivity, conductive polymers have been employed as differentiation substrates.

Polymer-based nanostructures

Several studies have shown the applicability of electrospun PLLA nanoscale fibers for neural tissue engineering and stem cell differentiation. Especially aligned PLLA fibers efficiently direct neural growth while also supporting NSC differentiation into neurons [140]. When co-polymerized with caprolactone and blended with collagen (PLCL/Coll), PLLA has also been shown to promote neurogenesis in hMSCs [141]. The addition of the natural collagen into the PLCL co-polymer further increases the biocompatibility of PLCL while also improving its mechanical properties favorable for nerve regeneration applications.

Caprolactone itself has also been fabricated into poly(ε-caprolactone) biodegradable fiber scaffolds to induce mouse ESCs [142] as well as adult rat NSCs [143] to differentiate towards the neuronal lineage. In both studies, the stem cells were seeded onto electrospun PCL fibers and stimulated with retinoic acid with surprisingly similar results. The cells were seen to generate all types of neural cells, including astrocytes, oligodendrocytes and early neurons. A comparison between aligned and randomly distributed nanofibers revealed the capability of the aligned scaffold to both guide the neurite outgrowth as well as discourage differentiation into astrocytes and oligodendrocytes. Both of these aspects would be important properties for a scaffold used for example to repair nerve injuries and to promote neuronal survival.

An even more recent approach to polymer-based scaffolds in neuronal regeneration is a possibility for controlled drug release from the biomaterial substrates. For example nerve growth factor (NGF) and retinoic acid have been added into PCL-scaffolds to further enhance its stimulative properties. To add NGF to a stem cell substrate, PCL was first co-polymerized with poly(ethylene glycol) to generate amine-functionalized block co-polymers [144]. This material was electrospun into nanofibrous meshes and then conjugated with NGF. When rat MSCs were cultured on the nanofibers, NGF was released from the matrix and found to clearly enhance neurogenesis. In a similar manner, also the encapsulation of RA within aligned PCL-fibers resulted in increased expression of neural markers in human MSCs [145].

Polyurethane and PES are also biocompatible polymers used in electrospinning to generate fibrous scaffolds. Human embryonic stem cells (hESC) grown on polyurethane fibers cultured with bFGF and EGF expressed positive neuronal markers after up to 47 days in culture [146]. The most interesting observation was done when comparing the positive markers seen from cells on the fiber scaffold and on a control substrate omitting the fibers. Although having otherwise identical differentiation condition, the lack of nanofibers induced the hESCs to preferentially differentiate towards glial cells, while the cells grown on the fiber substrate showed positive staining for both mature

neuronal as well as DA neuronal markers.

Similarly to polyurethane, also PES fibers coated with laminin promoted neuronal differentiation of adult rat NSCs when cultured with RA [147]. In this experiment, the diameter of the fibers was found to significantly affect the behavior of the cells. While polymer fibers with a diameter of 283 nm increased NSC differentiation into oligodendrocytes, fibers with 749 and 1452 nm drove elongation of the neurites along a single fiber axis and consequently promoted differentiation into neurons. In consensus with these results, also nanopillars fabricated from PDMS were observed to enhance the rate of neural differentiation by the increased pillar size from 35 to 400 nm [148].

As can be seen from these experiments, it is challenging to find the optimal parameters for the fabricated nanofibers and the optimal differentiation factors to chemically stimulate neurogenesis. Furthermore, while polymer materials have been proven to provide a biomimetic environment for maintaining survival of stem cells and promoting neurogenesis, most of them are still not suitable for a substantial amount of applications due to their lack of electrical conductivity. This shortage, however, could be overcome by using conductive polymers, a subgroup of organic polymers with metallic or semiconductive properties.

Conductive polymers

Several types of conductive polymers, such as polypyrrole (PPy) [149], polyaniline (PANi) [150] and poly(3,4-dioxyethylenethiophene) (PEDOT) [149, 151] have been fabricated into nanofiber-based coatings for neural tissue interfaces. All of these polymers have shown good electrical properties as well as capability to support neural adhesion and growth on the substrate surface. However, since these materials are fairly new in the field of biomaterials, some issues still need to be addressed in order to generate safe and efficient coatings for implantable devices.

While PANi has the advantage of an easy synthesis and low costs, the use of this polymer in biological applications is limited by its lack of flexibility and biodegradability, poor processibility and the evidence showing a chronic inflammation following implantation [152]. In contrast, PPy is easily modified to possess suitable properties, including larger surface areas, different porosities and excellent biocompatibility. However, it is very difficult to further process once synthesized, and is also mechanically rigid and brittle, non-thermoplastic and insoluble.

To date, PEDOT has shown the greatest promise in neural electrode applications due to the possibility of modifications as well as good electrical, chemical and environmental stability. [152]. An interesting application of PEDOT was demonstrated in an electrode interfaced with neural tissue by

in situ polymerization of PEDOT [153]. Once implanted, PEDOT formed filaments long enough to extend away from the electrode, bypass possible fibrous scar tissue and make contact with the healthy neurons around it. However, even PEDOT is not an entirely optimal material, facing some of the mutual obstacles among conducting polymers related to complicated processing and biocompatibility optimization.

In addition to biocompatibility issues, another major challenge related to conducting polymers is the stability of the deposited polymer layers. The problem of delamination has been observed to be an issue for both PEDOT and PPy -coatings as films but not as nanofiber structures [149]. While fabrication into nanostructures improves the adherence of the coating, it raises another concern about the effects of possible nanoparticle leakage into the surrounding environment. As long as the toxicity of nanoparticles is still under investigation, these materials are not allowed to be used in implantable medical applications.

Carbon nanotubes and -fibers

Carbon nanotubes (CNTs) are cylindrical nanostructures generated from graphene sheets wrapped onto themselves [154]. In neuroscience applications, the mostly used geometries are single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). SWCNTs are made up of a single graphene sheet closed at its ends by fullerene caps, whereas MWCNTs are comprised of several concentric graphene cylinders. In addition to their high conductivity, these carbon nanotubes also exhibit excellent mechanical strength and supposedly good biocompatibility.

One of the first experiments on carbon nanotubes as a substrate for stem cells studied culturing NSCs on SWCNT assembled with layer-by-layer poly(ethyleneimine) (PEI) thin films [155]. This piloting study confirmed the ability of carbon SWCNTs to support the differentiation of NSCs in the presence of a suitable differentiation medium. Since then, SWCNT-based scaffolds have emerged as effective tools not only to maintain, but also to promote stem cell and progenitor fate towards the desired neuronal lineage.

Human ESCs were first successfully differentiated into neuronal progenitors and neurons on CNT-derived matrices [156, 157]. In these experiments, CNTs were observed to direct the stem cell fate towards the neuronal lineage while having no negative effects on the cell viability. Furthermore, the mesh like surfaces seemed to enhance protein adsorption and cell attachment, whereas there were no apparent cytotoxic reactions from the cells [157].

Later, various studies showed the effect of SWCNT and MWCNT materials in guiding hMSCs towards neurogenesis [158–160]. Both SWCNTs [158] and MWCNTs [160] have been seen to upregulate neuronal markers from

hMSCs when cultured on the nanosubstrates without any agitation by an exogenous induction medium. Also MWCNTs fabricated into aligned sheet-like orientation directed hMSC morphogenesis and axon outgrowth inducing differentiation into neural cells under the influence of a neurogenic medium [159]. Recently, the trend in the neuroregeneration field has been shifting from these 2D nanosubstrates towards fabricating 3D matrices to best mimic the natural growth environment of the neural stem cells. One approach to generate 3D substrates is a hydrogel-based structure encapsulating CNTs, shown to present a non-toxic, neurogenic environment for MSCs [161].

The non-toxicity of the novel CNT-based substrates is not a matter of course, which is why so much effort is being put on finding solutions to shield the environment from possible nanoparticle leakages. As discussed previously with polymer-based nanostructured materials, the toxicity of these substrates is still debatable. So far, there has been no conclusive evidence of the toxicity, degradation and safety of carbon nanotubes [162]. However, it has been suggested that appropriate functionalization and route of administration can make carbon nanotubes both biodegradable and biocompatible.

In contrast to stem cells differentiated into neurons by chemical stimulation, there is currently no evidence of the electrophysiological function of the neurons generated by carbon-based topographical stimulation [5]. The expression of specific neuronal markers is a good indication of the desired direction of cell maturation, but is not a sufficient implication of electrically functional neurons. Thus, the next important step in utilizing nanomaterials in neuroregenerative applications would be the assessment of the functionality of the acquired neurons. Another challenge in this area is the synaptic integration of the new neurons into the existing neuronal network. Since nanoscale features evidently are able to guide axon and neurite outgrowth, they could provide an answer to this problem of complexity.

From this vast variety of experiments, it is difficult to conclude which features sizes, patterns or topographical cues would comprise the most effective way to stimulate stem cell differentiation. The main reason for this is the wide variance of material features, fabrication methods and coatings sometimes combined with a mix of differentiation factors, that are employed in directing the behavior of the cells. There would be a demand for more coherent and comprehensive series of studies that would address the effects of the material features and chemical factors separately in order to find the optimal cues for guiding stem cell differentiation into functional neurons. However, it seems that the combination of nanotopography and a mix of chemical cues could have the most favourable effect on cell behavior, providing that issues such as nanoparticle toxicity and stability of the soluble factors could be overcome.

4 Medical applications

Neurological dysfunctions such as stroke, traumatic brain injury, spinal cord injury, and a variety of neurodegenerative diseases are an enormous health issue world wide, and a lot of effort has been put into solving the problem of regeneration and functional restoration of the damaged central nervous system. Neural tissue engineering is an area of this research field, studying different biomaterials and their suitability to repair or support the injured neural tissue. Many types of materials can be fabricated to affect the survival, differentiation and functional activities of stem cells and neurons. These studies are also the basis for implementing the biomaterials into stem cell therapies, clinical application that aim to restore the damaged tissue by implanting stem cells and directing their growth and differentiation into the desired cellular lineages.

4.1 Regenerative neural tissue engineering

The area of tissue engineering in the central nervous system faces multiple challenges related to issues such as biocompatibility, stability and functionality of the implanted structures. Ideally, a repair system should be able to support regrowth of injured axons and possibly stimulate the generation of new neurons and their differentiation as well as promote their integration into the existing neuronal network [5]. In practice, these requirements establish a need for appropriate agents and structures to overcome the unfavorable and most often inhibitory environment for axonal regrowth, organization, synaptic remodeling and cell survival and differentiation.

4.1.1 Supporting scaffolds for neural interface

One of the pitfalls of the neuroregenerative research has been the limitations related to two dimensional cell cultures. While being suitable platforms for exploring the effects of various signaling factors and surface topographies,

the 2D structures lack the potential for real life *in vivo* applications. A three dimensional growth environment is essential for assessing the growth and behavior of neural tissue in a natural-like setup. Especially the formation of functional synaptic networks and electrophysiological function fail to be completed in 2D cell cultures [163]. Thus, there is a demand for more complex culture and transplant scaffolds for repairing neural tissue damage.

Although the field of 3D fabrication is a fairly new area of material science, several polymer-based scaffolds have already been designed for neural interface applications. A particularly useful method for designing 3D cultures employs the material group of hydrogels. These polymer-based materials offer both a solid support as well as an aqueous dispersion medium for neurons and stem cells [164]. In general, hydrogels have a low elastic modulus that can be further modified to simulate the stiffness of the neural tissue. These materials can also be tuned to improve their biocompatibility and degradation rate, which makes them a favorable choice for neural tissue constructs. In addition, hydrogel-based 3D implants enable mass transfer conditions similar to those of the natural tissue, which could help the cells to better adapt for implantation [163].

Modification of the hydrogel structures can also be used to further control the growth conditions of the cultured cells. The addition of nanotubes and -fibers into the hydrogel has been shown to serve as a guidance matrix for neuron growth and neurite extension [165]. The 3D hyaluronic acid hydrogel scaffold was integrated with aligned laminin-coated PCL-nanofibers. This combination material allowed directional control over the neurites, a property that could prove useful in engineered neural tissue applications.

Culturing stem cells on 3D hydrogel structures has already shown promise of enabling the type of cell behavior that has not been previously possible with 2D culture conditions. A recent study demonstrated the formation of neuroanatomical structures on an organoid culture system, on which the stem cells exhibited capability to self-assemble and to model human cortical development [166]. This approach could also be used as a tool for disease models, as it would allow creating specific dysfunctions in an environment accurately mimicking the human brain.

Although the 3D constructs have shown great promise in *in vitro* conditions, it is difficult to predict how they will perform in the inhibiting *in vivo* environment of the central nervous system [163]. However, the achievements in this field have already opened new possibilities for neural regeneration. The use of combination hydrogel scaffolds would enable embedding different signaling factors and nanoscale guiding systems to influence cell attachment, survival, differentiation and neurite extension. In addition, the embedding of nanostructures into a hydrogel matrix could be used to reduce the risk

of leaking of the nanoparticles into the surrounding tissues. The most effective grafts for neural regeneration would probably include both structural, scaffold-mediated cues as well as integrated stimulation factors.

In the future, it might be even possible to recreate some of the complex neuroanatomical structures, such as the cortex or spinal cord [163]. For example, the multilayered cortex could be formed by guiding the cell growth with layered nanofibers, so that the upper layers would promote intracortical connections and the lower layers direct the cell extension to develop into deep projections. This could be achieved by using induced pluripotent stem cells (iPS cells) or even MSCs, which would enable designing autologous cell grafts to replace damaged brain areas.

4.1.2 Stem cell therapies

The applicability of ESCs, iPS cells as well as MSCs has been assessed in multiple studies during the past twenty years. However, MSCs have recently been of particular interest in stem cell therapies for their multipotency and possibility to create autologous transplants with no rejection issues. In contrast to using embryonic cells for research, the use of MSCs does not involve any questionable ethical issues. In addition, the process of generating these cells is less complex and thus less expensive than that of iPS cells. The strategy of the stem cell therapy may be either differentiating stem cells *in vitro* into the desired cell lineage and subsequent transplantation into the affected region [167], or direct transplantation of the stem cells followed by *in vivo* differentiation [168]. The potential of different stem cell therapies have been studied in a variety of neurodegenerative diseases and brain injuries, such as ischemic brain damage, multiple sclerosis and Parkinson's and Huntington's disease.

One of the first applications of stem cell therapy has been the restoration of lost neurons in ischemic brain injury [169]. Ischemia is the condition in which the blood flow to the brain is disturbed as a result of a blockage or a hemorrhage. Thus the aim of the stem cell therapy in this context would be the replacement of the dead cells at the site of the injury. The effectiveness of stem cells has been assessed with multiple animal models using embryonic [170], neuronal [171] or mesenchymal stem cells [172–174]. In addition, a recent study showed an enhanced recovery in ischemic stroke patients after an injection of autologous MSCs [175]. While successful transplantation has been achieved with all of these different cell types, a great deal of effort is still required for the optimal clinical outcome [169].

Multiple sclerosis (MS) is a neurodegenerative, chronic autoimmune disease described by inflammation and deterioration of the myelin sheath of

motor neurons [176]. The clinical trials during the past few years have been focusing on ensuring the safety of the transplantation of stem cells into MS patients [177, 178]. The injection of MSCs into patients unresponsive to traditional medication has resulted in a decrease in characteristic lesions [178] and has also shown potential neuroprotective and regenerative effects [177]. Despite of these encouraging results, the capability of MSCs to differentiate into all types of neuronal cells *in vivo* is yet to be proven [177]. However, the transplantation procedure itself has been shown to be feasible as well as relatively safe [177, 178] with no serious adverse effects observed.

The stem cell treatments for Huntington's disease (HD) are still in animal testing, but have nevertheless shown promising effects in these disease models. HD is a progressive neurodegenerative disease characterized by degeneration of the striatum, with disabling and essentially fatal clinical course [179]. One pathological mechanism related to the neuron loss seems to be the dysfunction of astrocytic secretion of neurotrophic factors. Therefore, these NTFs, for example BDNF and GDNF, have been directly delivered into the affected sites to increase neuronal survival [180, 181]. As the transplantation of autologous MSCs had previously been proven to reduce the neurological symptoms of HD [182], a new strategy was soon developed to combine these two factors to create NTF-secreting stem cells [179, 183]. These studies confirmed the improved clinical performance of the engineered MSCs compared to native ones, thus establishing a new possible therapeutic approach to treating MS.

The search for stem cell therapies in Parkinson's disease has been ongoing for more than a decade, starting from animal models and proceeding into clinical trials in human patients during the past few years. In two different studies, ES cells were used to produce DA neurons via two different approaches. In the other experiment, DA neurons were derived from ES cells and transplanted into a mouse disease model [167], while the other one first transplanted the ES cells, followed by differentiation *in vivo* [168]. In both studies, the generated DA neurons showed correct functionality at the transplantation site.

ES cells were shown to be capable of spontaneous DA differentiation and to possess potential for implementation in clinical applications. However, the high potency of the ES cells comes at a price, as these cells have also been seen to form tumors after transplantation *in vivo* [168]. Thus, the use of ES cells is never straightforward, which is why the option of MSCs has become more favorable in generating DA neurons. MSCs have shown neuroprotective and anti-inflammatory effects both *in vitro* and *in vivo* [184, 185]. In addition, clinical trials conducted using bone marrow -derived autologous MSCs in PD patients have shown promising evidence of the effects of the treatment on the

condition of the patients [186]. Similarly to the engineered stem cells hoped to treat HD, new NTF-secreting cells are also designed to be transplanted to patients with Parkinson's disease in the new future [187].

Various trials in stem cell therapies have given hope for clinical implantation in untreatable neurological diseases. However, several hurdles are still to be overcome in the way to successful and safe stem cell transplantation into human patients. One challenge is to find the correct timing for the injection [169]. In animal studies, the stem cell injection is usually given directly after the disease model is generated, while this is not practical in clinical situations. Other issues requiring further research include the dosage to be used as well as the need of repeated dosages, the route of administration and possible use of scaffolds or other delivery structures. The fundamental challenge is to find a way to control the development of the stem cells in order to avoid tumor formation, while simultaneously create a functional subset of neurons with the ability to fire action potential and integrate into the existing neural network.

4.2 Conductive interfaces for neural stimulation

Brain electrodes have been developed for chronic stimulation in various neurological diseases including Parkinson's and Alzheimer's disease as well as sever depression and chronic pain. In these applications, both carbon nanotubes and polymer-based nanostructures have been shown to improve the performance of the electrode in *in vitro* and *in vivo* applications [149, 188]. Due to their higher surface area, the nanofibers lower the electrode impedance and increase the observed current [10, 151]. The long-term functionality is also enhanced by nanotubes designed to support neurite outgrowth and attachment to the electrode surface [149].

Although the implantable electrodes have already been applied to clinical practice [6], there are still some difficult issues especially related to the host response of the neural tissue [189]. The inflammation reaction results in a glial scar formation replacing the neuronal cells around the implanted electrode, leading to encapsulation and loss of function. This event also greatly reduces the number of neurons in the close vicinity of the electrode.

One approach to improve the biocompatibility of the electrode has been to determine the optimal mechanical properties for the material. A lot of research has focused on finding the most suitable physical dimensions and stiffness for the implantable device [190]. It seems that the size of the elec-

trode itself should be minimized, but should still exhibit high conductivity and moderate softness to mitigate the host tissue response.

Another strategy to reduce the inflammation effect of the implantation would be to tailor the material to actively guide neuronal growth and migration towards the electrode surface [188]. This could be achieved by for example using nanostructures together with integrated soluble factors to guide neurite growth or by introducing stem cells or progenitor cells at the implantation site to generate new neurons directly at the surface. If the differentiation of DA neurons from MSCs could be made more efficient and reliable enough, there could be a clinical window for this method to be implemented in simultaneous regeneration of the affected neural network and restoring the dopamine levels in the midbrain.

While the first clinically applied brain stimulation electrodes have proven successful, the research is still ongoing to further optimize the performance and meet the needs of the patients. An example of such an improvement is the concept of closed-loop neurostimulation [191, 192]. While the traditional device delivers stimulation at constant parameters, the closed-loop system would allow adaptation to the disease condition by simultaneously monitoring certain biological markers. For example in PD, responsive stimulation could reduce the fluctuations of the symptoms as well as increase the battery life, thus requiring fewer replacement procedures [192]. To become a reality, such applications need optimized material properties and surface structures as well as highly biocompatible electrodes to be able to both sense and stimulate the surrounding neurons.

5 Materials and methods

In this thesis work, the aim was to differentiate hMSCs into DA neurons on different brain electrode materials. Several experiments were conducted to modulate the protocol of Trzaska *et al.* [7] in order to succeed in differentiating hMSCs, as well as compare the effects of different electrode materials on the differentiation process. The first pilot experiments were done by growing C6, PC12 and hMSCs on glass coverslips to test the differentiation protocol, antibodies for immunofluorescence and quantitative PCR (qPCR) principles. In the main experiment, four different carbon-based materials were tested as a substrate for hMSC differentiation. The methods used to assess the cell differentiation were immunofluorescence staining and real-time quantitative PCR. A process diagram for the experiments is presented in Figure 5.1.

5.1 Sample preparation

The pilot experiments were conducted using glass coverslips (Menzel Gläser ⌀12 mm, Thermo Fisher Scientific) as a growth substrate for C6, PC12 and hMSC cells. In the electrode material experiments, the carbon materials used for hMSCs were silicon substrates coated with tetrahedral amorphous carbon (ta-C), ta-C coated with poly-D-lysine (PDL), ta-C coated with carbon nanodiamonds (abbreviated as vox) and vox functionalized with BDNF. All of these material types were tested with and without chemical stimulation (see Table 5.1).

The sample size was 1 cm x 1 cm for all material samples. The ta-C samples were prepared by first depositing a 20 nm Ti layer onto a conducting silicon wafer by magnetron sputtering, followed by a 7 nm ta-C film fabricated by filtered cathodic vacuum arc (FCVA) [8]. The nanodiamond-coating was then produced by a spraying technique, further described in the paper by Elomaa *et al.* [193]. The nanodiamond solution (5 wt-% in water) was diluted in ethanol 1:100 to prepare a solution with 0.05 wt-%. The spraying was done from a distance of 10 cm and the scanning was repeated ten times, the pressure being 3.5 bars.

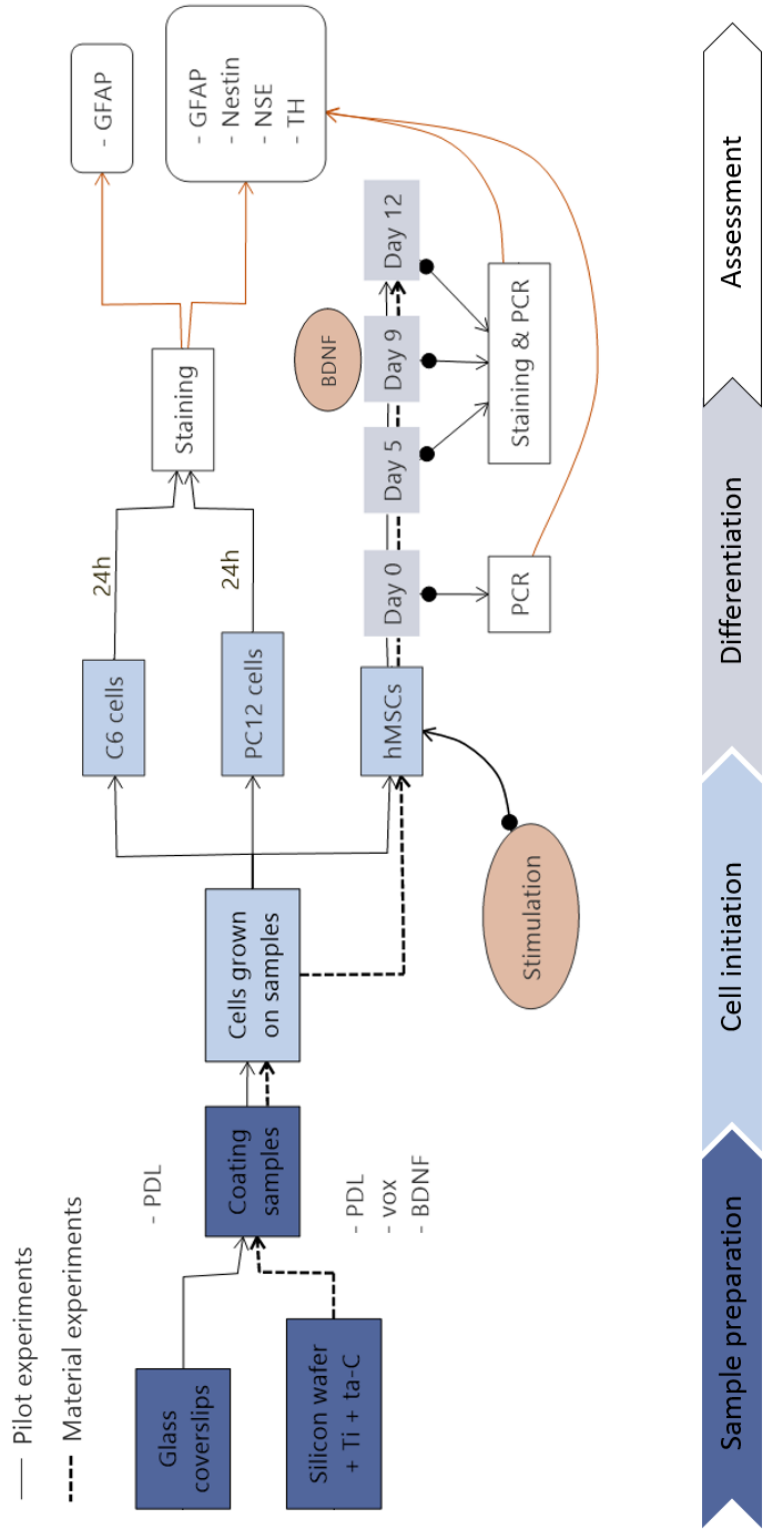

Figure 5.1: A process diagram for both the pilot experiments and the main experiments conducted in this work. The pilot experiments with C6, PC12 and hMSC cells are indicated with solid arrows, whereas the main material experiments with hMSCs are shown in dashed arrows. The color coding represents the different phases of the experimental protocol, consisting of sample preparation, cell initiation, differentiation and assessment.

Table 5.1: Different materials and coatings used in the electrode material experiments. The materials were tetrahedral amorphous carbon (ta-C), ta-C coated with PDL, ta-C coated with carbon nanodiamonds (vox) and vox functionalized with BDNF. All sample types were tested with and without chemical stimulation.

Material	Coating	Stimulation
ta-C	No	No
	No	Yes
	PDL	No
	PDL	Yes
ta-C + vox	No	No
	No	Yes
	BDNF	No
	BDNF	Yes

PDL-coating was used in all glass coverslip experiments as well as some of the material experiments to enhance the attachment of the cells onto the surface. All material samples were first sterilized in 80% ethanol for 15 minutes, after which the ethanol was removed and the samples washed with PBS (phosphate-buffered saline, VWR). 2 ml of poly-D-lysine hydrobromide (Sigma) was diluted in 38 ml serum-free medium (Dulbecco's Modified Eagle's Medium, DMEM). 0.7 ml of this solution was pipetted into the 24-well plate wells and 1.4 ml into the 12-well plate wells and the plates were coated for 1 h at room temperature (RT). The PDL was then aspirated and the wells were washed with PBS.

BDNF-protein was linked to the sample surface by an enzyme linkage system using a zero-length EDC-NHS crosslinker (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, N-hydroxysuccinimide, Sigma). 300 µl of a EDC-NHS solution (0.2 M/0.05 M) was incubated on the corresponding samples for 2 h at RT. The samples were washed with PBS, and BDNF (brain-derived neurotrophic factor, human, Sigma) diluted in di-water (50 ng/ml) was added and the samples were incubated for 1 h at RT.

5.2 Growing cells on samples

The cells used in these experiments were stored at -196°C at passages between 3 and 5 and cultured on Petri dishes with 10 cm of diameter (Corning Inc., New York, NY, USA). The C6-cells from a rat glial cell line (ATCC® CCL-107™, Manassas, VA, USA) were used to assess the feasibility of the GFAP antibody (see section 5.4). The cells were subcultured once (medium F-12 HAM Nut Mix (Sigma) supplemented with 15% horse serum (HS, Biowest), 2.5% fetal bovine serum (FBS, Gibco™ Thermo Fisher Scientific), 1% Penicillin Streptomycin (Thermo Fisher Scientific) before using them in the experiments. At approximately 70-80% confluence, the cells were detached using 2.5 mg/ml trypsin in PBS-EDTA (0.01 ml/cm^2) at RT for 5 min. They were then suspended in culture medium and transferred into three 24-well plate wells with coverslips coated with PDL (cell density 5.3 x 10^5/cm^2). The cells were allowed to adhere overnight at 37°C and 5% CO_2 before staining.

PC12-cells (ATCC® CRL-1721.1™, Manassas, VA, USA) derived from the rat embryonic neural crest were cultured to test nestin, NSE and TH antibodies in addition to GFAP (see section 5.4). The cells were subcultured once (medium F-12K Nut Mix (Gibco™ Thermo Fisher Scientific) supplemented with 15% HS, 2.5% FBS, 1% PS) and at approximately 70-80% confluence detached from the dishes by scraping. The cells were suspended in new medium and transferred into nine wells of a 24-well plate with PDL-coated coverslips (cell density 2.2 x 10^4/cm^2) and incubated overnight.

The differentiation protocol and the assessment methods using antibodies and qPCR were also tested on hMSCs cultured on PDL-coated glass coverslips before applying them to the material samples. Human bone marrow derived MSCs (Poietics™, Lonza, Walkersville, MD, USA) were thawed and cultured on Petri dishes with 10 ml of Lonza Mesenchymal Stem Cell Growth Medium, (MSCGM™) containing Mesenchymal Stem Cell Basal Medium (MSCBM™) supplemented with Mesenchymal Stem Cell Growth Supplement, L-glutamine and GA-1000 (Gentamicin/Amphotericin-B).

The obtained cells were subcultured once, allowed to proliferate for approximately 7 days and then detached using 0.25% trypsin in PBS-EDTA (0.035 ml/cm^2) at RT for 8-10 min. An equal volume of temperature equilibrated MSCGM was added, the suspension was transferred to a Falcon tube and the trypsin was removed by centrifuging at $600 \times g$ for 5 min. The cells were then resuspended in MSCGM and seeded onto the well plates (cell density 5.0 x 10^3/cm^2). For each three time points (see section 5.4), two replicates for each antibody were prepared into four 24-well plates. Each plate also contained a negative control sample. In addition, three replicates

per time point were assembled into one 12-well plate (without coverslips) for qPCR.

For the material experiments, hMSCs were obtained as described above. For each three time points (see section 5.4), the hMSC cells were transferred onto four 24-well plates containing nine samples of each material type, two replicates for each antibody plus negative control. The cells were seeded onto a total of 216 samples (12 well plates) with the density of 5.0 x $10^3/cm^2$ and incubated overnight.

5.3 Differentiation protocol

The differentiation of the hMSCs was conducted following the guidelines of the protocol of Trzaska *et al.* [7]. After the overnight incubation, the medium of the corresponding samples was changed to a differentiation medium consisting of 0.4 ml/0.82 ml Neurobasal Medium (Gibco^{TM} Thermo Fisher Scientific), 0.8 µl/1.6 µl B27 Supplement (0.5%, Gibco^{TM} Thermo Fisher Scientific), 2 µl/4.1 µl Shh (250 ng/ml, Sigma), 1.6 µl/3.3 µl FGF8 (100 ng/ml, Sigma) and 0.8 µl/1.6 µl FGF2 (50 ng/ml, Sigma) for the 24-well plate wells and 12-well plate wells respectively.

The wells were placed back into the incubator at 37 °C and 5% CO_2 and incubated for 9 days. At the 9 day time point, 0.2 µl/0.4 µl (24-well plate and 12-well plate respectively) BDNF (50 ng/ml, Sigma) was added into each well and the plates were incubated for additional 3 days (total of 12 days from the initial induction).

5.4 Immunofluorescence staining

For assessing the differentiation process, three time points, 5, 9 and 12 days in culture were used as checkpoints for indicative markers. The markers for both staining and qPCR experiments were chosen for their known specific expressions in neuronal precursors, mature neurons and DA neurons (Figure 5.2). The markers used in immunofluorescence experiments were glial fibrillary acidic protein (GFAP) [2], nestin [194], neuron-specific enolase (NSE/ENO2) [195] and tyrosine hydroxylase (TH) [196]. These markers were followed at the time points 0, 5, 9 and 12 in the pilot experiment, the 0 time point kept as a reference (Fig. 5.3). In the material experiments, Whole molecule control (WMC, Mouse IgG) was used as a negative control, and the markers were stained at time points 5, 9 and 12 (Fig. 5.4).

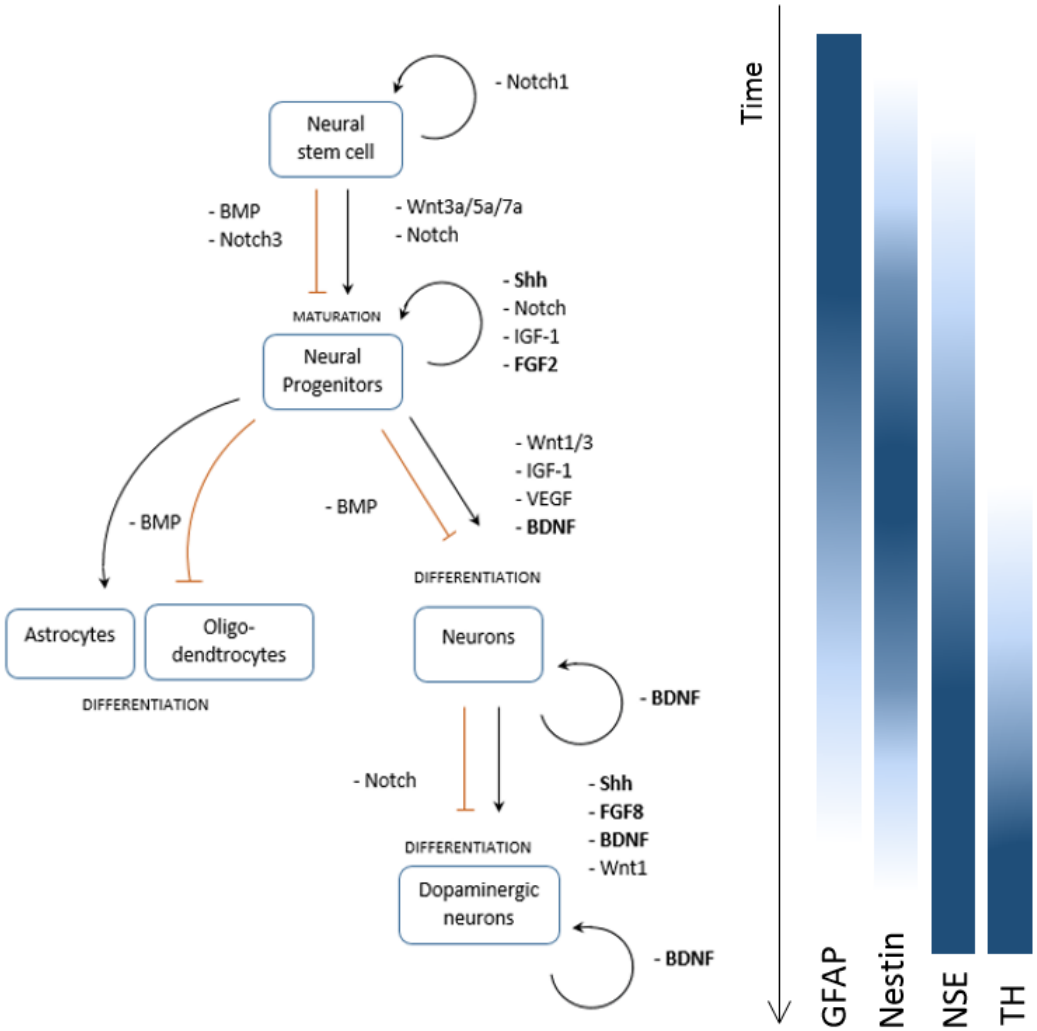

Figure 5.2: Immunofluorescence and qPCR markers expressed by the differentiating neuronal cells. The strength of the color indicated the expected strength of expression of the markers as the differentiation proceeds. The time runs vertically from the top towards the bottom of the image.

For immunofluorescence staining, the plates were fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes at room temperature and washed 3 x 10 minutes with PBS. The cells were then permeabilized with 0.5% Triton X-100/PBS solution for 10 minutes at RT and washed 3 x with PBS. Goat serum was used to block the cells with a solution of 90% bovine serum albumin (BSA) (0.1% in PBS) and 10% goat serum for 30 minutes.

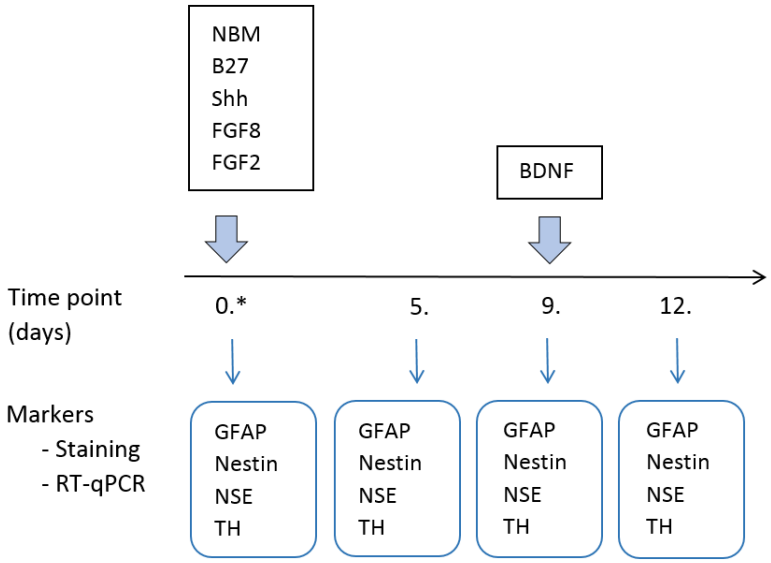

Figure 5.3: A timeline for the differentiation and immunofluorescence staining for hMSCs on glass coverslips as well as RT-qPCR for both the initial experiment and material samples. The differentiation medium containing NBM, B27, Shh, FGF8 and FGF2 was added at time point 0, followed by the addition of BDNF at time point 9. The assessment was conducted using immunofluorescence staining and RT-qPCR with markers GFAP, nestin, NSE and TH, keeping the 0. time point as a reference (*).

Primary antibodies against GFAP (Anti-GFAP mAb, Abnova), nestin (Anti-NES mAb, Abnova), NSE (Anti-NSE mAb, Abcam), Th (Anti-Th mAb, Abcam) and WMC IgG (Mouse IgG, Whole Molecule Control, Thermo Fisher Scientific) were diluted in PBS/BSA (0.1%) -solution. The dilutions used for these experiments were 1:50 for GFAP, nestin and NSE and 1:20 for TH and WMC. The samples were stained with 50 µl of primary antibody solution for 1 h at RT and washed 3 x with PBS.

The secondary antibodies, Alexa Fluor® 488 (Goat anti-Mouse IgG, Thermo Fisher Scientific) for the pilot hMSC experiment and Alexa Fluor® 568 (Goat anti-Mouse IgG, Thermo Fisher Scientific) for subsequent experiments, were diluted 1:200 in PBS/BSA (0.1%) -solution and the samples were incubated for another hour with 50 µl of this solution. After washing 3 x with PBS/BSA (0.1%), the cells were fixed onto the microscope slides with Vectashield Mounting Medium containing DAPI (4',6-diamidino-2-phenylindole) for staining the nuclei. The samples were imaged with a fluorescence microscope (Olympus BX51M, camera Leica DFC420).

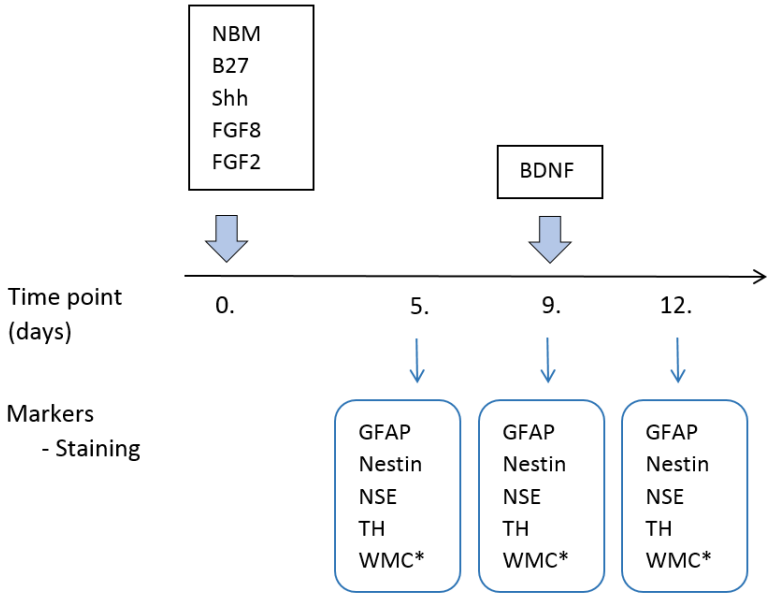

Figure 5.4: A timeline for the differentiation and immunofluorescence staining of hMSCs on material samples. The differentiation medium containing NBM, B27, Shh, FGF8 and FGF2 was added at time point 0, followed by the addition of BDNF at time point 9. The markers for staining, GFAP, nestin, NSE and TH were assessed at time points 5, 9 and 12, having WMC as a negative control (*).

5.5 Quantitative real-time PCR

The four markers followed by immunofluorescence were further assessed by qPCR: GFAP [197–199], nestin [197, 199], NSE [198] and Th [3] (see Fig. 5.3). At four time points 0, 5, 9 and 12 days in culture, the medium was removed from three replicates from the 12-well plate, the cells were lysed with RPE lysis buffer and the total RNA extraction was conducted using RNeasy Mini Kit (Qiagen Valencia, CA, USA) according to the manufacturer's protocol. The RNA obtained was counted and stored at -70°C. In order to amplify the RNA, complementary first-stand DNA (cDNA) synthesis was performed from total RNA using Maxima First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Four forward-reverse primer pairs were designed to further assess the expression of the neuronal markers GFAP, nestin, NSE and Th (Table 5.2). The corresponding sequences for the markers were searched in the National Center for Biotechnology Information (NCBI) Entrez search system, and the

primers were designed using the Applied Biosystems Primer Express software. The specificity was verified with the NCBI Primer-BLAST online tool and the primers were produced by Oligomer (Helsinki, Finland). Commercial RPLP0 and GAPDH primers were used as housekeeping genes.

Quantitative real-time qPCR was run for all cDNA samples using StepOnePlus^{TM} Real-Time PCR System instrument. A master mix for each gene was prepared from the following components: 12.5 µl Maxima SYBR Green/ROX qPCR Master Mix (2X, Thermo Fisher Scientific), 8.5 µl Nuclease-free water and 1 µl of both forward and reverse primers (concentration 5 µM). 2 µl of cDNA was added to 23 µl of the master mix. The real-time qPCR programme consisted of an initial denaturation step for 3 min at 95°C, followed by 45 cycles of denaturation at 95°C for 10s, annealing at 60°C for 30s and extension at 72°C for 30s. The programme was completed with a melting step with ramping to 95°C (0.5°C/step). The The results were analyzed using the relative quantitation method.

Table 5.2: Primers for assessing the hMSC differentiation with RT-qPCR using GFAP, nestin, NSE and Th as markers. RPLP0 and GAPDH were used as housekeeping genes. The NM codes, forward and reverse primer sequences, melting temperatures (T_m) and amplicon lengths are listed for each gene of interest.

Gene	NM code	Forward/Reverse Primer	T_m(°C)	Amplicon length (bp)
GFAP	NM_001131019.2	CACCGCAGCCCTGAAAGA GTTGCTGGACGCCATTGC	59.97 60.13	55
Nestin	NM_006617.1	AGCCCTGACCACTCCAGTTTAG CCCTCTATGGCTGTTTCTTTCTCT	61.68 60.08	128
NSE	NM_001975.2	TTGCTCTTGTCCCACGTGTCT CCCAGCACTATGCACAGTTCA	61.91 60.61	62
Th	NM_000360.3	CCGAGCTGTGAAGGTGTTTGA CGGGCCGGGTCTCTAGAT	60.81 60.20	128
RPLP0	NM_001002.3 NM_053275.3	GGCGACCTGGAAGTCCAACT CCATCAGCACCACAGCCTTC	62.11 61.31	149
GAPDH	NM_001975002046 NM_001289746 NM_001289745.1	AGTCAACGGATTTGGTCGTATTG TGGAATTTGCCATGGGTGGA	59.32 59.88	150

6 Results and discussion

The differentiation process of the hMSCs was visualized by immunofluorescence and further analyzed using real-time quantitative PCR. These methods were first tested by culturing C6 and PC12 cells and differentiating hMSCs on glass coverslips. The immunofluorescence results from the first experiments with C6 and PC12 cells are shown in Figures 6.1, 6.2 and with hMSCs in 6.3. The stained cells from the material experiments with eight different material types are presented in Figures 6.4-6.11. The amounts of RNA extracted from the samples are listed in Tables 6.1 and 6.2 and the qPCR relative quantification results are presented in Figures 6.13.

6.1 Immunofluorescence staining

The adequate functionality and specificity of the chosen antibodies were first examined on cultured C6 and PC12 cells. In glial-like C6 cells, the expression of GFAP was strongly visible as expected, and also differed from the control (Fig. 6.1). In contrast, the glial cell associated GFAP showed weaker fluorescence in PC12 cells, which represent a neural cell line (Fig. 6.2). In addition, also NSE showed lower expression than nestin and TH, but could still be distinguished from the control WMC.

The first hMSC differentiation experiment was conducted only up to the 9. time point and was then stopped due to unexpected technical issues. However, the staining results were obtained from the 9. time point samples confirming the desired specificity of the antibodies (Fig. 6.3). However, it was also seen that the differentiation had probably not been completed due to the early interruption of the process, since both NSE and TH showed only weak fluorescence. Nevertheless, these pilot experiments verified the applicability of the GFAP, nestin, NSE and TH antibodies in assessing the differentiation process of hMSCs.

The main experiment designed to study the differentiation of hMSCs on several electrode materials was successfully completed, and the immunoflu-

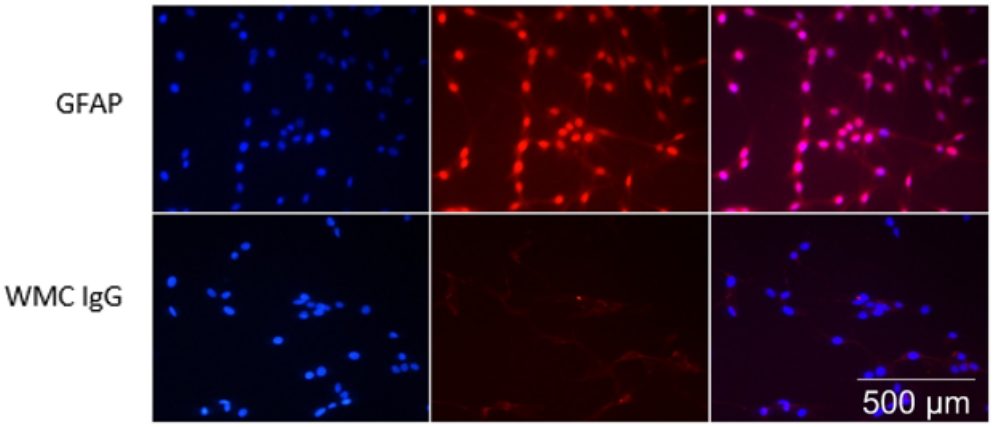

Figure 6.1: Immunofluorescence images of stained C6-cells. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The left column shows stained nuclei, the middle one stained actin and the right one the merged images for antibodies GFAP and negative control WMC IgG (Whole Molecule Control IgG).

orescence data was obtained according to the research plan using four antibodies GFAP, nestin, NSE and TH (see section 5.4). The results are shown in Figures 6.4-6.11.

On ta-C surfaces with no coating or stimulation, the hMSCs show good adherence, they are able to grow and proliferate and the material does not seem to have any toxic effect on them (Fig. 6.4). There is also no evidence of differentiation as would be expected due to the absence of any stimulation. This is also the case with ta-C materials coated with PDL (Fig. 6.5). In addition, it seems that there are no detectable differences between these two sample types suggesting an indifference of hMSC behavior to PDL-coating.

This is somewhat surprising, since PDL and PLL (poly-L-lysine) are frequently used in cell culture conditions to improve cell adherence onto the surface. However, Qian *et al.* [200] obtained similar results by comparing neuronal differentiation of hMSCs on different substrate coatings. It was concluded that PDL was the only coating among the tested substrates that did not support the growth and expansion of the hMSCs. Therefore, in the future it might be beneficial to replace PDL with a coating that has shown more positive effects on MSC growth and differentiation. For example, a soluble basement membrane extract known as Matrigel has been seen to both enhance the differentiation process in hMSCs as well as substantially improve cell expansion [200].

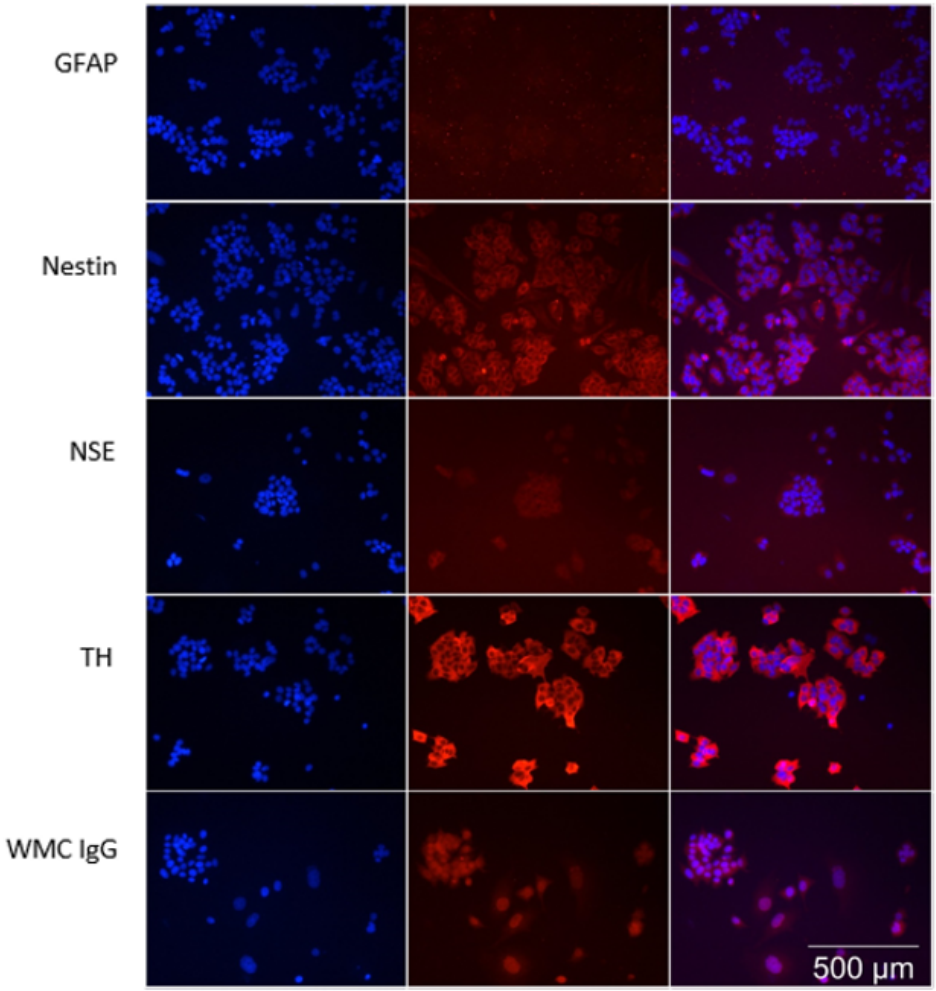

Figure 6.2: Immunofluorescence images of stained PC12-cells. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The left column shows stained nuclei, the middle one stained actin and the right one the merged images for each antibody GFAP, nestin, NSE, TH and negative control WMC IgG (Whole Molecule Control IgG).

In contrast to the untreated samples, the stimulated cells grown on ta-C exhibit distinctive morphology resembling neuronal shape both with and without PDL-coating (Fig. 6.6 and 6.7)). Especially samples stained with nestin antibody clearly show the elongated neurites in comparison with the more random shape of the unstimulated cells.

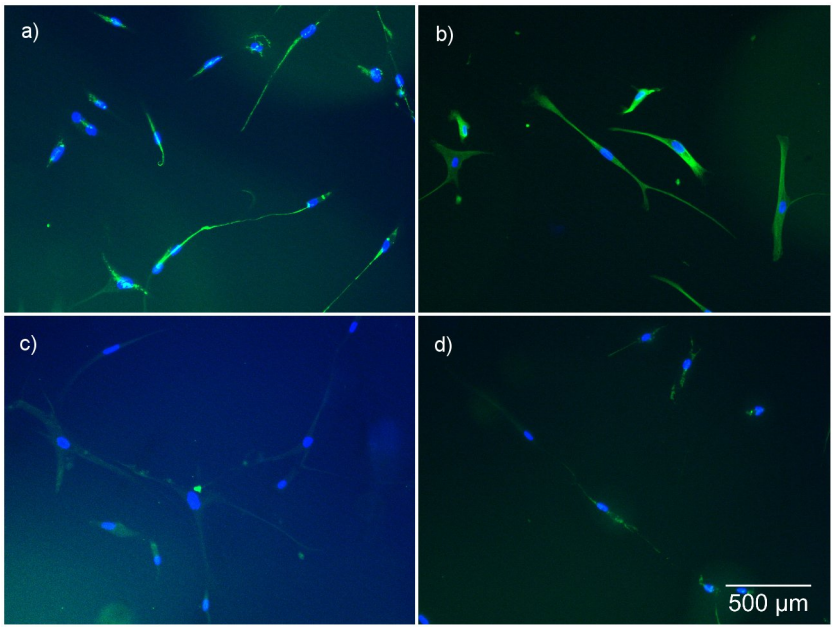

Figure 6.3: Immunofluorescence images of stained differentiating hMSC cells with a) GFAP, b) nestin, c) NSE and d) TH (9. time point). The nuclei are shown in blue (DAPI) and the antibodies in green (AlexaFluor® 488).

In general, there seem to be less cells on the stimulated samples, which would imply arrested proliferation and increased differentiation. This observation is also in agreement with another study that used hMSCs to generate mature neurons [112]. Moreover, the DAPI-stained nuclei have obtained a longer shape instead of the round ones observed in undifferentiated cells.

However, although the stimulated samples coated with PDL seem to express NSE at the 9. time point (Fig. 6.7), there is still no sign of TH expression on either of these material types, even at the 12. time point. In addition, the nestin expression does not diminish towards the last time point as would be expected if the cells were developing from progenitors into mature neurons. It seems as though the differentiation is still in progress and not yet fully completed, since some NSE is visible but TH is still to be developed.

In several studies inducing MSCs into DA neurons, the levels of TH have been clearly elevated already at day 7 [110], 12 [3] or 14 [112] in culture. However, in some protocols, the differentiation process has been prolonged for 14-30 days for neurons [141, 198] or even 35-42 days to generate DA neurons from rat MSCs [111]. Thus, it would be relevant to determine the optimal timeline for differentiation from hMSCs by following the process over a longer period of time and with regular checkpoints along the way.

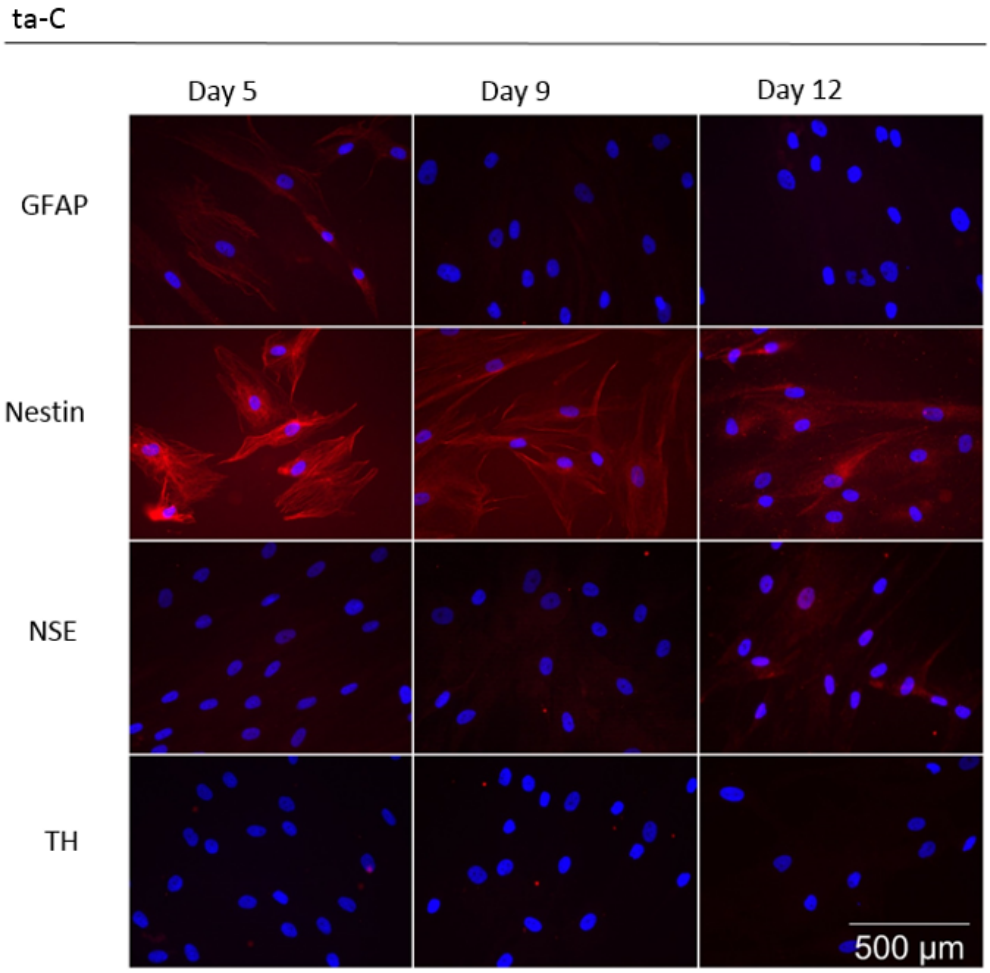

Figure 6.4: Immunofluorescence images of stained hMSCs on ta-C samples. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The left column shows stained nuclei, the middle one stained actin and the right one the merged images for each antibody GFAP, nestin, NSE and TH.

However, an extension in the differentiation protocol would also require multiple markers and methods to assess them. In addition, longer incubation periods would call for adjustments to the media volumes as well as replacing the differentiation media in several occasions to avoid drying of the cells and running out of nutrients.

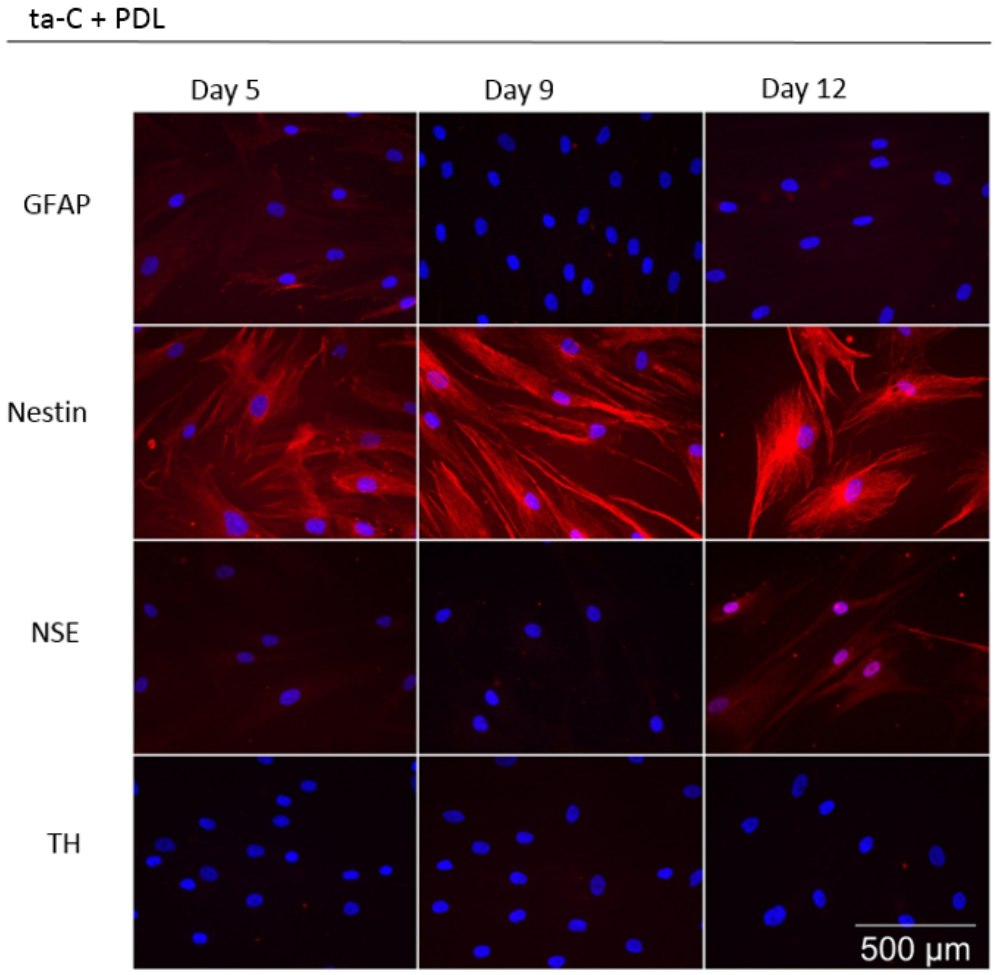

Figure 6.5: Immunofluorescence images of stained hMSCs on ta-C samples coated with PDL. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The left column shows stained nuclei, the middle one stained actin and the right one the merged images for each antibody GFAP, nestin, NSE and TH.

A similar overall cell behavior to those cultured on ta-C materials can also be observed in the cells grown on samples coated with carbon nanodiamonds. While the hMSCs cultured on vox-coated samples do not show signs of differentiation (Fig. 6.8), the morphological change in the stimulated cells is apparent already at the 5. time point (Figs. 6.10 and 6.11). Furthermore, the expression of NSE follows the predicted pattern showing transient increased levels at the 9. time point before decreasing again at the 12th.

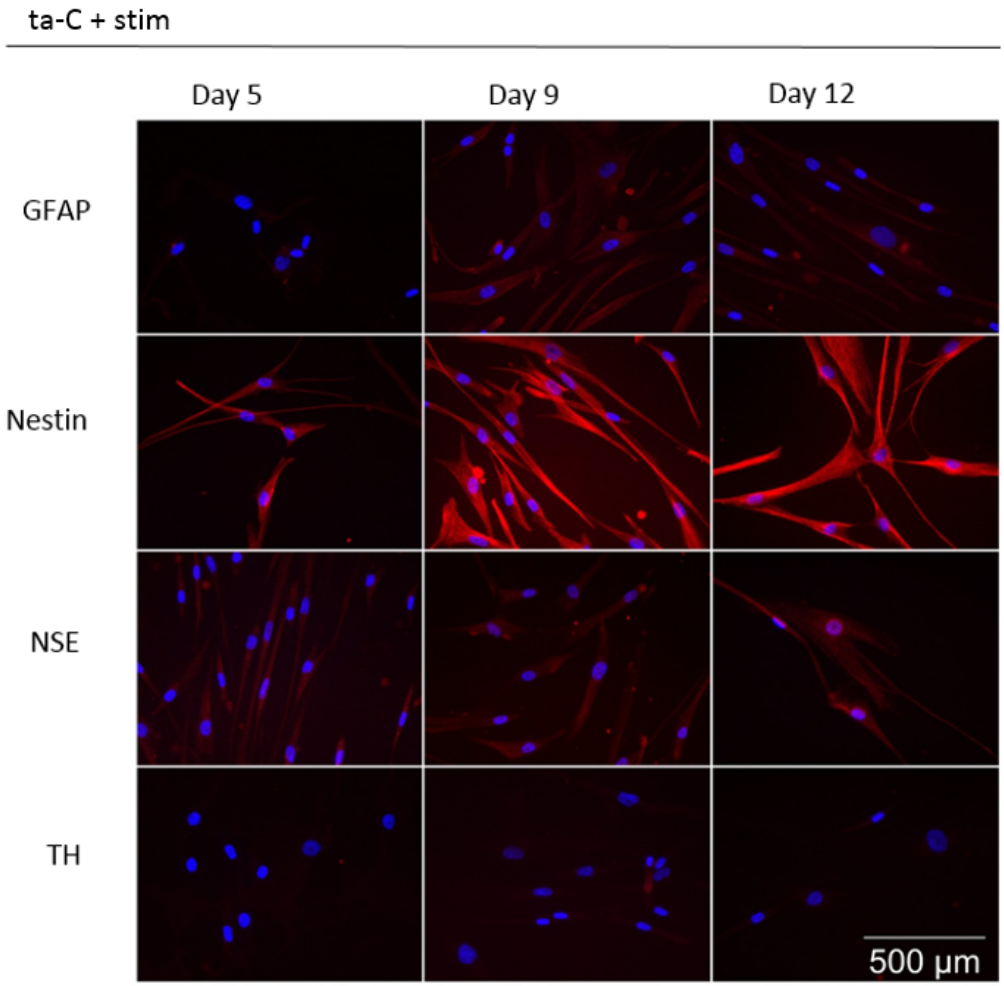

Figure 6.6: Immunofluorescence images of stained hMSCs on ta-C samples stimulated with differentiation medium. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The left column shows stained nuclei, the middle one stained actin and the right one the merged images for each antibody GFAP, nestin, NSE and TH.

An interesting remark is that the morphology of the cells cultured on vox surface seems to be shifted towards a neuron-like shape at the 12. time point (Fig. 6.8). This is in agreement with the known effect of nanotopographical cues on stimulating neurogenesis (see Section 3.3). The inducive effect is even more elevated on the vox samples functionalized with BDNF (Fig. 6.9). On these materials, there is also a slight NSE expression visible at the latest time point, suggesting an effect of the linked BDNF on the cell behavior.

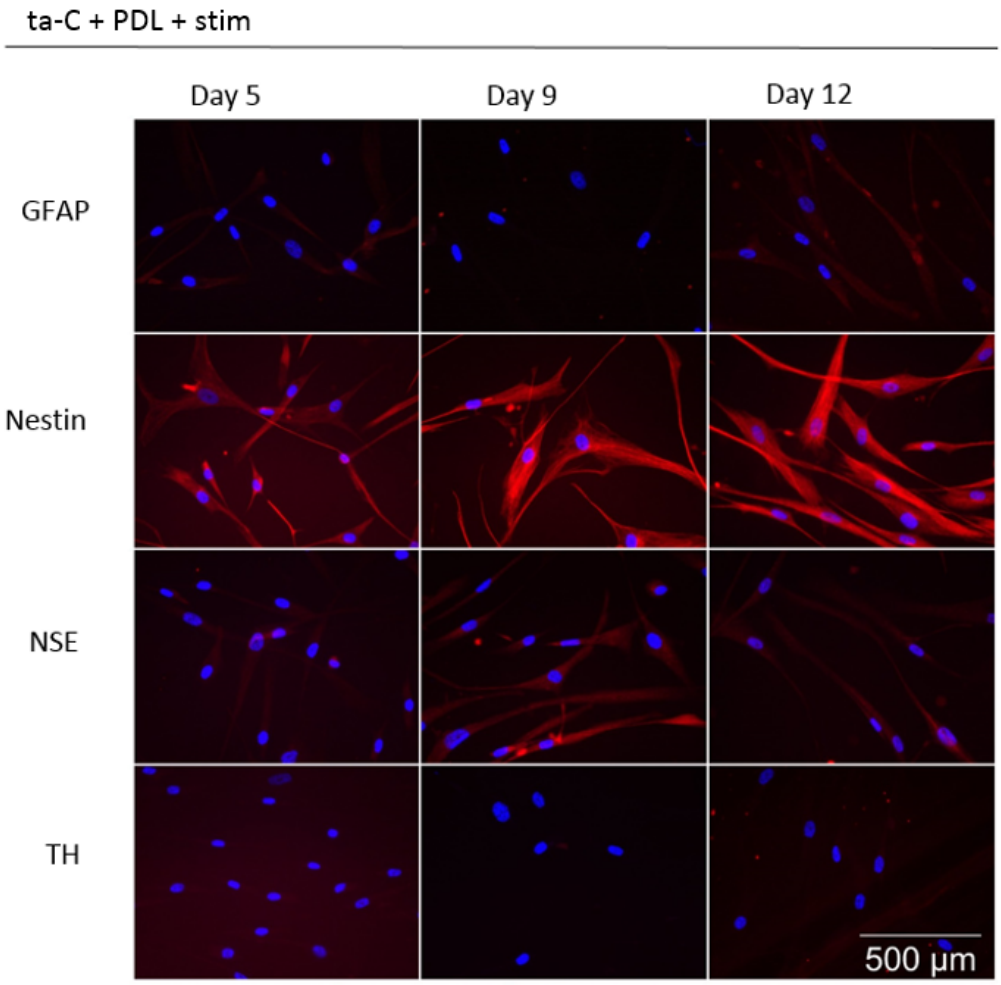

Figure 6.7: Immunofluorescence images of stained hMSCs on ta-C samples coated with PDL and stimulated with differentiation medium. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The left column shows stained nuclei, the middle one stained actin and the right one the merged images for each antibody GFAP, nestin, NSE and TH.

On the contrary, there are no significant differences in the cell behavior between the stimulated samples, either on vox-surfaces or vox functionalized with BDNF (Figs. 6.10 and 6.11). Nevertheless, coating ta-C samples with carbon nanodiamonds seems to have beneficial effects on the differentiation process. In addition, due to its multiple carboxyl sites, the vox-coating provides a useful platform for functionalization with various molecules such as BDNF.

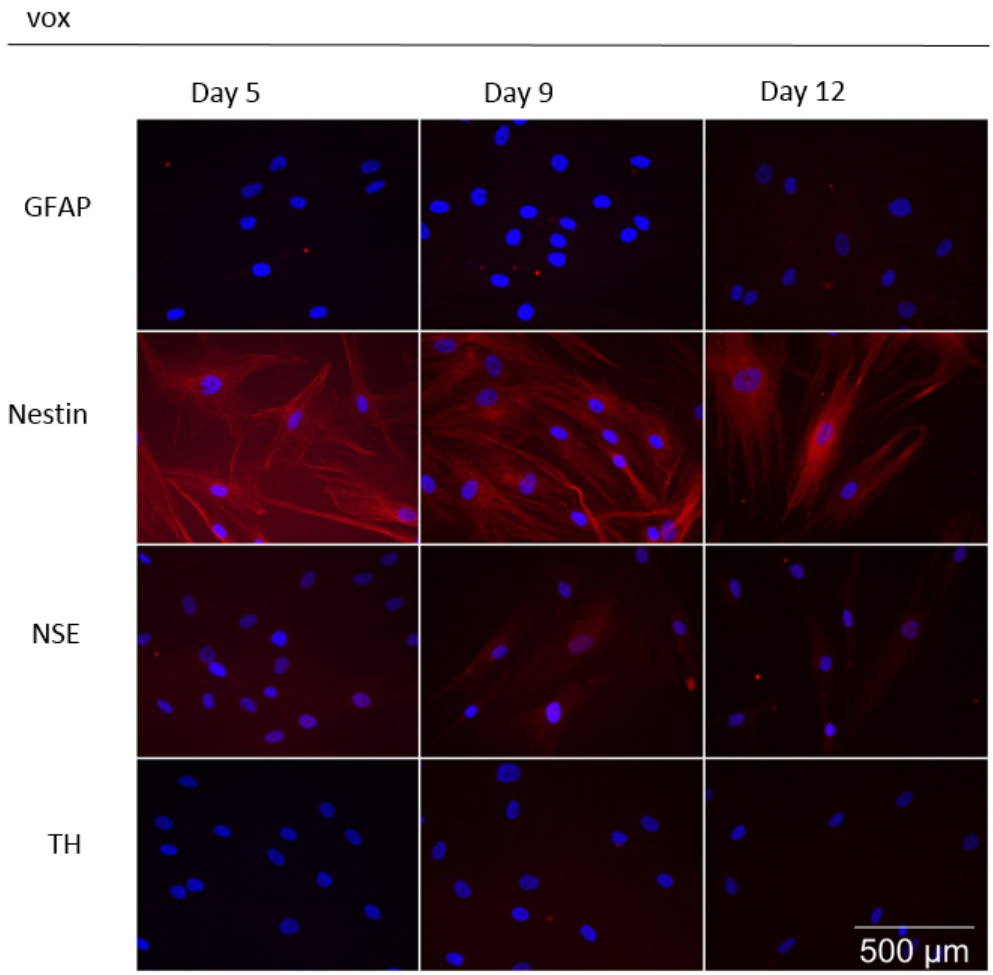

Figure 6.8: Immunofluorescence images of stained hMSCs on vox samples. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The left column shows stained nuclei, the middle one stained actin and the right one the merged images for each antibody GFAP, nestin, NSE and TH.

A rather surprising result is the constant expression of nestin in virtually all samples. Nestin is expected to be expressed by neuronal stem cells and developing neurons and gradually disappear as the cells maturate from progenitors into neurons. Although the fluorescence is stronger from the stimulated cells, it is also clearly seen in the untreated hMSCs.

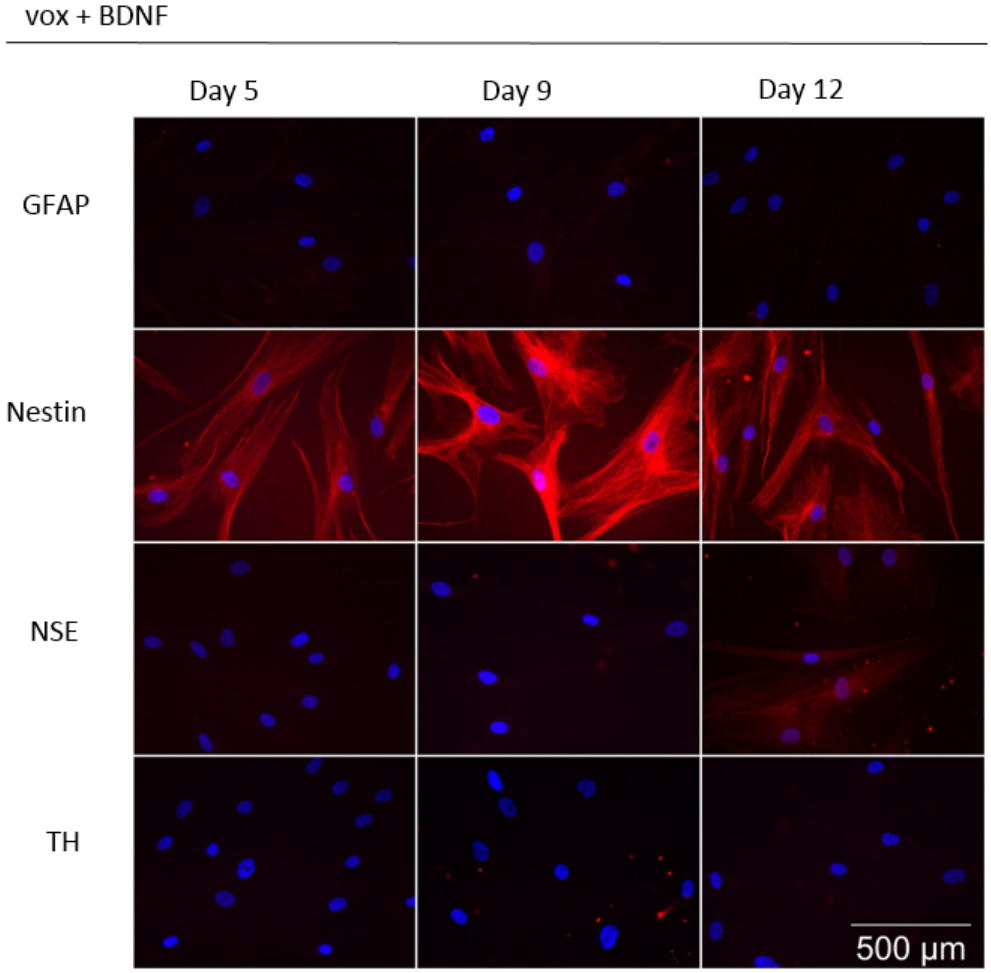

Figure 6.9: Immunofluorescence images of stained hMSCs on vox samples functionalized with BDNF. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The left column shows stained nuclei, the middle one stained actin and the right one the merged images for each antibody GFAP, nestin, NSE and TH.

It could be argued that the persistent expression of nestin is simply due to unspecific binding of the antibody, but when comparing the nestin expression of the stimulated cells to the negative control of vox-samples (Fig. 6.12), this explanation does not seem justified. Unexpectedly, the same observation was also done in another study assessing the specification of hMSCs into DA neurons [3]. In this study, further functional analysis of the stimulated cells showed that they exhibited neuronal precursor -like excitable properties

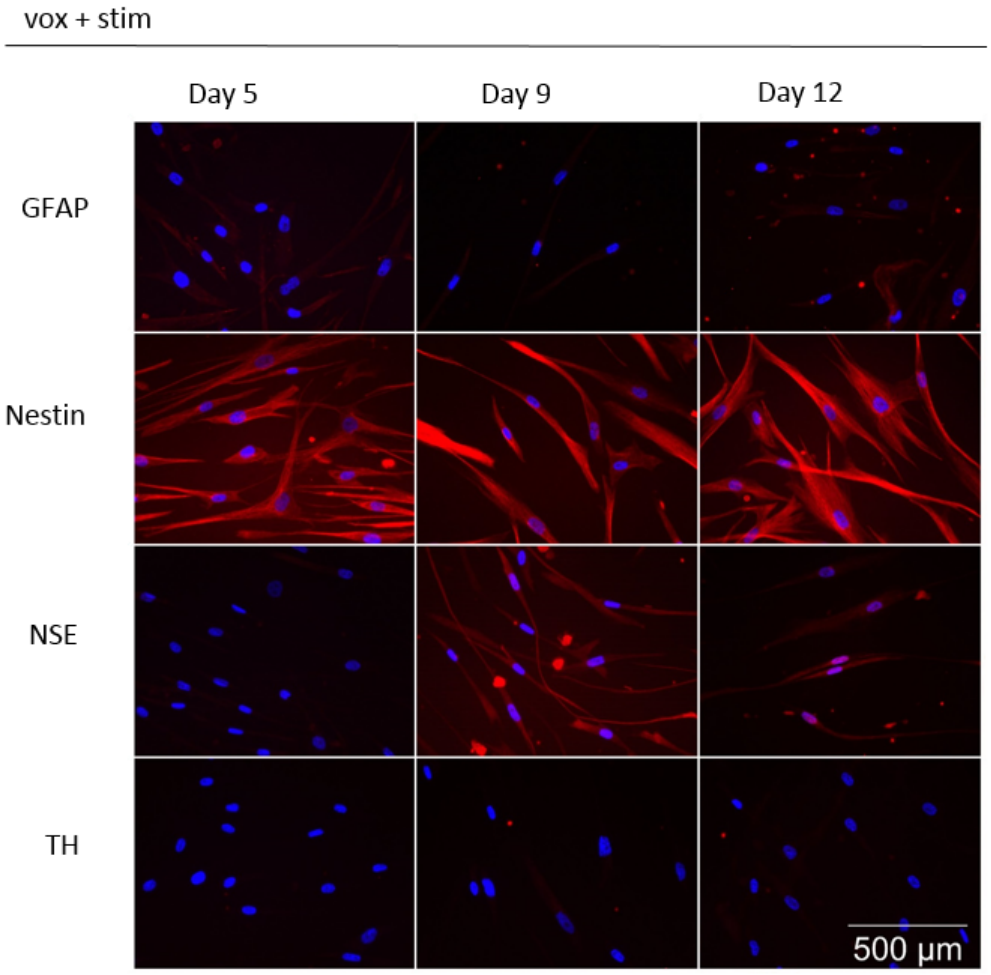

Figure 6.10: Immunofluorescence images of stained hMSCs on vox samples stimulated with differentiation medium. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The left column shows stained nuclei, the middle one stained actin and the right one the merged images for each antibody GFAP, nestin, NSE and TH.

rather than those characteristic to mature neurons. This would support the deduction that the cell maturation process is not yet completed.

On the other hand, other neuronal markers such as beta-tubulin III and NeuN have also been seen to be expressed by several cell types, although previously thought to be neuron-specific [11]. In addition, it has been seen that different factors such as cell stress or disruption of the cytoskeleton can

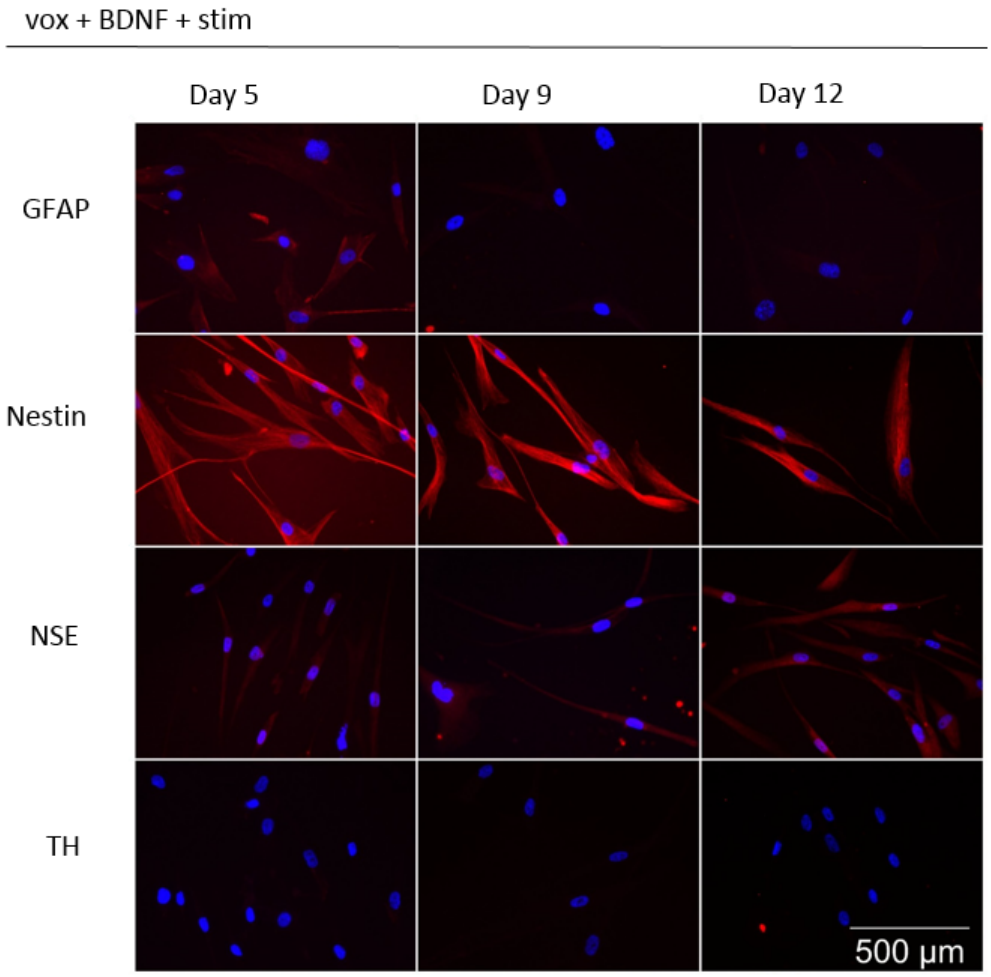

Figure 6.11: Immunofluorescence images of stained hMSCs on vox samples stimulated with differentiation medium. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The left column shows stained nuclei, the middle one stained actin and the right one the merged images for each antibody GFAP, nestin, NSE and TH.

affect cell morphology and gene regulation [201]. Thus, it is not sufficient to assess the differentiation by cellular markers alone, but also functional analysis is required to evaluate the electrophysiological properties of the generated cells.

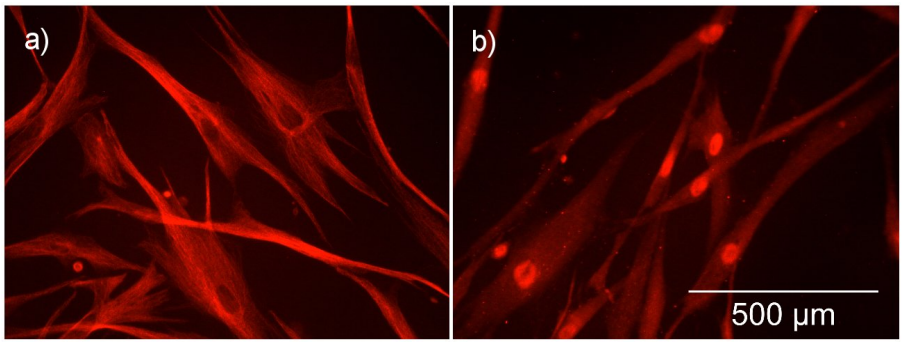

Figure 6.12: Immunofluorescence images of stained hMSCs on vox samples stimulated with differentiation medium. The nuclei are shown in blue (DAPI) and the antibodies in red (AlexaFluor® 568). The samples are nestin (a) and negative control (b).

6.2 Quantitative real-time PCR

The amounts of RNA (ng/µl) obtained from the samples of the pilot hMSC experiment are shown in Table 6.1. From each of these samples, 39.2 ng RNA was translated into cDNA and amplified with qPCR. The qPCR results are shown in Figure 6.13. The results are presented as RQs (relative quantitation), first standardized by subtracting the corresponding C_t values of the housekeeping gene RPLP0 from the C_ts of the sample and then normalized as the fold expression with respect to those of the 0. time point (RQ = 1, shown as a dashed line). The positive error bars represent the standard deviation of the three replicates and are also identical to the negative side (not shown for the clarity of the image). The RQs are calculated as the average of the three replicates for the markers GFAP, nestin and NSE. The C_t values for TH were either undetermined or above 30, making them unreliable.

Table 6.1: The amounts of RNA (ng/µl) from the cells cultured on PDL-coated well plates at time points 0, 5, 9 and 12. Three replicates A, B and C were obtained for each time point.

Material	Time point											
	0.			5.			9.			12.		
	A	B	C	A	B	C	A	B	C	A	B	C
PDL	2.9	8.7	16.6	14.3	28.0	17.6	4.4	25.1	28.1	3.1	2.9	2.8

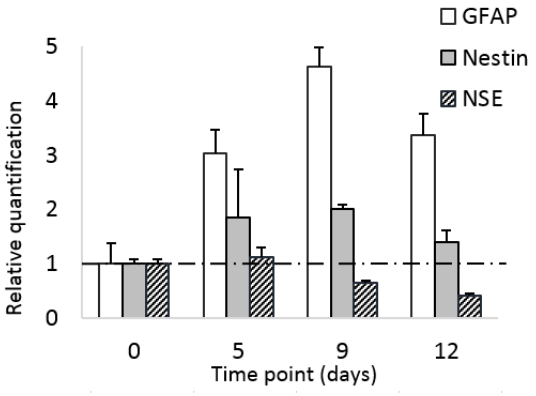

Figure 6.13: qPCR results from the first hMSC differentiation experiment. Relative quantification of markers nestin, NSE and GFAP. Results are standardized and normalized as the fold expression with respect to those of the 0. time point (RQ = 1, dashed line). The positive error bars show the standard deviation of the three replicates and are identical to the negative side. RQs are calculated as the average of three replicates.

According to these results, the expression of GFAP would be over four times more at time point 9 than at time point 0, which is not the outcome that was expected according to Figure 5.2. Similarly, the levels of NSE seem to decrease rather than increase towards the later time points. In contrast, nestin shows the correct profile with initial increase of expression followed by a drop at the 12. time point. However, the C_t values for GFAP were slightly above 30, which compromises the reliability of the results. It should also be noticed that the amount of cDNA might not have been sufficient for accurate qPCR amplification. Nevertheless, these first results provided an adequate basis for the subsequent experiments with electrode materials.

The amounts of RNA (ng/µl) obtained from the material samples from time points 0, 5, 9 and 12 are listed in Table 6.2. From these samples, 21 ng of RNA was translated into cDNA and amplified with qPCR. The RQ values from qPCR for each material type at all time points are presented in Figure 6.14. The RQs were calculated as described above.

On the ta-C samples, the stem cells were not under any stimulative influence, and thus would be expected to show low levels of neural markers and possibly stronger signals from GFAP. Indeed, GFAP seems to increase towards the 12. time point, although this is not definite due to the rather wide error bars. Similarly, also nestin, NSE and TH behave as expected within the error limits.

Table 6.2: The amounts of RNA (ng/µl) from each material type at time points 0, 5, 9 and 12. The sample materials were ta-C (1.), ta-C with stimulation (2.), PDL-coated ta-C with stimulation (3.) and vox functionalized with BDNF (4.). Three replicates A, B and C were obtained for each time point.

Material	Time point											
	0.			5.			9.			12.		
	A	B	C	A	B	C	A	B	C	A	B	C
1.	9.7	5.7	6.6	10.0	6.3	7.0	10.5	3.9	6.7	13.7	3.3	4.8
2.	6.1	4.1	11.1	3.2	15.9	4.4	5.0	2.8	1.9	5.4	8.3	4.1
3.	18.3	11.7	12.2	2.3	3.0	1.5	6.6	2.7	3.7	4.3	2.6	2.5
4.	4.2	13.7	4.2	2.4	3.1	3.3	13.1	4.6	5.7	3.3	2.4	1.8

The stimulated cells on ta-C materials also show expected marker expressions, although again with some large error bars. The expression of GFAP is decreased towards the later time points, which is consistent with the neuron-like morphology seen in the immunofluorescence staining. Moreover, the levels of NSE increase at day 12, also suggesting a shift towards neuronal differentiation. In contrast, as already observed from the staining results, the expression of TH is very weak if not negligible.

The results from the ta-C coated with PDL and stimulated with differentiation medium significantly differ from those without PDL-coating. The most predominant variation is seen in the GFAP expression, which is highly elevated on the PDL substrate, suggesting an increase in glial cell specification. However, the other strongly expressed marker is NSE, associated with neural lineage. The same observations can be seen from the vox BDNF samples. While nestin shows relatively constant expression for both material types, as was also seen in the fluorescence images, the unexpected burst of expression of GFAP is highly contradictory to the previously hypothesized expression profiles (see Fig. 5.2).

In general, the results from these qPCR experiments seem somewhat inconsistent with the immunofluorescence images and the expectations based on the known expression profiles of the markers (see Fig. 5.2). However, some of the expression levels are accompanied by relatively wide error limits, allowing several different interpretations of the expression profiles. The consistency between the samples could be increased by transferring the material samples into new wells before RNA extraction to ensure that the obtained

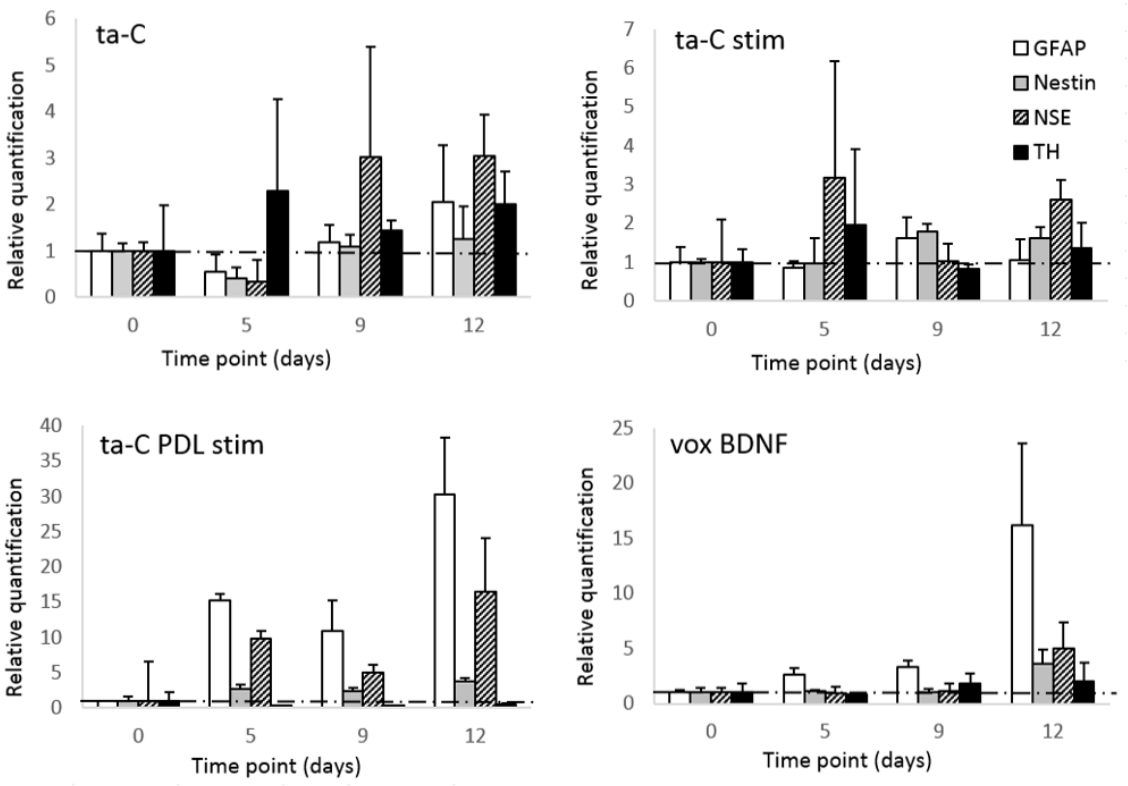

Figure 6.14: qPCR results from the hMSC differentiation experiment on electrode materials ta-C, ta-C with stimulation (ta-C stim), PDL-coated ta-C with stimulation (ta-C PDL stim) and vox functionalized with BDNF (vox BDNF). Relative quantification of markers GFAP, nestin, NSE and TH. Results are standardized and normalized as the fold expression with respect to those of the 0. time point (RQ = 1, dashed line). The positive error bars show the standard deviation of the three replicates and are identical to the negative side. RQs are calculated as the average of three replicates.

RNA originates solely from the cells grown on the samples. In addition, the size of the material samples greatly affects the amount of RNA isolated from the cells. While the sample size of 1 cm x 1 cm has been proven appropriate in osteogenesis of MSCs [202], it might not be sufficient for differentiating MSCs into neurons. The size of the samples becomes a limiting factor due to the reduced proliferation of the differentiating cells. Therefore, a bigger sample size could be used in future studies to obtain more reliable data from qPCR experiments.

The next step in developing the optimal protocol for differentiating hMSCs into DA neurons would be increasing the yield of cells expressing DA specific markers, and most importantly, finding the means to generate electrically functional neurons. Although hMSCs have already been successfully differentiated into neuron-like cells in multiple experiments, the ability to fire spontaneous or evoked action potentials from these cells is yet to be accomplished. It has been proven that cells expressing neuronal markers and even readily secreting dopamine might still show impaired electrical functionality in whole cell recordings [3]. Therefore, assessing these properties of the generated cells is essential in evaluating their maturity and suitability for medical applications.

7 Conclusions

In this thesis work, the process of adult stem cell differentiation into neurons was studied via two approaches: a literature review on the field of adult neurogenesis and an experimental assessment of a protocol for differentiating hMSCs into DA neurons. The literature study covered the main cellular pathways related to neuronal and DA differentiation *in vivo* as well as several methods for guiding stem cell behavior *in vitro*. In addition, the recent development in using stem cells and active materials in medical applications was discussed.

The experimental part was carried out by culturing hMSCs under a differentiation medium and following their development on several ta-C -based material types via immunofluorescence staining and real-time qPCR. The effects of PDL-coating, carbon nanodiamond-coating and BDNF-functionalization on the differentiation process were evaluated using cellular markers related to neurogenesis: GFAP, nestin, NSE and TH.

Several pilot studies were conducted previous to the main material experiments. These initial studies with C6, PC12 and hMSC cells confirmed the sufficient expression of the antibodies used in the immunofluorescence staining as well as the functionality of the qPCR primers. In the main experiment, the hMSCs were differentiated for 12 days in culture on four different carbon-based materials.

The results from the immunofluorescence staining indicate that while the stimulation medium seems to shift the fate of the hMSCs towards the neuronal lineage on all material types, the process might not be fully completed as suggested by the lack of TH expression. Moreover, nestin was visible on all samples including the stimulated cells at the 12. time point. This was partly explained by another study observing nestin expression from undifferentiated hMSCs [3]. However, in the stimulated cells the persistent nestin levels further imply that the differentiation process is still in progress at the time point 12, thus raising a question of the adequate amount of time required for complete maturation.

The qPCR results from the cells cultured or stimulated on ta-C showed

expected expression profiles for all markers, although with relatively wide error limits. On the stimulated ta-C samples, the elevated NSE levels at the last time point were in agreement with the staining results, corresponding to the slight detected fluorescence and neuron-like morphology of the cells seen from the images. On the contrary, the marker profiles from ta-C coated with PDL revealed an undesirable effect of the PDL-coating. The highly increased GFAP-levels indicated a presence of glial-like cells rather than neurons.

A similar effect was also seen from the cells grown on the vox BDNF materials, although these also expressed the neuron-specific NSE. From the combined results of the stainings and qPCR, it could be deduced that the nanotopography of the substrate seems to guide the hMSCs towards the neuronal lineage, and this effect could be further enhanced by the BDNF functionalization. However, a general problem in the qPCR experiments was found to be the insufficient amount of RNA obtained from the samples. This was most likely due to the small sample size combined with a differentiation-related decrease in the amount of cells per sample. Thus, bigger samples would be necessary in order to obtain more reliable qPCR results from neurogenesis experiments with hMSCs.

For future experiments, it will be important to strive for improving the efficacy of the hMSC differentiation protocol to gain higher yields of mature DA neurons from the stem cells. This could be achieved by optimizing the timeline for the differentiation process, as well as combining the chemical stimulation with nanotopographical cues to potentially enhance the commitment of the stem cells towards the neuronal lineage. Another essential goal is the successful generation of electrically functional neurons and the use of proper assessment methods to confirm the capability of the cells to fire action potentials.

The developments in the research on neurogenesis in the adult brain pave the way for deeper understanding of the mechanisms governing the fate of the stem cells *in vivo* and thus enable the breakthroughs in the stem cell experiments conducted in cell laboratories. In applications such as active neural scaffolds and closed-loop neurostimulation, a variety of questions remain unsolved, especially related to the tissue response and biocompatibility of the implants. The use of stem cells to replace damaged neural tissue or nanostructured surfaces to guide the regrowth of neurites could offer new approaches in tackling these challenges, potentially enabling new strategies in treating incurable neurodegenerative diseases.

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