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ADULT NEURAL STEM CELLS IN NEUROINFLAMMATION

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To my wonderful family

ABSTRACT

Adult neural stem cells (NSC) can be isolated from the subventricular zone (SVZ) of the lateral ventricles, the subgranular zone (SGZ) of the hippocampus and the central canal of the spinal cord. These cells are thought to have regenerative potential and pose important therapeutic possibilities in neuroinflammatory conditions such as Multiple Sclerosis (MS). The aim of this thesis was to investigate the function of these cells during neuroinflammation. To that end we employed both *in vitro* primary cultures of NSC and the experimental autoimmune encephalomyelitis (EAE) model.

I. NSC generate neurons in demyelinated spinal cord lesions. In order to monitor NSC behaviour in EAE we labelled the endogenous NSC by injecting a lipophilic dye, DiI in the ventricle system of Dark Agouti rats. These rats were immunized to develop EAE and injected with BrdU to identify proliferating cells. We report that NSC proliferated and migrated to demyelinated lesions in the spinal cord of EAE diseased rats, where some of the cells started to express βIIItubulin or NeuN. Ultimately, these NSC-derived neuronal-like cells could generate overshooting action potentials.

II. Nitric oxide (NO) suppresses NSC-derived neurogenesis. In this study we used NSC cultures to analyse the effects of nitric oxide (NO) on NSC proliferation and differentiation. NO is produced within EAE and MS lesions and has been correlated with disease exacerbation in MS. We report that exposure of NSC to pathophysiological concentrations of NO diverted their differentiation potential from neurogenesis towards astrogliogenesis. Using immunocytochemistry we could demonstrate a lower percentage of βIIItubulin-IR neurons but a higher percentage of O4-IR oligodendrocytes in NO-exposed cultures. The higher rate of gliogenesis in these cultures was also confirmed by western blotting for the astrocyte-specific protein GFAP and activated STAT1, a transcription factor involved in gliogenic differentiation. Moreover, the pro-neurogenic determinant neurogenin-2 was down-regulated subsequent to NO exposure, constituting a potential mechanism for the NO-mediated down-regulation of neurogenesis.

III. High Mobility Group Box Protein 1 (HMGB1) expression correlates with inflammation in MS and EAE. In the third study we focused on the expression of the cytokine HMGB1 and its receptors RAGE, TLR-2 and TLR-4 in MS and EAE. Our interest in HMGB1 is related both to its cytokine function in inflammatory diseases and to its possible involvement in cell migration and differentiation. We detected cytoplasmic translocation of HMGB1, indicative of active release, in microglia and macrophages located in MS lesions. Moreover, the expression of HMGB1 and its receptors in immune cells isolated from the cerebrospinal fluid (CSF) of MS patients was significantly higher compared to cells from CSF of control patients. In EAE, an increased transcript level of HMGB1 correlated with higher disease severity. Finally, microglia cells could translocate HMGB1 to the cytoplasm, implying their ability to actively release this protein and indicating their potential contribution to inflammation.

IV. TLR-2 and TLR-4 agonists induce TNFα release from NSC. TLR-2 and TLR-4 recognise bacterial moieties and can also ligate HMGB1. TLR activation in innate immune cells leads to release of inflammatory agents aimed at clearing invading pathogens. The connection between TLR and NSC originates from the *Drosophila* ortholog, *Toll*, which participates in neuronal patterning. Immunocytochemical investigations of primary NSC cultures revealed the presence of both TLR-2 and TLR-4 on these cells. Moreover, the expression of these receptors was differentially regulated by inflammatory conditions and cytokines. Agonist-induced TLR activation was not involved in differentiation or proliferation of NSC. Activation of these receptors prompted NSC to express the pro-inflammatory cytokine TNFα at both mRNA and protein levels.

In conclusion, we demonstrated that inflammatory conditions can both promote and inhibit the ectodermal differentiation capacity of NSC, but also to yield them unexpected immune features.

Keywords: neural stem cells, inflammation, EAE, NO, neurogenesis, HMGB1, TLR, TNFα

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TLR activation induces TNFα production from adult neural cell/progenitor cells.
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CONTENTS

LIST OF ABBREVIATIONS

bFGF	Basic Fibroblast Growth Factor
bHLH	Basic Helix-Loop-Helix
BrdU	5′-bromodeoxy-uridine
BMP	Bone Morphogenetic Protein
CBP	CREB(cAMP response element-binding)-binding protein
CD	Cluster of Differentiation
cGMP	Guanosine 3',5'-cyclic Monophosphate
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CSF	Cerebrospinal Fluid
DETA-NONO:ate	Diethylenetriamine-NONO:ate
DiI	1,1′-dioctadecyl-6,6′-di(4sulfopentyl)-3,3,3′,3′tetramethylindocarbocyanin
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
EGF	Epidermal Growth Factor
GC	Guanylate Cyclase
GFAP	Glial Fibrillary Acidic Protein
HMGB1	High Mobility Group Box Protein 1
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
LIF	Leukemia Inhibitory Factor
LTA	Lipoteichoic Acid
MS	Multiple Sclerosis
MyD88	Myeloid Differentiation factor 88
NeuN	Neuronal Nuclei
NF-κB	Nuclear Factor kappa B
NO	Nitric Oxide
NSC	Neural Stem Cell
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PBMC	Peripheral Blood Mononuclear Cells
PCNA	Proliferating Cell Nuclear Antigen
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
RAGE	Receptor for Advanced Glycosylated End products
RNA	Ribonucleic Acid
Shh	Sonic Hedgehog
SGZ	Subgranular Zone
SOX	Sry-containing HMG box
STAT	Signal Transducers and Activators of Transcription
SVZ	Subventricular Zone
TNF	Tumor Necrosis Factor
TLR	Toll Like Receptor

INTRODUCTION

When I started my PhD studies in 2002, the discovery of an adult neural stem cell was relatively new and still presented a hot topic of controversy. For a new student the multitude of reports on this subject were, mildly said, confusing. Much of the contradicting information available stood more clearly if the following experimental aspects were considered:

1. Embryonic *versus* adult model
2. The anatomical location of studies, i.e. rostral *versus* caudal
3. The studied organism, i.e. animal model *versus* human
4. The environmental conditions, i.e. healthy *versus* pathological

This doctoral thesis is based on studies of adult neural stem cells and their behaviour within an inflammatory environment. We have employed both *in vitro* models of adult neural stem cell cultures (papers II and IV) and animal models of neuroinflammation in which endogenous stem cells can be studied *in vivo* (papers I and III). In this *Introduction* I will introduce the reader both to the field of stem cell research and to the immunological mechanisms thought to be involved in the development of neuroinflammatory diseases such as Multiple Sclerosis (MS). At the end of the *Introduction* I will combine the two fields and discuss what it is known to date about the role and behaviour of neural stem cells (NSC) during neuroinflammation.

NEURAL STEM CELLS

A HISTORICAL VIEW

By the end of the 19th century, Ramón y Cajal had described the different developmental phases of a neuronal cell (Ramón y Cajal, 1913). These developmental features were not seen in the brain in the adult individual, and together with the findings of Koelliker (Koelliker, 1896) and His (His, 1904), a dogma emerged. This dogma, which ruled the neuroscience field for more than 100 years, stated that the adult mammalian brain was a fixed structure with no possibility of renewal. During the intervening time there were some findings suggesting mitosis in various parts of the brain, including the lateral

ventricle walls (Levi, 1898, Hamilton, 1901, Allen, 1912), but because of the poor reliability of the methods, they were ignored.

In the late 1950s the method of [^{3}H]-thymidine autoradiography was introduced. Animals were injected with radioactively labelled thymidine which integrates in the Deoxyribonucleic Acid (DNA) during the S-phase of the cell cycle, thus being present in all cells that had undergone cell proliferation. During the 1960s Smart and Altman presented evidence for ^{3}H thymidine-labelled cells in the subventricular zone, in the neocortex, the dentate gyrus and the olfactory bulb of young rats (Smart, 1961, Altman, 1962, Altman, 1963, Altman and Das, 1965, Altman and Das, 1966, Altman, 1969). Later, Michael Kaplan and colleagues combined ^{3}H thymidine labelling with electron microscopy. These authors could show ^{3}H thymidine-labelled cells that had the ultrastructural characterisation of a neuron (i.e. dendrites and synapses) in the cerebral cortex of adult rats (Kaplan and Hinds, 1977, Kaplan, 1985). Kaplan also demonstrated mitosis in the subventricular zone (SVZ) of macaque monkeys (Kaplan, 1983). Kaplan's work had small impact and according to Charles Gross this could partly be attributed to the contradictory results of Pasko Rakic (Gross, 2000). Rakic had conducted studies in adult rheus monkeys without finding any evidence for formation of new neurons (Rakic, 1985a, Rakic, 1985b).

At last, in the 1980s the experiments of Nottebohm and colleagues re-opened the discussion of neurogenesis in the adult brain. Nottebohm observed that thousands of new neurons were added per day to the part of the brain controlling seasonal bird song. They also demonstrated that the new neurons were ^{3}H thymidine-labelled (i.e. newly generated), had synapses and responded to sound by generating action potentials, i.e. were functionally integrated in the neuronal network (Goldman and Nottebohm, 1983, Paton and Nottebohm, 1984, Burd and Nottebohm, 1985).

The field was further revolutionised by the introduction of BrdU (5-bromo-3-deoxyuridine) a synthetic thymidine analogue that could be detected using immunotechniques (Nowakowski et al., 1989). The advantage of this is that the identity of the newly generated BrdU labelled cells could be investigated with markers of specific cell types. Kuhn, Gage and colleagues were among the first to use BrdU stereology in the study of adult neurogenesis in rodents (Kuhn et al., 1996, Kempermann et al., 1997b). By the early 1990s Altman's claim of neurogenesis in the dentate gyrus was confirmed. Finally, BrdU labelling of new neurons was demonstrated in the dentate gyrus of the marmoset (Gould et al., 1998), macaque (Gould et al., 1999, Kornack and Rakic, 1999) and ultimately in humans (Eriksson et al., 1998).

After 100 years there is enough compelling evidence to break the dogma of Cajal, Koelliker and His. There are cells in the adult brain that have the characteristics of stem cells - that is self-renewal and multipotency. Moreover, these cells can differentiate into new neurons that are functionally integrated into pre-existing networks of the adult brain (Winner et al., 2002, Song et al., 2002).

More questions arise though. What is the adult NSC location and identity? What molecular factors influence their proliferation and differentiation? How are the properties of these cells changed when the niche is altered during inflammatory conditions? Finally, how can these cells be used as therapeutics?

The regenerative niches in the adult brain are developmental remnants governed by embryonic cues. Much information about the adult NSC can therefore be gained by studying the generation of the nervous system.

A DEVELOPMENTAL VIEW

A stem cell is defined as a cell that can self-renew and give rise to progeny belonging to different cell lineages. The potency of a stem cell depends on the range of its differentiation potential. Thus embryonic stem cells generating all types of cells in the body are totipotent, while NSC are multipotent and only differentiate into neurons, oligodendrocytes and astrocytes (Reynolds et al., 1992, Johansson et al., 1999b).

The NSC have temporal and spatial memory. The formation of the cells in the central nervous system (CNS) begins with generation of neurons and ends with generation of astrocytes and oligodendrocytes (reviewed by (Gotz, 2003). This sequence of events is mimicked *in vitro* cultures. When NSC are isolated from the neurogenic period they first generate neurons and then astrocytes (Qian et al., 2000, Morrow et al., 2001). NSC from the adult brain are also more gliogenic than neurogenic. As an example of spatial memory NSC from the SVZ differentiate into GABAergic neurons *in vitro*, which is reminiscent of the *in vivo* situation (Moe et al., 2005).

The development of the nervous system starts with the formation of the neural plate and the neural tube, which is comprised of a single layer of proliferating neuroepithelial cells. This proliferating area forms the ventricular zone (VZ). Neuroepithelial cells are the primordial NSC and can give rise to all cell types in the CNS (Williams and Price, 1995). Upon initiation of neurogenesis the neuroepithelial cells generate and are replaced by the radial glia. So in fact all CNS cells arise from the radial glia. The morphology of radial glia is polarised and they extend the whole way from the lumen to the pial surfaces. The molecular markers expressed by these cells are

characteristic of both neuroepithelial cells and astrocytes, including nestin, prominin-1 (CD133) glial fibrillary acidic protein (GFAP), astrocyte-specific glutamate transporter (GLAST) and the Ca^{2+} binding protein S100β. During mid-gestation a second germinal zone arises, the SVZ. In the adult the VZ disappears and is replaced by the ependymal layer, while the SVZ persists and harbours the adult NSC review by (Gotz and Huttner, 2005)

NSC can divide in two different ways: by symmetrical division two identical daughter cells are generated, while asymmetrical division generates a stem cell and more committed progeny. Even during the very early stages of development most of the progenitor cells are restricted to either neurogenic or gliogenic fates. It is not yet entirely clear why neurogenesis happens first and why the gliogenic progenitors only start to divide and mature when neurogenesis is completed. However, there is a small population of cells, around 10-20% of the progenitor cell pool, which can generate both neurons and oligodendrocytes or astrocytes. This multipotent population, vastly diminished to a few percent, can persist into adulthood and constitute a source of adult NSC (Williams et al., 1991, Williams and Price, 1995) review by (Gotz, 2003).

THE IDENTITY OF ADULT NEURAL STEM CELLS

Adult NSC can be isolated from the SVZ of the lateral ventricles, the subgranular zone (SGZ) of the hippocampus and the spinal cord.

The identity of NSC has been debated for a long time. To begin with, Reynolds and Weiss (Reynolds et al., 1992) isolated an EGF-responsive cell from the striatum of adult mice. This population could self-renew and generate so-called *neurospheres* and also differentiate into neurons and astrocytes. The striatum comprises the lateral ventricles and the SVZ where proliferating cells in the adult had been previously reported. Johansson and colleagues reported that the ependymal cells had stem cell properties. Other laboratories described a cell in the SVZ layer that accounted for the stemness in the area (Morshead and van der Kooy, 1992, Morshead et al., 1994, Chiasson et al., 1999, Gritti et al., 1999).

The SVZ architecture, described by Alvarez-Buylla and colleagues (Doetsch et al., 1997) consists of a ciliated ependymal layer facing the ventricle. Underneath there are proliferating migrating neuroblasts (type A cells), a transit amplifying population of rapidly dividing cells (type C cells) and slowly proliferating astrocytes, the B cells. In a series of experiments Doetsch and Alvarez-Buylla identified adult NSC in the SVZ. They observed that after depletion of fast proliferating A and C cells, the B type astrocytes started to proliferate and regenerated the entire SVZ architecture. By specific retroviral labelling of the B cells, they could also demonstrate that these cells

generated neurospheres *in vitro*. Thus the adult NSC were identified as the B cell astrocytes (Doetsch et al., 1999a, Doetsch et al., 1999b). This finding is not entirely surprising since the embryonic stem cell during development, the radial glia, also has astrocytic properties. Actually, Merkle and colleagues (Merkle et al., 2004) using a cre-lox tracking strategy demonstrated that developmental radial glia develop into adult SVZ B type astrocytes.

Neurogenesis in the SVZ starts with the proliferation of a B cell astrocyte. This generates the transit amplifying population of C cells, which in turn give rise to the A-type neuroblasts. The neuroblasts migrate via the rostral migratory stream to the olfactory bulb where they differentiate into GABAergic interneurons. This process has also been described in primates (Pencea et al., 2001) and humans (Bedard and Parent, 2004, Curtis et al., 2007). Besides the cell types mentioned, the SVZ niche is also comprised of blood vessels, microglial cells and extracellular matrix.

The second germinal centre in the adult brain, the SGZ of the hippocampus, also contains astrocytic stem cells. These have long radial processes and are sometimes referred to as *type I progenitors*. These cells give rise to the fast proliferating D cells or *type II progenitors*, which migrate into the granule cell layer and differentiate into glutamatergic granule neurons (Seri et al., 2001, Laywell et al., 2000). Again here the niche is closely associated with capillaries and the importance of this will be discussed in a later chapter. Neurogenesis in this area is related to memory and learning and is affected by environmental enrichment (Kempermann et al., 1997b, Kempermann et al., 1998), physical exercise (van Praag et al., 1999) and various hormones (Cameron et al., 1998, Gould and Tanapat, 1999, Tanapat et al., 1999).

Spinal cord development differs from that of the brain. Here the neurons are directly derived from the neuroepithelial cell pool, while the radial glia cells appear at the end of neurogenesis. Although the spinal cord lacks an SVZ, NSC are present and can be isolated from adult individuals, (Weiss et al., 1996, Shihabuddin, 2002). The identity of spinal cord NSC has not yet been clarified but some reports indicate a population of cells close to or within the ependymal layer (Adrian and Walker, 1962, Frisen et al., 1995, Johansson et al., 1999a). Since the spinal cord is considered a non-neurogenic area (no formation of new neurons when the development is completed), the function of the NSC in normal conditions remains to be elucidated. However, as a consequence of spinal cord injury the NSC proliferate and migrate to the site of injury (Frisen et al., 1995, Johansson et al., 1999a). Additionally, in demyelinated lesions of an animal model of Multiple Sclerosis these cells can differentiate into oligodendrocytes (Picard-Riera et al., 2002, Brundin et al., 2003) and cells with neuronal properties (*Paper I*). Thus the competence of the NSC of the adult spinal cord is evident during altered conditions. The

otherwise quiescent NSC then activate their proliferation and differentiation machinery.

THE NICHE

There are both extrinsic and intrinsic factors that regulate NSC proliferation and differentiation. Some of these factors act to maintain the "stemness" of the cells, such as Sox1, 2 and 3, Notch, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Others induce cell cycle exit and differentiation, such as BMP, CNTF, LIF, BDNF, NGF, thyroid hormone-3 and 4, among others. Many of these factors already act during development.

EGF and bFGF

The best characterised factors keeping stem cells in an undifferentiated state are EGF and bFGF. The latter is involved in early developmental processes of the nervous system such as neural plate formation, proliferation of neuroepithelial cells and patterning of the nervous system review by (Dono, 2003). Not until later during development does an EGF-responsive cell type appear in the SVZ (Reynolds et al., 1992). This EGF responsiveness is thought to coincide with the initiation of astrogliogenesis since EGF signals through STAT3, a known gliogenic transcription factor (Burrows et al., 1997, Viti et al., 2003)

The NSC from adult brain and spinal cord proliferate in response to both EGF and bFGF (Weiss et al., 1996). For example, infusion of EGF or bFGF into the brain increases the proliferation of progenitor cells in the SVZ (Craig et al., 1996, Kuhn et al., 1997). Moreover, addition of EGF *in vitro* reverses the fated progenitor cells to a more undifferentiated state (Doetsch et al., 2002).

Numb

Numb is a plasma membrane-associated cytoplasmic protein see review by (Cayouette and Raff, 2002) involved in fate-decision of NSC (Verdi et al., 1996). As a consequence of asymmetrical division, Numb is parted differently between dividing cells inducing different fates in the daughter cells. Numb blocks Notch signalling (Zhong et al., 1996) and it is thought to be involved in generation of different neuronal lineages (Zilian et al., 2001). Numb is also expressed in the adult SVZ where it was demonstrate to maintain ependymal wall integrity and neuroblasts survival (Kuo et al., 2006).

Notch

Notch is a single-pass transmembrane protein active during development and in maintaining tissue homeostasis in adult. In mammals there are four different Notch receptors that bind to five different ligands: Delta-like 1, 3 and 4, and Jagged-1 and -2. The ligands are also transmembrane proteins (Artavanis-Tsakonas et al., 1999). Notch1, Jagged1 but not Delta-like 1 are expressed in the adult SVZ (Stump et al., 2002). During development dysfunctional or absent Notch signalling results in earlier induction of neurogenesis, implying its negative effect on this process. The activation of Notch in NSC promotes either self-renewal or astrogliogenesis, both achieved by blocking neurogenesis.

When cells start to differentiate into neurons they express higher levels of the Delta receptor. The neighbouring cells receive signals via Notch and either remain undifferentiated or adopt an alternative fate, i.e. that of astrocytes. The intracellular mechanisms induced by ligand binding results in cleavage of the Notch intracellular receptor domain. This translocates to the nucleus where it binds to the co-repressor RBP-J, thereby converting it into a co-activator. This initiates the transcription of Hairless and enhancers of split (Hes) 1 and 5 genes. The Hes block pro-neuronal factors and subsequently neurogenesis (Nakamura et al., 2000).

Sox

Sox proteins are transcription factors belonging to the High Mobility Group (HMG) family of proteins and bind to specific DNA sequences located in the minor groove of the DNA helix (Lefebvre et al., 2007).

There are twenty different Sox proteins described in vertebrates. Sox 1, 2 and 3 are all preservers of the stemness state. The underlying mechanism involves inhibition of pro-neuronal genes such as neurogenin (Bylund et al., 2003). Sox 8, 9 and 10 are involved in oligodendrogenesis (Stolt et al., 2002, Stolt et al., 2003, Stolt et al., 2004) while Sox 21 promotes neurogenesis (Sandberg et al., 2005).

Sonic hedgehog

During development Sonic Hedgehog (Shh) is released by the floor plate and notochord contributing to neuronal patterning (review by (Ulloa and Briscoe, 2007). It also promotes oligodendrogenesis through induction of the bHLH transcription factors genes *Olig-1* and *Olig-2* (Zhou et al., 2000, Lu et al., 2000).

In the adult SVZ Shh is involved in maintenance and proliferation of the NSC (Machold et al., 2003, Ahn and Joyner, 2005).

Noggin

Noggin is expressed both during development and in the adult SVZ where it is produced by ependymal cells (Lim et al., 2000). Noggin blocks the signalling and differentiation of Bone Morphogenetic Proteins (BMPs) (see below).

Bone Morphogenetic Proteins

The BMP belong to the TGFβ superfamily and regulate the differentiation of NSC. Early during development the BMP are neurogenic, while later on and in the adult they promote astrogliogenesis. Signalling via BMP receptors leads to the activation of serine/threonine kinases that phosphorylate and activate the signal transducing proteins smads. The smads heterodimerise and translocate to the nucleus where they interact with other transcription factors such as signal transducers of activated transcription factors (STATs), co-activators p300/CBP complex or co-suppressors (Nakashima and Taga, 2002). BMP are produced by cells in the SVZ but their gliogenic effect is inhibited by noggin released from the ependymal cells (Lim et al., 2000).

CNTF and LIF

The IL-6 family members CNTF and LIF are potent inducers of astroglial differentiation. They share a common receptor subunit, gp130. Receptor dimerisation leads to activation of tyrosine kinases in JAKs, which activate STATs, especially STAT1 and 3. Other signalling pathways are involved, leading to activation of transcription factors such as NF-kappa B, AP-1 and NL-IL-6 (C/EBP-β) that in turn interact with the p300/CBP co-activator (reviewed by (Nakashima and Taga, 2002).

Homeobox proteins

Homeobox protein are well characterised in the patterning of the nervous system. Each location in the developing nervous system is divided into domains with specific combinations of homeobox protein expression. This diverse expression decides the cellular position along the ventral-dorsal, anterior-posterior and medial-lateral axes. In the adult CNS members of the Dlx homeobox protein family are expressed in the SVZ. Dlx-2 is expressed in C-type cells in the SVZ and is involved in generation of GABAergic neurons. *In vitro* upon stimulation with EGF, Dlx-2 expression is downregulated, see review by (Gangemi et al., 2004).

Basic helix loop helix family of proteins

The bHLH protein family of transcription factors is characterised by a motif consisting of two α-helixes connected by a loop. The bHLH transcription factors can either be inhibitory (such as HES, Hey and ID families) or induce transcription (such as the pro-neural bHLH, mash 1 and neurogenins 1 and 2).

The pro-neuronal bHLH proteins form heterodimers with E-proteins and bind to E box promoter elements, thereby inducing transcription. The pro-neuronal genes mash-1 and neurogenins are expressed transiently at the phase of induction of neurogenesis and disappear in mature neurons. They can therefore be detected in neurogenic areas such as the SVZ and SGZ, but not in mature neurons. There is also a spatial divergence of the two genes, mash being detected in the ventral part of the telencephalone and neurogenin in the dorsal part. Mash and neurogenin interact with the p300/CBP co-activator to induce a second wave of neuronal genes, such as NeuroD and the Math family of proteins which induce terminal differentiation of neurons. In contrast, bHLH Olig1 and 2 promote oligodendrocyte differentiation reviewed by (Kageyama et al., 2005).

As mentioned previously, Hes is a downstream mediator of Notch signalling. It mediates self-renewal or induces astrogliogenesis by blocking neurogenesis. This is achieved through two mechanisms. Hes forms dimers with members of its own class and bind to N-box promoter elements. After DNA binding Hes recruits co-repressors that inhibit the transcription of pro-neuronal target genes. Another mechanism is to directly interact with pro-neuronal proteins and hinder their induction of transcription.

The Id (inhibition of differentiation) proteins sequester the E-proteins that are needed for pro-neuronal bHLH function. Id proteins have DNA-binding motifs but they lack activation domains. Consequently, they act as dominant negative transcription factors by binding E-proteins and thus inhibiting the activation of transcription reviewed by (Ross et al., 2003).

Fight for fate: signal transduction convergence

The fate of a cell is determined by the net effect of converging signal transduction pathways activated simultaneously. This can be exemplified by the p300/CBP co-activator. Gliogenic signals such as CNTF, LIF or Notch and neurogenic signals such neuronal bHLH converge at the level of p300/CBP co-activator. Hes, and STAT1 and 3 interact with p300/CBP and induce the transcription of the astrocytic gene G*fap*. This interaction is disrupted by neurogenins which will sequester the co-activator and induce the transcription of neuronal genes. The fate of a cell is thus determined by the balance between

gliogenic *versus* neurogenic mediators (Miller and Gauthier, 2007). The development of the nervous system is divided into a neurogenic period followed by a gliogenic period. Thus when the concentration of neurogenic determinants decreases, astrogliogenesis takes over.

This is just one example of factors and signal transduction pathways involved in fate decision. The neurogenic niches are much more complex *in vivo*. Recently, the effects of endothelial-derived factors such as VEGF (Li et al., 2006, Xiao et al., 2007) and nitric oxide (Moreno-Lopez et al., 2000, Packer et al., 2003, Matarredona et al., 2004) on the NSC pool are emerging. In neuroinflammation the level of complexity is further increased by all the factors released by immune cells.

CELLS OF THE NERVOUS SYSTEM

The nervous system consists of neurons and supporting cell types morphologically characterised as macroglia and microglia. The macroglial cell pool consists of astrocytes and oligodendrocytes.

ASTROCYTES

Astrocytes, the most abundant cell type in the CNS, have multiple functions. They are involved in ion homeostasis, uptake of neurotransmitters, metabolic support for neurons, blood brain barrier formation and immune reactions. Following CNS injury astrocytes form the glial scar, which protects the rest of the intact tissue against spreading of the injury. They are also the source of different trophic factors such as NGF, BDNF, CNTF and EGF (see review by (Ridet et al., 1997). During development the radial glia act as stem cells and provide scaffolding for migrating neurons (Gotz and Huttner, 2005). In the adult brain the CNS stem cell has been identified to be of astroglial origin (Doetsch et al., 1999a).

OLIGODENDROCYTES

The oligodendrocytes are the myelinating cells in the CNS. In contrast to its peripheral counterpart, the Schwann cell, one oligodendrocyte can myelinate several neuronal axons. Oligodendrocyte maturation into myelinating cells starts post-nataly after both neurogenesis and astrogliogenesis is completed and neuronal networks have been established. The transcription factors involved in oligodendrocyte differentiation are not as well characterized as for

neurogenesis. The bHLH factors Olig1 and 2, the homeobox factor Nkx2.2 and the Sox proteins 8, 9 and 10 have all been shown to be involved. Several extrinsic factors also influence glial differentiation: bFGF, IGF-1, PDGF, thyroid hormone, CNTF, LIF, BMP, Shh and Notch (Nicolay et al., 2007).

The terminal differentiation of oligodendrocytes is characterized by myelin formation. Myelination is promoted by the thyroid hormones 3 and 4 and is regulated by neuronal features such as axonal diameter (Voyvodic, 1989) and surface molecules such as PSA-NCAM (Charles et al., 2000) . Myelin enables the salutatory conduction of electric impulses in the neurons. It also provides trophic support for neurons and creates a physical barrier towards detrimental factors.

In demyelinating diseases such as Multiple Sclerosis (MS), the origin of remyelinating oligodendrocytes is intensely debated (Chandran, 2006). Some studies report that mature oligodendrocytes can contribute to remyelination (Prineas et al., 1993), however, the best candidates are NSC and oligodendrocyte progenitor cells (OPC) (Chari and Blakemore, 2002). One of the reasons for the inefficient remyelination of the plaques is that the required signals regulating the migration of OPC into the lesions are hampered. An example of this pathological signalling is the changed balance between Semaphorin 4 and 5, resulting in a loss of attracting signals for OPC (Franklin, 2002, Williams et al., 2007)

MICROGLIA

For a long time the microglial cells "small glia," were as the name denotes considered to be of ectodermal origin. In 1938 Rio Hórtega proposed that microglial are of mesodermal origin, a notion which is now generally accepted (Rio-Hortega, 1937). The consensus to date is that microglial cells migrate into the CNS during development and are trapped there upon blood brain barrier formation. The microglial cell pool is renewed throughout life by division of already resident cells. During normal conditions microglial cells have homeostatic functions, taking care of the dead cells and screening the local environment for any sign of pathogen intrusion. Indeed, microglia are also called "the macrophage of the brain" since they demonstrate features important for peripheral macrophage function. Some of these features are: expression of scavenger receptors, pattern-recognition receptors, antigen presentation molecules (MHC class II), co-stimulatory molecules, production of various cytokines and other factors (NOS and ROS). Microglia have been considered as the bad agents of inflammation, having a default response which is always detrimental to surrounding tissue. The activation mode of microglia is plastic and as with their peripheral counterparts, their response is

dependent on the stimuli provided. In normal conditions microglia have a ramified shape. Upon activation they adopt a more rounded shape that facilitates migration towards the site of injury. In MS and its animal model counterpart EAE, the role of microglia in neuroinflammation has been extensively studied and reports suggest both a beneficial and detrimental role in development of these diseases (Jack et al., 2005, Hanisch and Kettenmann, 2007).

Since there are microglial cells present in the SVZ niche it is of major importance to know how inflammation will influence these cells and in turn how microglia themselves will influence the stem cell pool. Butovsky and colleagues demonstrated that microglia activated with interleukin (IL)-4 trigger neurogenesis in NSC cultures (Butovsky et al., 2006b). In contrast, IFNγ-stimulated microglia induce oligodendrogenesis (Butovsky et al., 2006b).

THE IMMUNE SYSTEM

Haematopoiesis defines the formation of white and red blood cells. In the adult, all cells are born in the bone marrow, where the niche is composed of stromal cells. Stromal cells are non-haematopoietic cells such as fat, endothelial, fibroblast cells and macrophages that produce different factors such as GM-CSF, M-CSF and the interleukins (IL)-3,-4,-5,-6 and -7. Affected by combinations of the different factors, the haematopoietic stem cell differentiates into progenitor cells that further generate all cells in the blood (Kuby, 1997). It is interesting that several receptor/signal transduction pathways found in the NSC niche, such as Notch and IL-6 are also active in fate determination of the haematopoietic system. The aim of the following chapters, however, is to give an introduction to the functions of mature immune cells.

CELLS OF THE IMMUNE SYSTEM

The cells of the immune system develop from a mesodermal stem cell, which diverges early into myeloid and lymphoid branches. The myeloid progenitor cell gives rise to most of the cells of the innate immune system: macrophages/monocytes, neutrophils, basophils, eosinophils, dendritic cells and mast cells. The lymphoid lineage generates the lymphoid cells, i.e. B and T cells, NK-cells and NK-T cells.

The first line of defence against invading pathogens is constituted by the innate immune system. One characteristic of innate immune cells is the expression of pattern recognition receptors, such as toll-like receptors (TLR) that bind to pathogen associated molecular patterns (PAMPs). The PAMPs are conserved moieties such as carbohydrates or lipoproteins derived from various microbes (Pandey and Agrawal, 2006). Encounter of pathogens directly triggers a defence response aiming at killing and clearing out the infection. The response comprises release of noxious factors such as NO, reactive oxygen species (ROS) and different chemokines and cytokines such as IL-23, IL-12, IL-6, TNF-α IL-1β or type 2 interferons (IFN) (Konat et al., 2006). Besides different systemic effects some of these factors activate and attract the members of acquired immunity, B- and T-cells, to the site of inflammation. The acquired immunity has epitope specificity and life-long memory.

The cell type responsible for directing the whole immune response towards a certain specificity is the $CD4^+$ T-cell. There is also another type of T-cell ($CD8^+$ T cell) which is cytotoxic and involved in killing of modified cells. Here, I will only focus on the $CD4^+$ T-cell.

T CELL ACTIVATION

Naive T-cells require two signals to be activated. The first signal is transmitted via the T cell receptor (TCR) when it interacts with major histocompatibility complex (MHC) classII molecules carrying an antigen (Rosenthal and Shevach, 1973, Zinkernagel and Doherty, 1974). MHC class II molecules are expressed on the surface of professional antigen presenting cells (APCs) such as dendritic cells or macrophages. The second signal is mediated via interaction between co-stimulatory molecules B7-1 and B7-2 on the APC and CD28 on the T cell. The requirement for both signals constitutes a means of avoiding erroneous T cell activation and subsequent immune reaction. Any naive cells interacting with MHC without receiving the second signal enter a state of non-responsiveness, so called *anergy* (Alegre et al., 2001). The second signal acts in a reciprocal fashion, also activating the APC itself to produce cytokines that will further influence T cell differentiation. APC-derived IL-12 skews the T-cell differentiation towards a T helper (TH)1 subtype which is characterised by release of IFNγ, IL-2 and TNF. IL-10 differentiates the T cells into a TH2 subtype which releases IL-4, IL-5, IL-6 and IL-10. The cytokines of the TH1 and TH2 subsets are mutually inhibitory, i.e. the TH1 subset inhibits the production and activity of the TH2 subset and *vice versa*.

The TH1 TH2 paradigm proposed by Mosmann and Coffman (Mosmann et al., 1986) has been forced to be readdressed by the discovery of the TH17 subset (Langrish et al., 2005, Park et al., 2005a). In spite of much incongruence, the immunological reaction in both EAE and MS has for a long time been attributed to IFNγ-producing TH1 cells. Now the role of the TH1 cells is partially substituted by the IL-17, IL-6 and TNF producing T-cells, the so-called TH17 subset (Chen et al., 2006). The function of IL-17 is not entirely clear but there is evidence for its involvement in human autoimmune disease and it has been detected in patients with MS and systemic lupus erythematosus (Matusevicius et al., 1999, Wong et al., 2000, Kebir et al., 2007).

TOLERANCE

The term immunological tolerance is related to the ability of T-cells to distinguish between self and non-self. Tolerance is first established in the thymus where T-cells recognising self-antigens are depleted. In the thymus, there is an ectopic expression of almost all molecules in the body displayed on MHC molecules on the surface of thymic medullary epithelial cells (Anderson

et al., 2002). Normal T-cell function requires that the T-cell can recognise self MHC molecules. Thus T-cells carrying T-cell receptors that can bind to MHC, survive. (Kisielow et al., 1988, von Boehmer et al., 1988) In contrast, T-cells that bind too strongly to MHC molecules alone or in context with self-antigens are depleted (Kappler et al., 1987, McDuffie et al., 1988). These selection processes in the thymus aim to provide for a mature T cell population that is self-MHC restricted and self-tolerant (Sprent et al., 1988). However, this system is not fully effective. All individuals have autoimmune cells in the circulation that in concert with other susceptibility determinants can break the tolerance of the immune system and cause autoimmune diseases.

To keep T-cells that have escaped central tolerance in check in the periphery several mechanisms have developed, such as regulatory APCs and T-cells that dampen immune responses (Steinman et al., 2003). Additionally, some tissues such as the testis, the eye and the brain have historically been described as being immune privileged. This means that the antigens in these compartments are sequestered and cannot be accessed by the immune system. When tolerance is broken the immune system reacts against self-antigens, leading to development of autoimmune responses and consequently autoimmune diseases.

MULTIPLE SCLEROSIS AND EAE

Autoimmune diseases can either be systemic, such as systemic lupus erythematosus, or organ-specific, such as insulin-dependent diabetes, rheumatoid arthritis or multiple sclerosis. In the organ-specific autoimmune diseases the immune response is elicited against an antigen derived from the specific organ affected.

MULTIPLE SCLEROSIS

Multiple Sclerosis is a demyelinating, inflammatory disease first described during the 19th century by Cruveilier and Charcot (Cruveilhier, 1841, Charcot, 1868). The main characteristic of the disease is destruction of the myelin sheath, leading to ineffective conduction of electric impulses in the affected neurons. This in turn leads to a plethora of neurological deficits. 80% of the patients experience a disease course characterised by recurrent periods of worsened symptoms followed by full or partial recovery, so called Relapsing Remitting MS, RR-MS. After a period of time the disease develops into a secondary progressive phase when the patients get gradually worse (SP-MS). Even in the early phase of disease there are subclinical lesions and loss of neurons which gradually contribute to into accumulating deficits (Silber and Sharief, 1999) Even though the myelin is the primary target of the autoimmune reaction, axonal degeneration accounts for the mounting disability. There are many possible causes of axonal injury in MS (see review by (Comston, 1999). Neuronal death can be a result of exitotoxicity, where microglial cells have been reported to produce glutamate (Takeuchi et al., 2006). Neurons can also be a target for the inflammatory milieu after losing the shielding myelin sheath. However, neuronal death has also been observed in spite of intact myelin sheath (Evangelou et al., 2000). Due to the variability in lesion site the disease has different manifestations in different patients and can be quite difficult to diagnose. Disease history or image evidence of minimum two attacks affecting more than one anatomical site is strongly suggestive of MS (Poser et al., 1983, McDonald et al., 2001). The diagnostic criteria also require exclusion of other possible diseases such as neuro-borreliosis, tumors or cerebral SLE. Oligoclonal bands can be detected in the CSF in 90% of the patients but these bands are also found in other diseases such as CNS infections.

In MS peripheral activation of T cells is followed by secretion of proinflammatory cytokines subsequent to antigen restimulation (Olsson et al., 1990). What activates autoimmune T cells? The initiating event causing breakage of tolerance in MS is not known. The consensus is that self-reactive T

cells are activated in the periphery and migrate to the CNS where they initiate an inflammatory cascade. Several events have been proposed for activation of these cells in the periphery: **1**) molecular mimicry, whereby T cells are activated by pathogen-derived moieties "mimicking" the sequence of myelin peptides; **2**) bystander activation whereby strong inflammatory conditions facilitate T cell activation **3**) myelin debris released from the CNS subsequent to injury or a as a primary degenerative event. Upon activation the T-cells express adhesion molecules and can enter the CNS. Here they are reactivated by resident microglia and start releasing chemokines and cytokines, thereby recruiting more immune cells from the periphery. Both in MS and its animal model counterpart, Experimental Autoimmune Encephalomyelitis (EAE), macrophages, T- and B-cells are involved in the pathological events in the CNS, including demyelination.

The demyelination pattern in different patients can have various causes. Lassmann and colleagues have identified 4 different patterns of demyelination, of which the first and second can be found in EAE (Lassmann, 1999). In the first pattern the demyelination occurs as a consequence of macrophage activation and the release of noxious action such as NO, ROS and TNFα. The second pattern is caused by autoantibodies and complement while the third pattern is characterised by degradation of the myelin associated glycoprotein, MAG and finally, the fourth is caused by non-apoptotic oligodendrocyte death (Lassmann, 1999). Actually, oligodendrocyte death can occur prior to macrophage activation, implying that this might be one of the initial events triggering an inflammatory reaction in the periphery.

MS is a complex disease where both genetic and environmental factors contribute to an individual's risk of developing the disease. In regard to environmental factors, several microbial agents including herpes simplex virus, *Chlamydia pneumoniae* and Epstein-Barr virus have been associated with MS (Sundstrom et al., 2004, Buljevac et al., 2005). In addition to this, patients can experience worsening of symptoms in the aftermath of an infection (Buljevac et al., 2002). The northern part of Europe and North America has the highest incidence of MS, being more than 1 per 1000 individuals. Different genes have been associated with MS, some of them are the *MHC* haplotype (Jersild et al., 1973, Olerup and Hillert, 1991), *Mhc2ta* (a co-activator for MHC class II transcription) (Swanberg et al., 2005) and the *IL7* receptor (Lundmark et al., 2007). The combination of the different susceptibility alleles accounts for the heterogeneity of the disease in different patients which also complicates the design of a proper treatment.

MS: a neural stem cell disease?

NSC recruitment to the demyelinated areas has been observed both in the brain and spinal cord of EAE-diseased animals (Picard-Riera et al., 2002, Brundin et al., 2003). Spontaneous remyelination occurs in MS, where so-called *shadow plaques* are formed (Prineas and Connell, 1979, Prineas et al., 1993). Here the myelin is thin and the myelin sheath is disproportionate to the axonal diameter. A recent publication reported that as in EAE, human NSC are mobilised from the SVZ and give rise to OPC in the periventricular lesions (Nait-Oumesmar et al., 2007). Interestingly, these lesions are less likely to be remyelinated than those located in the subcortical or deep white matter (Patrikios et al., 2006). Since periventricular lesions predominate in MS (Charcot, 1868) a question arises. Could it be that the close proximity between these prevalent inflammatory, demyelinating lesions and the SVZ leads to depletion of the NSC pool which in turn accounts for a lower reparative capacity evident in MS?

EXPERIMENTAL AUTOIMMUNE ENCEPHALLOMYLITIS

Interestingly, EAE has its roots in the field of virology. During the late period of the 19th century Pasteur and colleagues vaccinated patients against rabies. As a vaccine they used dried homogenates of spinal cords from rabbits infected with rabies virus. Some patients developed paralytic symptoms ranging from mild to fatal. Later, Rivers experimented with uninfected spinal cord homogenate which he injected into rhesus monkeys and could still induce paralysis in some of the animals. To increase the incidence of disease he had to administer more injections. With the introduction of Complete Freund's adjuvant in 1942 the requirement for multiple injections was reduced and the EAE induction protocol got its modern form, see review by (Baxter, 2007).

EAE has been induced in different animal species such as rabbits, dogs, guinea pigs and also rats and mice. The common formula is composed of Freund's adjuvant, which is a paraffin oil used alone (*incomplete*) or containing killed *Mycobacterium tuberculosis* (*complete*). Spinal cord homogenate or different myelin peptides are used as antigens. The pathology and disease course depends on animal species and the antigen used. In the rat, the susceptibility to EAE differs greatly between strains, a phenomenon that is coupled to the type of MHC haplotype. The resemblance to MS is also variable. For instance, when immunising Lewis rats with myelin basic protein (MBP) peptide they develop a monophasic disease with diffuse infiltration of immune cells in the brain, but without demyelination. (McFarlin et al., 1973) In contrast, Dark Agouti (DA) rats immunised with myelin oligodendrocyte oligoprotein (MOG) develop a relapsing-remitting disease with demyelinating lesions

predominantly occurring in the spinal cord. The immune cells apparent in the lesions are mostly macrophages/microglial and T-cells and as in MS the activity of the lesions ranges from inflammatory, demyelinated to remyelinated shadow plaques (Storch et al., 1998).

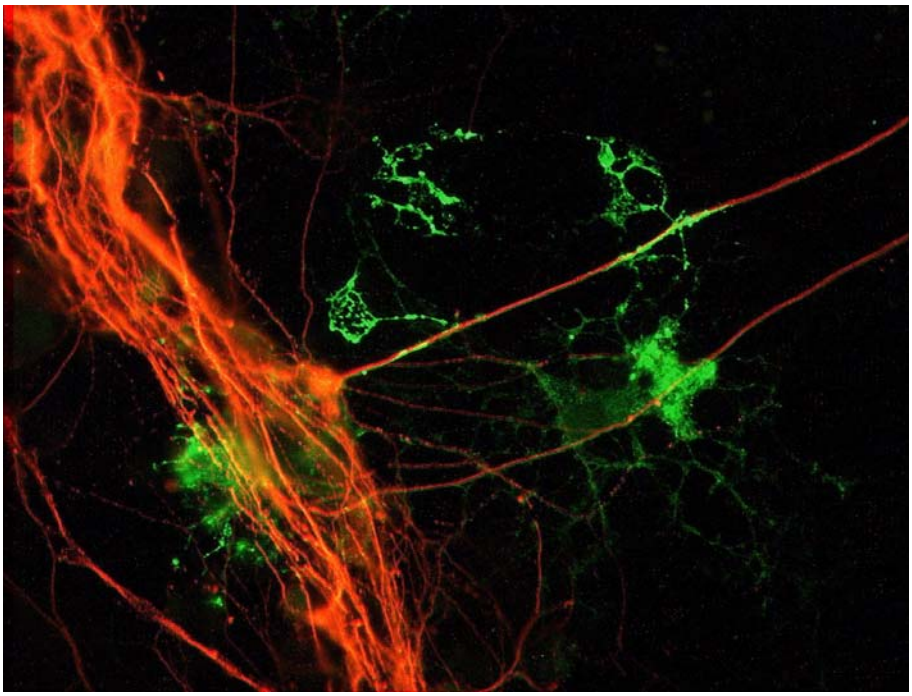

Figure 1. Myelinating oligodendrocytes. Immuno-labeling of co-cultures consisting of neurons (βIIItubulin, red) and oligodendrocytes (myelin basic protein (MBP) green). Covacu *et al*., unpublished data.

NEURAL STEM CELLS IN NEURO-INFLAMMATION

During normal conditions NSC from the regenerative areas of the brain, the SVZ and the SGZ, generate new neurons that integrate into pre-existing neuronal circuits of the olfactory bulb and hippocampus, respectively. These processes proceed throughout adult life since the need of maintaining olfaction and memory creates an "environmental pressure" engaging the NSC pool. The migration, proliferation and differentiation of the NSC into these areas are well controlled by a well established niche. What happens after a CNS trauma is that new needs arise and new cues are released from the damaged areas, changing the niche and the behavior of the NSC. For instance, the normally quiescent spinal cord stem cells proliferate and migrate in response to spinal cord injury (Johansson et al., 1999a) Paper I). What is also very important to consider is that these cells can be induced to exit their niche and migrate for long distances into "uncharted territories" governed by new extrinsic cues. Migration along established pathways in the SVZ and the SGZ are regulated by signals such as PSA-NCAM (Doetsch et al., 1997), reelin (Frotscher, 1998, Nakajima et al., 1997) slit (Nguyen-Ba-Charvet et al., 2004), nitric oxide (Moreno-Lopez et al., 2000) and ephrin (Conover et al., 2000).

In the case of CNS damage the NSC are subjected to migrational cues released from the inflammatory foci. NSC have been demonstrated to respond to stem cell factor (SCF) (Erlandsson et al., 2004), monocyte chemoattract protein 1 (MCP-1)(Widera et al., 2004) and stromal derived factor-1 (SDF-1) (Imitola et al., 2004b, Takeuchi et al., 2007) and express their binding receptors kit, CCR2 and CXCR4. EGF also stimulates migration in NSC (Boockvar et al., 2003) and intra-nasal administration of heparin-binding-EGF (HB-EGF) increased migration to demyelinated regions in the corpus callosum (Cantarella et al., 2007). Moreover, hepatocyte growth factor (HGF) (Takeuchi et al., 2007, Heese et al., 2005), fibronectin (Tate et al., 2002), sphingosine-1-phosphate (Kimura et al., 2007), and IL-8 (Beech et al., 2007) are also responsible for NSC homing to the damaged areas.

Migration and differentiation of NSC has been demonstrated in various types of lesions following hypoxic and ischemic injury (Park et al., 2004), middle cerebral artery occlusion (Fukunaga et al., 1999), traumatic brain injury (Riess et al., 2002) and demyelination (Picard-Riera et al., 2002, Brundin et al., 2003, Nait-Oumesmar et al., 2007).

Inflammatory reaction, good or bad?

The primary role of inflammation is to create a noxious milieu leading to clearance of pathogens. When inflammation does not subside the result can be severe secondary tissue damage. Well established niches such as the SGZ are disturbed by inflammatory factors and consequently the ongoing neurogenesis in this area is impaired. Does this mean that inflammation is always detrimental to neurogenesis? Or is it only detrimental to well established regenerative areas in which the perfect balance of factors contributing to neurogenesis is disturbed? Different studies by Monje (Monje et al., 2002, Monje et al., 2003, Monje et al., 2007) and Ekdahl (Ekdahl et al., 2003) have demonstrated that inflammation initiated by irradiation or with intracranial injections of LPS impaired hippocampal neurogenesis. Moreover, the culprit of this reaction was identified to be the microglia. In contrast to these findings microglia have been shown to support migration of NSC and depending on their type of activation microglia skew NSC differentiation towards either oligodendrogenesis (Butovsky et al., 2006a, Butovsky et al., 2006b) or neurogenesis (Aarum et al., 2003, Butovsky et al., 2006b, Ziv et al., 2006b). The contradictory results might be attributed to variations in the microglial cell pools located in the SVZ and SGZ, contra the non-nurogenic areas. Indeed, the microglia are more abundant in the SVZ than in the SGZ, but they are less prone to re-activation than are microglia in non-neurogenic areas (Goings et al., 2006).

When it comes to the effect of individual cytokines on NSC proliferation and differentiation the reported results are equally ambiguous. TGFβ, involved in differentiation of T regulatory cells but also that of TH17 cells (Bettelli et al., 2006, Veldhoen et al., 2006) impairs NSC proliferation and neurogenesis both *in vitro* and *in vivo* (Buckwalter et al., 2006, Wachs et al., 2006). In contrast, Battista and colleagues reported a beneficial role of microglial-derived TGFβ in these processes (Battista et al., 2006). TNFα, a pivotal pro-inflammatory cytokine in both MS and EAE, gives the same dichotomous results, either promoting (Wu et al., 2000, Widera et al., 2006) or inhibiting NSC proliferation (Wong et al., 2004, Sheng et al., 2005, Iosif et al., 2006) and differentiation (Liu et al., 2005). However, IFNγ, another major cytokine found in neuroinflammation seems to be overall neurogenic (Wong et al., 2004, Song et al., 2005).

In conclusion, the effect of inflammation on the NSC can have different effects depending on type (i.e. dominating cytokines) and location of the inflammatory reaction.

Beyond the pressure of ectodermal fate: new roles for NSC in neuroinflammamtion

Traditionally, the therapeutic capacity of NSC has been mostly studied in regards to their differentiation potential and substitution of damaged cells. This view of NSC is changing. Reports have revealed that NSC constitutively express neurotrophic factors, thereby exerting trophic support for damaged neurons (Ourednik et al., 2002, Hagan et al., 2003, Lu et al., 2003b). During inflammatory conditions the NSC express co-stimulatory molecules (B7-1 and B7-2) and are thus able to interact with immune cells (Imitola et al., 2004a).

Moreover, the result of intraventricularly and intravenously injected NSC has been demonstrated to reduce demyelination, axonal loss and glial scar formation and as a consequence amelioration of the disease severity of EAE (Pluchino et al., 2003, Ben-Hur et al., 2003, Einstein et al., 2003, Pluchino et al., 2005, Einstein et al., 2006, Einstein et al., 2007). The NSC express adhesion molecules and chemokine receptors and can thus home to the damaged CNS (Pluchino et al., 2005) where they either migrate to demyelinated areas and differentiate into OPCs (Pluchino et al., 2003) or gather around perivascular regions (Pluchino et al., 2005, Einstein et al., 2006) or in the lymph nodes (Einstein et al., 2007). In the perivascular spaces the NSC remain undifferentiated for long periods of time, expressing immunomodulatory molecules and neurotrophic factors (Pluchino et al., 2005). In the lymph nodes NSC exert an inhibitory effect on the proliferation and activation of encephalitogenic T-cells (Einstein et al., 2007).

DOUBLE AGENTS: NITRIC OXIDE, HMGB1 AND TOLL LIKE RECEPTORS

Three of the publications in this thesis focuses on nitric oxide (NO), high mobility group box protein 1 (HMGB1) or toll like receptor (TLR) 2 and 4 and the implications of these different factors in NSC biology and neuroinflammation. Interestingly, all three of them have dual roles in stem cell biology acting in one way on the basic functions of these cells but influencing them in a different way during inflammation. Here is a brief introduction to each one of them.

NITRIC OXIDE

Before 1987, NO was not known to be a biological mediator. The research leading to its discovery began with experimentation of the way sympathetic activation leads to relaxation of blood vessels. Furchgott and colleagues described that sympathetic activation of the endothelium induced the release of a factor that diffused to the smooth muscles and promoted their relaxation, the factor being named endothelial derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). The identity of EDRF as NO was first revealed in 1987 by two independent groups (Ignarro et al., 1987, Palmer et al., 1987). Later, it was shown that NO is synthesised in the endothelium (Palmer and Moncada, 1989). The NO generating pathway, where an enzyme converting L-arginine to L-citrulline and NO, nitric oxide synthetase (NOS), was initially described in 1990 (Radomski et al., 1990).

There are three different isoforms of NOS: endothelial NOS (NOS I), neuronal NOS (NOS III, expressed in the nervous system, kidney, sarcoplasmatisc reticulum and mitochondria in the skeletal muscle, (Grozdanovic, 2001), and inducible NOS (iNOS or NOS II expressed in various immune cells such as microglia/macrophages and neutrophils (Stuehr et al., 1989, Abramson et al., 2001). The best characterized signal transduction pathway is through activation of soluble guanylate cyclase, leading to intracellular increase of cyclic guanylate monophosphate (cGMP) (Arnold et al., 1977).

In the central nervous system nNOS was first described by Garthwaite and colleagues (Garthwaite et al., 1988). NO is involved in long-term potentiation and neuronal plasticity in the hippocampus (Haley et al., 1992). It mediates NANC (non-adrenergic non- cholinergic) transmission in the

gastrointestinal tract and promotes erectile function (Leone et al., 1994). Upon massive NMDA receptor activation nNOS is activated and the released NO leads to neuronal cell death. At low levels binding of NO to the cytochrome c oxidase reversibly blocks mitochondrial respiration, lowering ATP levels and hindering apoptosis (Beltran et al., 2000). However, when extensively produced it blocks the respiration chain and leads to necrosis of neuronal cells see review by (Boje, 2004).

This duality of NO effects is also evident during immune reactions, where the enzyme responsible for excessive NO production is predominantly iNOS. The iNOS isoform was first identified in macrophages stimulated with INFγ and LPS (Stuehr et al., 1989). Being trascriptionally regulated and calcium-independent it produces 1000 fold more NO compared to the other NOSes, reviewed by (Forstermann et al., 1998). Since it is involved in innate immune reactions, NO is rapidly produced via iNOS and leads to clearance of pathogens. Moreover, NO can react with reactive oxygen species (ROS) forming the highly reactive compound peroxynitrite (Radi et al., 1991). During chronic inflammatory conditions when it is extensively produced NO can cause tissue destruction. It induces cell death in neural progenitor cells (Cheng et al., 2001), in mature oligodendrocytes (Merrill et al., 1993) and in neurons (Boje and Arora, 1992), thereby contributing to excitatory amino acid-induced injury (Dawson et al., 1993, Dawson et al., 1994, Bal-Price and Brown, 2001). NO production leads to nitrosylation and peroxynitrosylation of proteins, and can also cause DNA damage (Kwon et al., 1991) or may interfere with DNA repair (Wink et al., 1991). The level of NO production correlates with disease activity in various neuroinflammatory conditions such as bacterial meningitis (Kornelisse et al., 1996), systemic lupus erythematosus (Brundin et al., 1998), traumatic brain injury (Clark et al., 1996) and in multiple sclerosis (MS) (Johnson et al., 1995, Brundin et al., 1999, Danilov et al., 2003). In EAE it has dual roles and NO blockage can both ameliorate (Cross et al., 1994, Danilov et al., 2005) or worsen the disease (Ruuls et al., 1996, Gold et al., 1997) since it might adopt immunosuppressive functions (Kahl et al., 2004). Moreover, iNOS expression has been detected in lesions of rats with viral brain infections or with EAE (Koprowski et al., 1993), and in biopsies from MS patients (Bo et al., 1994, Brosnan et al., 1995).

Finally, NO has recently been described as a regulator of NSC function (Packer et al., 2003, Matarredona et al., 2004). Since blood vessels are a part of the SVZ and SGZ niches, NO is produced in close vicinity to NSC.

NO has a cytostatic function in the mammalian CNS (Packer et al., 2003, Moreno-Lopez et al., 2004, Matarredona et al., 2004) and in the developing CNS the expression of nNOS regulates neurogenesis (Moreno-Lopez et al., 2004). Several groups have demonstrated that neurotrophic factors induce NO production, which in turn acts upon target cells in a

paracrine or autocrine fashion inducing cell cycle exit and initiation of differentiation (Peunova and Enikolopov, 1995, Poluha et al., 1997, Cheng et al., 2003).

The regulatory function of NO in the regenerative areas of the adult CNS becomes evident in studies in which NOS inhibition results in excessive expansion of the neural stem/progenitor cell pool (Packer et al., 2003, Moreno-Lopez et al., 2004, Matarredona et al., 2004). Another function for NO in the adult CNS has been described in the rostral migratory stream (RMS), where progenitor cells from the SVZ migrate to the olfactory bulb and differentiate into neurons. Nitrergic neurons are located in close proximity to the RMS and the NO generated is proposed to regulate the migration and proliferation of progenitor cells into this area (Moreno-Lopez et al., 2000).

HIGH MOBILITY GROUP BOX PROTEIN 1

High Mobility Group Box protein 1 (HMGB1) is a nuclear, non-histone binding protein ubiquitously expressed in all nucleated cells. It interacts with the minor groove of the DNA helix (Yu et al., 1977) enabling bending of DNA and facilitating transcription (Giese et al., 1991). HMGB1 can be post-trasnslationally modified and hyper-acetylation of its nuclear localisation sites is thought to mediate its active secretion (Bonaldi et al., 2003). HMGB1 can be released from cells in two different ways: passive release occurs from necrotic cells (Scaffidi et al., 2002), while active secretion is provided by activated immune cells such as macrophages (Wang et al., 1999), microglia (paper III), NK cells (Semino et al., 2005) and neutrophils (Ito et al., 2007). Extracellular HMGB1 forms a positive feedback loop and further stimulates the immune cells to produce more cytokines, including TNF, IL-1, IL-6, IL-8 and macrophage inflammatory protein (MIP)-1α and MIP-1β (Andersson et al., 2000). The cytokine action of HMGB1 was first discovered in 1999 by Wang and Tracey (Wang et al., 1999). These authors detected HMGB1 in sera from septic patients and mice exposed to endotoxin and could ameliorate the symptoms by administering HMGB1- specific antibodies (Wang et al., 1999). Since then a multitude of reports has revealed the involvement of HMGB1 in various disease states such as hemorrhagic shock (Ombrellino et al., 1999), Rheumatoid Arthritis (RA) (Kokkola et al., 2002), acute lung injury (Ueno et al., 2004), cancer (Flohr et al., 2001, Kuniyasu et al., 2002), Alzheimer's disease (Takata et al., 2003), atherosclerosis (Kalinina et al., 2004) and malaria (Alleva et al., 2005).

HMGB1 has been reported to bind to three different receptors TLR-2 and TLR- 4 (Park et al., 2004, Park et al., 2005b, Yu et al., 2006) and the receptor for advanced glycosylated end products (RAGE) (Hori et al., 1995). The HMGB1 interaction with RAGE is the best described and has its origin in

neurobiology. HMGB1, also known as amphoterin, mediates neuronal outgrowth (Merenmies et al., 1991, Hori et al., 1995) and cell migration (Fages et al., 2000, Degryse et al., 2001) by signalling through RAGE.

Interestingly, HMGB1 acts as a chemoattractant for mesoangioblasts (vessel-associated stem cells) (Palumbo et al., 2004) and promotes neurogenesis in RAGE-transfected embryonic stem cells (Huttunen et al., 2002). Moreover, it interacts with Oct-4 (Butteroni et al., 2000), an embryonic stem cell determinant involved in maintaining pluripotency (Takahashi and Yamanaka, 2006, Takahashi et al., 2007). Thus, HMGB1 is not only involved in inflammatory reactions but could potentially play a role in modulating NSC function.

TOLL-LIKE RECEPTORS

Toll was first identified in Drosophila where it is involved in neuronal patterning during development and in immune functions in the adult fly. In 1997 Janeway and Medzitov cloned the cDNA of the human homolog for Toll (Medzhitov et al., 1997). In the same report the authors also demonstrated that human Toll receptor activation led to activation of NF-κB and the expression of the cytokines IL-1, IL-6 and IL-8. The functional connection between TLR and a pathogen-derived antigen was made by Poltorak and Beutler in 1998 (Poltorak et al., 1998). These authors discovered that mutations in the *Tlr4* gene rendered the mice unresponsive to endotoxin, concluding that TLR-4 is a receptor for LPS. To date, thirteen different TLR have been identified in mouse and ten in human and most studies have been focused on TLR function in the immune system.

TLR are pattern recognition receptors (PRR) binding to conserved pathogen moieties and activating the innate immune response. They are single span receptors located either on the cell surface or in endosomal compartments (only TLR- 3, -7, -8 and -9). The receptors function as dimers, either homo-dimers such as TLR-4 or hetero-dimmers, such as TLR-2 which associates with either TLR-1 or TLR-6. Different TLRs bind to various groups of antigens, for example TLR-4 binds to lipopolysacharide (LPS), TLR-2 to lipoproteins and TLR-9 to unmethylated CpG moieties. The intracellular portion of the receptors is comprised of a TLR IL-1 receptor (TIR) domain which recruits different adaptors. One such adaptor is MyD88 which activates two different signalling pathways, one of them being NF-κB, and leads to transcription of various cytokine genes. There is also an alternative pathway, called the MyD88-independent pathway, recruiting IRF transcription factors and leading to transcription of type I interferons. This pathway is engaged by

TLR-3 and TLR-4, which also signals through the MyD88-dependent pathway. The IFNs often act autocrinely and induce a second wave of cytokines see review by (Pandey and Agrawal, 2006).

Other functions of TLR include skewing of hematopoiesis towards innate immune differentiation (Nagai et al., 2006). Additionally, mesenchymal stem cells express various TLR and their proliferation and differentiation is regulated by TLR agonists (Pevsner-Fischer et al., 2007).

It is important to mention that TLR have endogenous ligands, often released during inflammatory conditions, such HMGB1 (Park et al., 2004)and heat shock proteins (Vabulas et al., 2002). TLR are also thought to be involved in regulating immune responses in EAE (Zekki et al., 2002, Hansen et al., 2006) and are widely expressed in the CNS during this disease condition.

AIMS

The addition of NSC to the field of neuroinflammation is relatively new. The stem cell field has focused on the identification and characterisation of these cells during normal conditions often in pre-existing neurogenic niches. The aim of this thesis is to elucidate the function and behaviour of NSC in inflammatory conditions and in the new-formed "niches" of inflammatory lesions.

RESULTS AND DISCUSSION

PAPER I

In a previous publication from our group it was demonstrated that NSC from the spinal cord migrated towards demyelinated lesions where they differentiated into oligodendrocytes. Encouraged by these results, we addressed whether these cells have the ability to generate neurons. Rats received intracranial injections with DiI in order to label the NSC-containing ependymal layer lining the ventricles and spinal cord canal. After a period of recovery the animals were immunised for EAE and after onset of disease BrdU was administered via subcutaneous injections. Using histochemical analysis we could demonstrate that DiI labeled cells migrated out from the central canal of the spinal cord into demyelinated lesions where they expressed neuronal markers such as βIIItubulin and NeuN (Geisert and Frankfurter, 1989, Mullen et al., 1992). Double labeling for proliferation markers such as BrdU or proliferating cell nuclear antigen (PCNA) (Daidoji et al., 1992, Rankin et al., 2004) suggested that these neurons were newly formed. Moreover, we could demonstrate that these cells had the ability to generate overshooting action potentials (AP) characteristic of immature neurons, *see figure 2*.

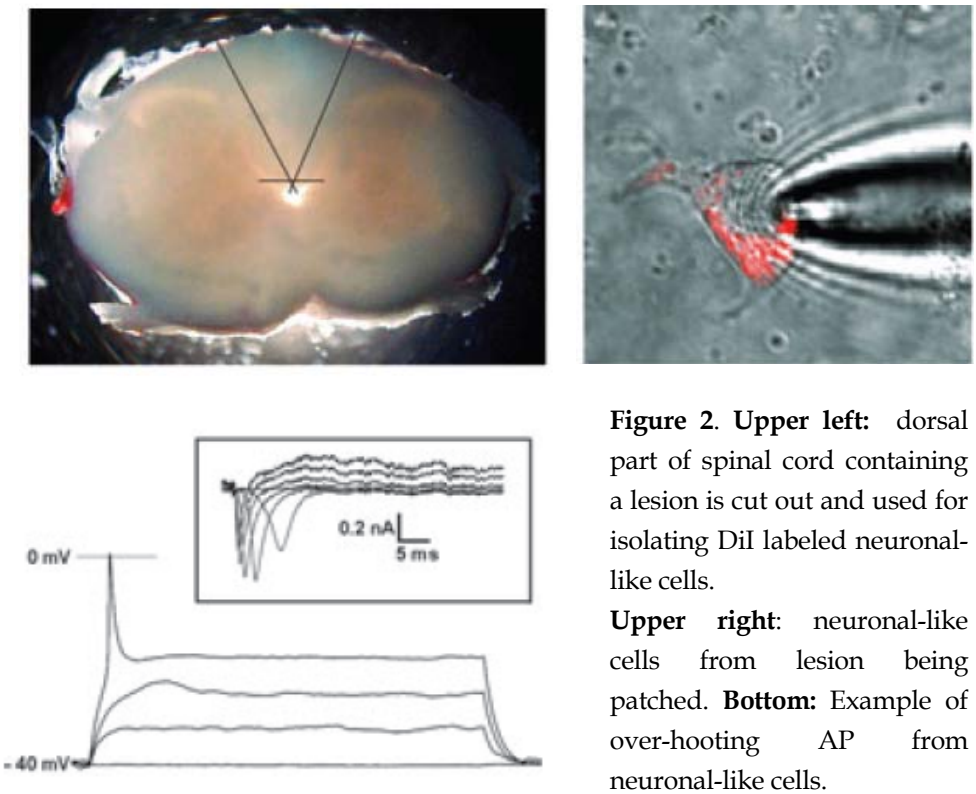

Figure 2. **Upper left:** dorsal part of spinal cord containing a lesion is cut out and used for isolating DiI labeled neuronal-like cells.
Upper right: neuronal-like cells from lesion being patched. **Bottom:** Example of over-hooting AP from neuronal-like cells.

The hallmark of a newly born neuron is that it has recently divided and expresses proteins specific for neuronal cells. To monitor the proliferating NSC *in vivo* the rats were injected once daily with BrdU. The concentration used was fairly low (Kuhn and Cooper-Kuhn, 2007) in order to avoid unwanted BrdU side-effects including cytotoxicity. BrdU labelling can also occur as a consequence of DNA repair (Rakic, 2002, Kuhn and Cooper-Kuhn, 2007). However, the low BrdU concentration further diminished the risk of detecting cells undergoing DNA repair since the extent of nucleotide incorporation is usually much lower than that during cell proliferation. Moreover, the cells were also positively labelled for PCNA, further confirming that proliferation was the underlying mechanism for BrdU incorporation. The newborn neurons were positive for βIIItubulin and NeuN, antigens expressed in immature and mature neurons, respectively. These are post-proliferative cells, i.e. the BrdU incorporation must have happened at an earlier developmental stage. Indeed, the DiI labelling of these neurons confirmed their spinal cord stem cell origin.

The spinal cord white matter is comprised of myelinated nerve cell tracts. Prior to our study, one sole report from Abbadie and colleagues (Abbadie et al., 1999) demonstrated the existence of neuronal somas in the dorsal part of the uninjured spinal cord white matter. While these neurons were oriented along the nerve tracts in a saggital manner, the newborn neurons we identified were detected in cross-section.

The neurogenic non-permissiveness of the spinal cord is well documented and axonal injuries are not regenerated. Under normal conditions the spinal cord seems to support the survival and maturation of fate-committed neuronal progenitor cells but this permissiveness is drastically lowered upon contusion injury (Cao et al., 2002). Moreover, spinal cord engrafted NSC are committed to a gliogenic fate (Cao et al., 2001), further proving the non-neurogenicity of this region. Adult NSC have been isolated from the spinal cord and their multipotency has been proven both *in vitro* (Weiss et al., 1996, Represa et al., 2001) and *in vivo* when transplanted int different hippocampal areas (Shihabuddin et al., 2000). The non-neurogenic milieu is thus provided by the spinal cord itself. This milieu is comprised of different repulsive signals inhibiting neuronal outgrowth such as nogo proteins (Chen et al., 2000) and other myelin peptides (DeBellard et al., 1996) that are released as a result of injury. In this regard it is interesting that we determined a negative correlation between the number of newly formed neurons and the extent of demyelination in the lesions. However, less demyelination could actually reflect a lower inflammatory profile of the lesion, which in turn might benefit neurogenesis. There are studies reporting both detrimental and beneficial effects of inflammation on neurogenesis (Ekdahl et al., 2003, Monje et al., 2003, Ke et al., 2006), the lesion characteristics being the determining factor.

The way inflammation affects the NSC niche can be exemplified by Notch. During normal conditions, Notch signalling is crucial for maintaining the stem cell pool in the spinal cord (Akai et al., 2005), but after injury it is upregulated and involved in neurogenesis blockade (Yamamoto et al., 2001). The same event is reported for BMPs (Ara et al., 2007, Fuller et al., 2007). Glial scar formation is a typical consequence of neuroinflammation that inhibits neuronal regeneration. It is composed of a packed layer of cells including astrocytes, microglial and oligodendrocyte progenitor cells (Fawcett and Asher, 1999) and provides a mechanical barrier between damaged and undamaged tissue. Although the glial scar is able to secrete neurotrophic factors such as FGF and NGF (Ridet et al., 1997) it impedes neuronal outgrowth just by physically being in the way. Astrocytes *per se* do not inhibit neurogenesis since astrocytes from neurogenic niches have been demonstrated to support neurogenic differentiation of NSC (Song et al., 2002) and astrocyte-derived cytokines such as IL-6 and IL-1β have proven neurogenicity (Barkho et al., 2006).

In the lesions analysed in our studies there is a profound infiltration of immune cells, among them macrophages. Microglia /macrophages can have beneficial effects by clearing myelin debris and promoting a permissive area for neuronal growth. Moreover, they can support neurogenesis (Aarum et al., 2003) and during EAE they release bFGF (Liu et al., 1998, Gehrmann et al., 1996), thus providing proliferative cues for spinal cord derived NSC (Shihabuddin et al., 1997). Besides microglia, T-cells have also been demonstrated to release neurotrophic factors during EAE (Hammarberg et al., 2000, Muhallab et al., 2002). Finally, two recent studies reported that new neurons were formed in either intact or injured spinal cord supported by T-cells (Shechter et al., 2007, Ziv et al., 2006a). Additionally, Ke Y and colleagues observed increased migration, proliferation and differentiation of NSC following acute spinal cord injury (Ke et al., 2006).

The final functional proof of a neuronal phenotype is its capacity of generating action potentials. Since terminal differentiation does not always result in functional maturity (Westerlund et al., 2003) we investigated whether the newborn neurons possessed the ability of firing action potentials. Patch-clamp recordings from lesion-isolated neuronal-like DiI labelled cells were performed. In order to avoid contamination with "old" neurons, we dissected away the grey matter and isolated the area of demyelination. The stringent requirements for the definition of a newly formed neuron posed methodological difficulties and limited the number of recordings. The difficulties resided in finding lesions containing DiI labelled cells with neuronal morphology which was still viable after the isolation procedure and which still retained a patchable membrane. In spite of these limitations we observed cells that had tetrodotoxin (TTX) –sensitive voltage-gated sodium

channels and that could fire overshooting action potentials. Similar immature action potentials had been reported by others from SVZ- and SGZ-derived progenitor cells (Liu et al., 1999).

In conclusion, NSC can differentiate into neuronal-like cells in the demyelinated spinal cord of the adult rat. The conditions favouring neurogenesis in this type of lesion have to be dissected out in order to allow for a more detailed study of this phenomenon and ultimately to investigate whether these neurons are incorporated into the pre-existing neuronal circuits.

PAPER II

The rationale for this study also originates from a previous report from our group in which we demonstrated that increased levels of NO derivatives in the CSF of MS patients correlated with disease exacerbation (Brundin et al., 1999, Svenningsson et al., 1999). Among other things, a *de novo* synthesis of NO could account for the increased levels of nitrite in the CSF (Danilov et al., 2003). Since MS lesions have been shown to contain NO producing cells (Bo et al., 1994) and that lesions are often found close to or within the SVZ (Charcot, 1868), we decided to study the effects of NO on NSC differentiation.

NSC were isolated and grown from the lateral ventricles of DA rats. The cells were then exposed to different concentrations of DETA-NONO:ate, an NO-donor. The effects were analysed using immuno-cytochemistry, western blotting and real-time RT-PCR. We demonstrate that when exposed to pathological levels of NO, NSC differentiated into astrocytes and oligodendrocytes but generated significantly fewer neurons. Moreover, NO down-regulated mRNA expression of the neuronal genes *Neurogenin-2* (*Ngn-2*) and *βIII-tubulin,* but not of the astrocytic gene *Gfap*. Astrogliogenesis, detected by western blotting for GFAP, also increased following NO exposure. This effect was mediated through down-regulation of *Ngn-2* with subsequent activation of the JAK-STAT pathway, as assessed by western blotting for phosphorylated STAT-1.

Why DETA-NONO:ate?

There are different commercially available NO-donors with half-lives ranging from hours to a few minutes. We chose DETA-NONOate which has a half-life of approximately 22 hours, thus facilitating a more stable NO exposure, which mirrors the actual *in vivo* situation. The decomposition of this donor is dependent on temperature and pH, but the release of NO occurs spontaneously at normal pH values and physiological temperature (Keefer et al., 1996). To determine the exact NO concentrations released in our particular experimental set-ups we used a Clark-type electrode. Since the NO levels

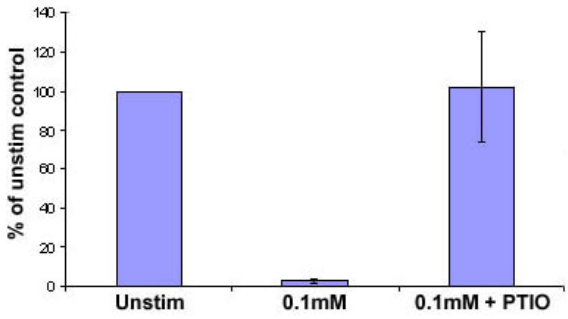

Figure 3. Lower percentage of neurons in cultures exposed to 0.1mM of DETA-NONO:ate. PTIO, an NO scavenger abrogates this effect.

in the brain during normal conditions range between 10-100nM (Shibuki, 1990) everything above this level was considered to be pathophysiological. PTIO, an NO scavenger, was used to prove that the effects detected were NO specific and not due to side-effects of the carrier compound, *see figure 3.*

Role of NO: depleting or directing?

There are two possible explanations for the decreased percentage of neurons in the NO exposed cultures. NO can either deplete a population of previously committed neuronal progenitors or alternatively direct the NSC fate from neurogenesis towards gliogenesis. In comparison to astrocytes, neurons display much higher sensitivity to NO-induced apoptosis (Almeida et al., 2001, Almeida et al., 2004). However, this has been demonstrated in terminally

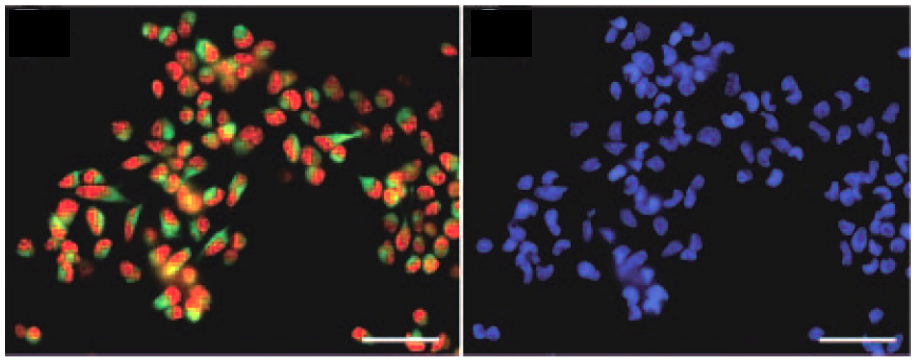

Figure 4 depicts NSC immuno-labeled for, to the left, Sox-2 (red) and nestin (green). To the right, DAPI counter-stained nuclei (blue).

differentiated mature neurons and not in progenitor cells that might be present in our cultures. Moreover, thanks to their ability to produce neurotrophic factors, neural progenitor cells are more protected than mature neurons from NO-induced cytotoxicity (Hsieh et al., 2003). Nevertheless, we decided to investigate the immaturity of the cultures both prior to and post-NO exposure. Since there are no NSC-specific determinants we analysed the combined expression of the proteins sox-2 and nestin which have both been reported to be expressed in NSC (Johansson et al., 1999a, Komitova and Eriksson, 2004). After performing both immunocytochemistry and flow cytometry we determined that 99% of the cells expressed both nestin and sox-2., *see Figure 4.* Moreover, the results from the flow cytometry analyses revealed a small decrease in the nestin/sox-2 double positive population following exposure to NO. A corresponding increase was evident in the sox-2 population, suggesting that these cells were still in an immature state, *see Figure 5.*

Another finding supporting the "diversion-of-fate" hypothesis is the compensatory increase of the percentage of glial cells in the NO-exposed cultures. Both astrocytes and oligodendrocytes were more abundant in

exposed cultures, in which we also detected increased activation levels of STAT-1. As discussed in the *Introduction*, STATs are involved in promoting astroglial fate where both Notch (Ge et al., 2002, Kamakura et al., 2004), LIF (Bonni et al., 1997, Rajan and McKay, 1998) and BMP (Gross et al., 1996, Rajan et al., 2003) engage the JAK/STAT signalling pathway. Sufficient JAK /STAT activation promotes the switch from neurogenic to gliogenic periods during development (He et al., 2005). Through blocking or activating the JAK/STAT pathway, He F and colleagues could either inhibit or promote astrogliogenesis (He et al., 2005). Neurogenic bHLH transcription factors suppress astrogliogenesis either by sequestering the CBP/p300 complex or by blocking the STAT transcription activation (Sun et al., 2001). Interestingly, the expression of Ngn-2 in our experiments was downregulated subsequently to NO exposure which explains the upregulation of phosphorylated STAT-1. So what we actually observed was a mimicked image of the sequential events during development, triggered in our experimental set-up by the action of NO.

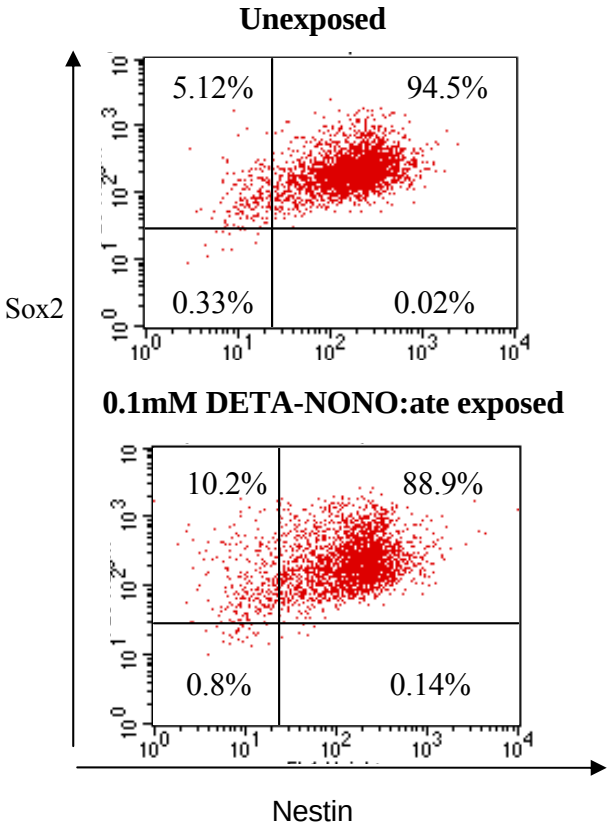

Figure 5. Flow cytometry analysis showing the distribution of sox2^{pos} and nestin^{pos} NSC populations before (upper chart) and after (lower chart) NO exposure.

The dichotomy of NO-mediated effects is even evident in the context of neurogenesis. Some groups have demonstrated pro-neurogenic effects of DETA-NONOate (Lu et al., 2003a) while others observed an increase of neurogenesis as a result of blocking NOS activation (Romero-Grimaldi et al., 2006, Fritzen et al., 2007). We also observed a tendency of increased neurogenesis at levels lower than 500nM of NO (0.1mM of DETA-NONOate), i.e. at NO levels found during normal conditions.

This dichotomy can partly be explained by activation of various signal transduction pathways at different NO concentrations (Thomas et al., 2004). For example, adenylate cyclase and cGMP signalling are engaged at NO levels below 50nM, while hypoxia inducible facor (HIF)1 and p53 activation are evident at higher (over 100nM) NO levels. We could determine that the blockade of neurogenesis was not mediated via guanylate cyclase activation since administration of ODQ (a specific blocker of guanylate cyclase) did not antagonise the NO effect.

Another possible way of action of NO is through its regulation of the intracellular redox milieu. The redox state of a cell influences its choice between proliferation and differentiation, demonstrated in an OPC culture model (Smith et al., 2000). Moreover, NO regulates the activation of redox sensitive transcription factors including NF-kB, AP-1, oct-1 and Zn-finger containing factors. It also modulates the stability of HIF and p53 and acts epigenetically by altering methylation or histonedeacetylation (Kroncke, 2003). Interestingly the NO-sensitive HIF and p53 converge at the CBP/p300 level review by (Zhou and Brune, 2005) linking NO signalling to the regulation of NSC fate.

Finally, Notch receptor expression was mildly downregulated in NO-exposed cultures, while its downstream mediator, hes was not affected. Interestingly, apurinic endonuclease 1/redox factor-1 (Ape1/Ref-1), a bHLH transcription factor has been recently proven in nematode to act downstream from Notch (Neves and Priess, 2005). Ref-1 works as a redox transcription factor maintaining other transcription factors in active state. Among these factors are NF-κB, HIF and p53 reviewed by (Fishel and Kelley, 2007). Moreover, in the nematode, ref-1 governs the delineation of neuronal subtypes (Lanjuin et al., 2006). It will be interesting to see if ref-1 plays any specific role in mammal neurogenesis and how it is influenced by NO present in the neurogenic niches.

Obviously there are many possible candidates for mediating NO-induced down-regulation of neurogenesis and more study of this issue is underway in our group.

PAPER III

Since blockage of RAGE ameliorates EAE (Yan et al., 2003), we considered it relevant to investigate HMGB1 expression in MS. Immunohistochemical analysis of autopsy sections of brains from MS patients revealed cytoplasmic HMGB1 in microglia and macrophages located within active lesions. Extranuclear localisation of HMGB1 has been associated with its active secretion (Bonaldi et al., 2003) but it is not exclusive evidence for its extracellular release. Using western blotting we detected much higher levels of HMGB1 in the CSF of MS patients compared to patients with non-inflammatory conditions (NIC). We then compared the mRNA expression of HMGB1 and its receptors RAGE, TLR-2 and TLR-4 in mononuclear cells from CSF (MC-CSF) and blood (PBMC) from MS patients and NIC. We determined a significant increase of the expression of both HMGB1 and its receptors in MC-CSF from MS patients compared to in NIC. In contrast, in PBMC we only detected a significant upregulation of RAGE and TLR-2. One explanation for this difference might be that the CSF cells are closer to the inflammatory foci and are subsequently more activated. Another explanation could be different cellular compositions in the CSF and blood which will thus respond differently to inflammation in regards to expression of HMGB1 and its receptors. For example, macrophages/monocytes are more easily induced to express TLR-2 than TLR-4 (Matsuguchi et al., 2000). Moreover, TNFα, a pivotal cytokine in MS and EAE has been demonstrated to downregulate TLR-4 expression in monocytes (Tamandl et al., 2003). In addition, the pool of inflammatory factors probably differs between the CSF and the periphery, which in turn will lead to different results.

Next we analysed the expression of HMGB1 and its receptors in EAE. By analysing sequential sections with immunocytochemistry and *in situ* hybridisation for HMGB1 we could conclude that an upregulation of HMGB1 mRNA correlated with active inflammation in demyelinated spinal cord lesions. Moreover, ED1 positive cells, i.e. macrophages and microglia, within active lesions exhibited a cytoplasmic localisation of HMGB1. At the time we performed this study microglia were not known to translocate HMGB1. Using microglial cultures we could demonstrate that upon classical stimulation with IFNγ and LPS these cells were able to translocate HMGB1 to the cytoplasm as well as macrophages do.

HMGB1: damage or repair?

The contribution of HMGB1 to inflammation is obvious in sepsis (Wang et al., 1999) and experimental RA (Kokkola et al., 2003) since antibodies against

HMGB1 ameliorate the symptoms of these diseases. Neuroinflammation, however, is a more complicated matter. First, the different compartments, CNS and the periphery, are very different in terms of cellular composition and immunological response. A cytokine that might have adverse effects in the periphery might be protective in the CNS and *vice versa*. The picture is even more complicated when one cytokine has diverse effects in the same cell type. One such example is TNFα, that on the one hand causes oligodendrocyte death (Merrill et al., 1993) and on the other promotes oligodendrocyte maturation (Arnett et al., 2001). This might partly explain why treatment with antibodies directed against TNFα resulted in some cases of exacerbation in MS, excluding its use as a treatment. In contrast, in RA the same treatment is successful. To regard HMGB1 solely as a detrimental cytokine and aiming at blocking it's action in MS would probably thus be a mistake.

As mentioned in the *Introduction*, HMGB1 is a pro-inflammatory cytokine but it can also support neuronal outgrowth, this mainly being mediated through RAGE. HMGB1 also binds to TLR-2 and TLR-4 and associates with various matrix molecules such as fibronectin, chondroitin sulfate and heparin sulfate. Both chondroitin sulfate and heparin sulfate constitute the side chain molecules on different proteoglycans. Proteoglycans with heparin sulfate side chains are called *syndecans*. Syncecans are expressed in the brain both in the adult and during development and are involved in synapse formation, see review (Rapraeger, 2001), and together with pleiotrophin and HMGB1 in neurite outgrowth (Rauvala and Pihlaskari, 1987, Merenmies et al., 1991, Salmivirta et al., 1992). Moreover, syndecans are expressed on the surface of NSC (Nagato et al., 2005). Via their heparin sulfate side chains syndecans are also important in potentiating FGF-2 signaling (Rapraeger et al., 1991) and heparin is added to NSC cultures to prevent FGF-2 degradation (Caldwell et al., 2004). Both FGF-2, pleiotrophin and HMGB1 (Milev et al., 1998) bind to another proteoglycan, *neurocan*, that is a constituent of the extracellular matrix in the CNS. Neurocan expression is most evident during developmental stages in the CNS, but again is expressed in response to injury (McKeon et al., 1999, Asher et al., 2000) and see review by (Rauch et al., 2001).

Thus the ability of HMGB1 to bind to proteoglycans brings it close to factors involved in NSC proliferation (FGF-2) and differentiation (pleiotrophin). Pleiotrophin is produced in NSC cultures (Furuta et al., 2004) and it inhibits FGF-2 signaling, thereby promoting NSC differentiation (Hienola et al., 2004). Additionally, it is involved in neuronal extension in hippocampal neurons (Raulo et al., 2005) and promotes differentiation of embryonic stem cells into dopaminergic neurons (Jung et al., 2004).

In conclusion, we reported that HMGB1 and its receptors were expressed in MS both in the periphery and in the CNS and that their

expression correlated with active inflammation. These results were also confirmed in EAE.

In light of these facts, the next obvious question was how HMGB1 affects NSC function.

INTERMEZZO

NSC cultures were thus exposed to various concentrations of recombinant HMGB1 protein ranging from 0.01μg/ml to 10μg/ml and their proliferation, survival and differentiation was analysed. The proliferation of NSC slightly decreased after addition of HMGB1. Since this might be an indication of cytotoxicity we determined the level of cell death using TUNEL staining. We observed that HMGB1 did not induce remarkable levels of apoptosis, with the exception of the highest HMGB1 concentration used, see *Figure 6*.

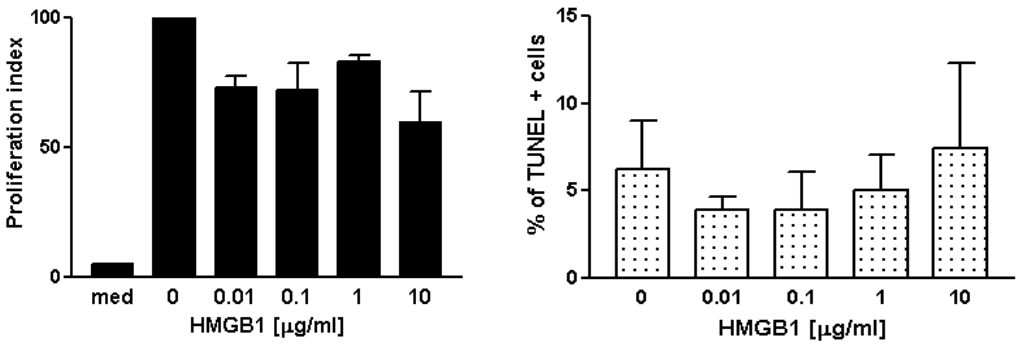

Figure 6. The graph to the left shows the proliferation index of cultures exposed to different concentrations of HMGB1. The graph to the right shows the corresponding levels of TUNEL positive cells in the same cultures. All cultures were grown with EGF except the medium control. Med=medium.

Differentiation is often preceded by cell cycle exit, resulting in lower levels of proliferation. To investigate whether decreased proliferation was an indication of differentiation, NSC were exposed to HMGB1 and the mRNA levels of βIIItubulin (a neuronal cytoskeleton protein) and GFAP (expressed in astrocytes) were measured using real-time RT-PCR. The transcription levels of these genes were not affected by HMGB1. Moreover, at the protein level we could sporadically detect an increase of βIIItubulin, but this outcome varied between experiments and different protein preparations.

In our experimental set-up HMGB1 seemed not to effect NSC proliferation or differentiation. One explanation for these results could be a lack of HMGB1 receptors or ineffective HMGB1 receptor signalling in NSC. We thus determined to examine the expression of RAGE and TLR-2 and -4 and their regulation in NSC, which resulted in paper IV.

PAPER IV

The main findings of this study reveal that NSC express TLR-2 and TLR-4 and that the level of thieir expression is modulated by inflammatory conditions. The reason for choosing to investigate these two receptors in particular has its grounds in our study with HMGB1, a ligand for both TLR-2 and TLR-4 (see *"Intermezzo"*).

Immunocytochemical analysis of NSC cultures revealed that a majority of the cells were positive for TLR-2, TLR-4 and Sox-2 antigens. This immunolabeling was much more intense for TLR4 than for TLR2, which was confirmed by flow cytometry analysis. The weaker TLR-2 immunoreactivity could reflect a lower level of expression, which coincides with studies of immune cells (Matsuguchi et al., 2000).

We next examined the involvement of the two TLR in NSC differentiation and proliferation. We separately exposed NSC cultures to the TLR-4 agonist LPS and to the TLR-2 agonists pam3cys and lipoteichoic acid (LTA). HMGB1 was also used and represented an endogenous ligand for these receptors. We could record neither an effect on the proliferation or differentiation of NSC. The differentiation was only investigated by analysing the transcriptional levels of neuronal- and astrocytic-specific genes and in order to draw a definitive conclusion protein levels have to be monitored. In relation to this, a recent publication by Rolls and colleagues (Rolls A 2007) reported a neurogenic potential for TLR-2 while TLR-4 seemed to provide inhibitory signals both for proliferation and neurogenesis. Besides the differences in methodology, the discrepancies between our results can potentially reflect variations between species. Indeed, with regard to neurogenesis, Kempermann and colleagues had previously described variations of NSC function in different mouse strains. (Kempermann et al., 1997a). In addition, Rolls and colleagues also detected the expression of these TLR *in vivo*, excluding the possibility of an *in vitro* artefact and thereby supporting our observations.

There are other indications pointing to the involvement of TLR in stem cell biology. In *Drosophila* embryogenesis the Toll signalling pathway indirectly interacts with a BMP-4 ortholog, the decapentaplegic (Araujo and Bier 2000). Recent evidence from mammalian studies reveal a direct interaction between BMP and TLR signalling pathways via an inter-linking factor called ecsit (Xiao 2003). Knockdown of ecsit leads to dysfunctional signalling of both Toll and BMP. Whether this interaction has a functional role in adult NSC differentiation and proliferation remains to be seen. It will also be interesting to understand exactly how TLR regulate mammalian neurogenesis and if this process remains unchanged during neuroinflammatory conditions.

Since the frame of our studies is neuroinflammation, we next decided to determine if and how inflammatory factors influence the expression of the TLR on NSC. Both inflammatory cerebrospinal fluid (CSF) from MS patients and supernatants from activated macrophages induced differential expression of TLR-2 and TLR-4. Since TNFα and IFNγ are pivotal cytokines in driving neuroinflammatory reactions we focused on the specific effects of these cytokines on TLR expression. Both IFNγ and TNFα induced the expression of TLR-2, while TLR-4 expression was only induced by IFNγ. The positive effect of IFNγ on TLR-4 expression was overcome by TNFα. To explain these observations we looked into studies performed on immune cells and the phenomenon referred to as endotoxin tolerance. Endotoxin tolerance provides a means of limiting immune responses. This involves blockage of TLR-4 signalling when cells previously stimulated with LPS are re-challenged with LPS again. TNFα and IL-1, which constitute the first wave of cytokines released upon TLR-4 signalling, are thought to mediate this endotoxin irresponsiveness (Li et al., 1994, Rieser et al., 1998). In addition to this negative effect on TLR-4, these cytokines also upregulate the expression of TLR-2, providing the cells with a wider range of pathogen recognition and higher combatant ability.

The TNFα-mediated dampening of TLR-4 might also have another explanation. As mentioned in the *Introduction*, TNFα induces NSC proliferation (Widera et al., 2006) which in turn seems to be controlled by TLR-4 signalling (Rolls et al., 2007) Hypothetically, TNFα might down-regulate the further upregulation of TLR-4 expression in order to enable its proliferative effects on NSC.

One important point to be discussed here is the potential contamination with microglia, which are known to express TLR, see review by (Carpentier et al., 2007). Microglia are also constituents of both SVZ and SGZ niches and might survive the culture procedures leading to contamination of our results. Indeed, we identified a population of CD11b expressing cells constituting below 1% of the stem cell culture. To exclude that the TLR were of microglial origin, we re-confirmed the expression and cytokine regulation of TLR in a sorted NSC culture devoid of $CD11b^+$ cells. It is inevitable not to ponder on the origin of these CD11b expressing cells. In order to confirm their microglial origin positive CD11b staining is insufficient. Since NSC have the ability to express so many immune-related surface molecules (Imitola et al., 2004a, Pluchino et al., 2005), it might be possible that the $CD11b^+$ cells are actually NSC. During the cell sorting procedure we collected the $CD11b^+$ population for further investigations; however, the number of cells was too low to allow any reliable experimentation. Using several microglial markers such as *Griffonia simplicifolia* B4 isolectin, F4/80, and Mac-1, Papavasiliou and colleagues have revealed the presence of microglia cells in embryonic NSC cultures (Papavasiliou et al., 1996). Interestingly, these cells could be derived

from a single neurosphere but whether the microglia actually originated form the NSC remains elusive. Others reports revealed the incapacity of NSC to generate microglia (Levison et al., 2003), while again there is one study suggesting that microglia might be a potential source of neurons, oligodendrocytes and astrocytes (Yokoyama et al., 2004). How unlikely that might sound, it is still important to characterise the origin and function of these CD11b expressing cells and also the microglia cells in the neurogenic niches.

Finally, in response to TLR-specific agonists NSC were able to express cytokine mRNAs including IL-1 and TNFα. IFNβ transcripts were not detected which might imply that the MyDD88-independent pathway was not activated. Moreover, we could also measure the levels of TNFα protein in the cell culture supernatants, confirming that transcription had lead to translation and release of protein. The ability of NSC to produce cytokines has also been reported by others (Klassen et al., 2003).

The next step in this study would be to investigate the *in vivo* production of NSC-derived TNFα. The ability of NSC to produce pro-inflamamtory cytokines can have unexpected consequences for the local immune response. As mentioned earlier TNFα has very diverse effects on NSC and on other CNS cells and it is difficult to predict the net effect of this cytokine *in vivo*. In a worse-case scenario, the NSC pool could be seriously damaged, and duly also the reparative capacity of these cells. It is also possible that the "inflamed environement" primes the stem cell towards an immune cell-like fate. In the best-case scenario TNFα and other cytokines might be important in rendering the NSC more responsive to inflammatory conditions. Finally, the role of TLR in regulating NSC homeostasis in normal conditions might change during inflammation. Sustained and increased TLR-4 transcripts have been detected in inflamed CNS in areas including the vasculature, parenchymal microglia and most importantly the ventricular ependyma (Chakravarty and Herkenham, 2005, Zekki et al., 2002), directly implicating the NSC in the inflammatory response evident during these conditions.

CONCLUDING REMARKS

My PhD project was initially entitled "Studies of regenerative processes in neuroinflammatory disorders". The term "regenerative processes" was referring in part to neural stem cells which had the generally accepted or expected role of repair and regeneration. In light of the latest findings described in paper IV, we decided to change the title to a more "plastic" one, i.e. "Adult neural stem cells in neuroinflammation".

In the first two papers we studied the NSC from a more "traditional" perspective, i.e. differentiation potential and regenerative ability. The questions we asked were:

I. Are NSC able to generate neurons in a classical non-neurogenic area such as the spinal cord? At that time, most of the researchers would probably just categorically say "no!" This is not surprising considering the vast pieces of evidence with regard to the non-neurogenicity and non-accessible milieu of the spinal cord. A damaged spinal cord is irreparable and unfortunately that is still the case today. However, there is a difference between trying to cure a damaged spinal cord and just exploring the possibility of neuronal differentiation. We had in a previous study seen that NSC proliferate, migrate to the lesion site and differentiate into oligodendrocytes and in the current study we could observe the formation of cells with neuronal-like features. Thus some cues in the injured spinal cord can support neurogenesis and functional maturation of neurons. To date, other studies have confirmed our observations (Ke et al., 2006, Ziv et al., 2006a), i.e. formation of new neurons in the inflamed spinal cord.

II. What is the effect of pathological NO levels on NSC in an inflamed CNS? In normal conditions NO is produced in small doses throughout the CNS but as discussed in the "*Results and Discussion*", different levels of NO can yield different outcomes. We even observed this phenomenon in our own cultures. When exposing NSC to lower levels of the NO-donor we observed a tendency of increase in percentage of neurons. However, at pathological concentrations, i.e. above 500nM NO, the neurogenesis is impaired followed by a compensative increase in gliogenesis, i.e. formation of both astrocytes and oligodendrocytes. In the context of multiple sclerosis, formation of new oligodendrocytes stimulates the thought of remyelination; however, oligodendrocytes might potentially also contribute to the formation of the glial scar.

So how do these two findings fit together? NO blocks neurogenesis and is present in the inflammatory lesions where we detect neurogenesis. One thing to consider here is the temporal aspect. Fulminant NO production might not coincide with formation of new neurons. Alternatively, because of its reactivity NO might be sequestered by surrounding tissue to the extent of forming the right ambiance for neurogenesis. To study the role of NO in the lesion neurogenesis, my colleague (Alexandre Danilov) performed NO blockage studies in EAE (Danilov et al., 2005). The much lower extent of inflammation in animals treated with a NOS inhibitor resulted in almost lack of lesions and provided a poor material for elaborating the effect of NO in lesions. Further studies are needed to establish the function of NO as a fate determinant during inflammation *in vivo*.

The last two studies focus on the function of the cytokine HMGB1 and its receptors, RAGE, TLR-2 and TLR-4 in MS, EAE and NSC.

We asked:

III. What is the expression of HMGB1 in EAE and MS? We could demonstrate that increased HMGB1 expression correlated with active disease. HMGB1 was present at higher levels in the CSF from MS patients and was more often found in the cytoplasm of immune cells in active lesions than in inactive ones. Even if HMGB1 has been proven to induce production of pro-inflammatory cytokines, its function in axonal growth cannot be omitted. However, we did not detect any effect of HMGB1 on NSC proliferation and differentiation. Since this protein binds to three different receptors different effects might be mediated through the different receptors.

IV. Do NSC express TLR and what is their function in these cells? We determined that NSC express TLR-2 and -4 and that their activation leads to cytokine production from these cells. HMGB1 could also induce cytokine production but further experimentation must be carried out to establish which receptor is responsible for this effect. - expression is further enhanced during inflammation and could thus overcome signalling mediated via RAGE.

In conclusion, we demonstrated that inflammatory conditions can both promote and inhibit the ectodermal differentiation capacity of NSC, but also to yield them unexpected immune features. The plasticity of the NSC has much more significance than I previously believed. I started out by following the mainstream of NSC research and now I am exploring the immunological features of NSC. Since immunology was the major subject of my Masters project I could almost entitle this thesis: "Making an illicit U-turn, going back to immunology."

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