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BRAIN REPAIR AFTER IRRADIATION OR ISCHEMIA: ROLE OF NEURAL STEM CELLS AND MICROGLIA

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The cover image shows neural stem cells (green) migrating across microglia (red) in culture. Image taken by Ahmed Osman

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BRAIN REPAIR AFTER IRRADIATION OR ISCHEMIA: ROLE OF NEURAL STEM CELLS AND MICROGLIA

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To

my

Parents

Brother and Sisters

Wife and Kids

Science demands a lot of time; therefore a researcher needs to devote or to deport.

Ahmed Osman

ABSTRACT

Brain injury has devastating consequences for the affected individual, and causes a burden for society. This is largely due to the fact that the mammalian brain has a limited capacity to regenerate. Recent discoveries in neurobiology revealed that the mammalian brain, including humans, is harboring neural stem cells (NSCs) that daily generate new neurons in the hippocampus and the olfactory bulb that contribute to maintain learning and memory throughout life, a process known as adult neurogenesis. When the brain is exposed to an injury or damage, these NSCs migrate to the damaged area, raising the possibilities of spontaneous recovery. Therefore, in this thesis we aimed to study the role of NSCs in brain repair, particularly after injuries caused by irradiation and ischemia. As the brain injury is often associated with inflammation, we also wanted to study the impact of microglia, the resident immune cells in the brain, on NSC biology.

Irradiation is used as an efficient tool to treat primary brain tumors and metastases. However, it leads to long-lasting cognitive decline in the cancer survivors. As a possible mechanism, depletion of hippocampal neurogenesis by irradiation has been proposed as a cause for the cognitive decline. As a restorative intervention, transplanted brain-derived NSCs into the irradiated brain have shown to generate neurons and astrocytes, and subsequently improve cognitive performance. However, such an approach might demand a clinically relevant source of cells, preferentially taken from the same patient. We therefore proposed the enteric neural stem/progenitor cells (ENSPCs), which are present in the enteric nervous system, as a source for cells that could be utilized to treat central nervous system (CNS) pathologies. Here, we transplanted the ENSPCs into the hippocampus of young irradiated mice. Our results displayed that ENSPCs showed poor survival in the brain, remained undifferentiated, triggered neuro-inflammation, and did not restore irradiation-induced loss of hippocampal neurogenesis.

Brain ischemia is caused by insufficient blood flow that leads to inadequate oxygen and nutrient supply, eventually resulting in neuronal death. After ischemic damage, NSCs increase in numbers and migrate toward the lesioned area, however whether they replace lost neurons is still controversial,

especially in the cortex. Here our aim was two-fold: First, we wanted to look at the temporal and spatial response of migrating neural progenitors when the brain undergoes cortical stroke; and second to assess the cortical neurogenesis after ischemia. To promote the survival of the neural progenitors in the injury site, and subsequently cortical neurogenesis, we interrupted the caspase–mediated cell death with a pan-caspase inhibitor. We induced cortical ischemia using the photothrombotic stroke model, and found that neural progenitors migrate to the injured cortex for at least one year after the onset of the lesion. Neural progenitors were migrating along the corpus callosum fiber tract to reach the damaged cortex. We also observed that cortical neurogenesis was very rare, and caspase inhibition, contrary to our expectations, did not enhance this process. Interestingly, we found that caspase inhibition even diminished the ischemia-induced NSC response to stroke, and that appeared to be associated with a reduced pro-inflammatory profile, but not anti-inflammatory profile. Hence, caspase inhibition warrants caution when intended for neuroprotection after CNS injury.

After brain injury, microglia become activated and they are abundant in the injury site. Depending on the activation signals, microglia can become either pro-inflammatory (neurotoxic, M1 phenotype) or anti-inflammatory (neuroprotective, M2). We therefore wanted to investigate the impact of either microglial phenotype on NSC survival, proliferation, migration, and differentiation. Our results revealed that factors associated with the pro-inflammatory phenotype were cytotoxic and triggered astrocytogenesis, while factors released associated with the anti-inflammatory phenotype promoted NSC migration.

LIST OF SCIENTIFIC PAPERS

I. **Osman A. M.**, Zhou K., Zhu C., Blomgren K. Transplantation of enteric neural stem/progenitor cells into the irradiated young mouse hippocampus. 2014. *Cell Transplantation* **23**:1657-1671.

II. **Osman, A.M.**, Porritt M.J., Nilsson M., Kuhn H.G. 2011. Long-term stimulation of neural progenitor cell migration after cortical ischemia in mice. *Stroke* **42**:3559-3565.

III. **Osman, A.M.**, Neumann S., Kuhn H.G., and Blomgren K. Caspase inhibition impaired the neural stem/progenitor cell response after cortical ischemia in mice. (Manuscript).

IV. **Osman, A.M.**, Rodhe J., Shen X., Joseph B., Blomgren K. The anti-inflammatory microglial secreteome promoted neural stem cell survival and migration, but reduced astrocytogenesis. (Manuscript).

Additional papers not included in the thesis:

I. Persson Å.*, **Osman A.M.***, Bolouri H., Mallard C., Kuhn H.G. 2013. Radixin expression in microglia after cortical stroke lesion. *Glia* **61**:790-799 (*equal contribution).

II. Lindwall C., Olsson M., **Osman A.M.**, Kuhn H.G., Curtis M.A. 2013. Selective expression of hyaluronan and receptor for hyaluronan mediated motility (Rhamm) in the adult mouse subventricular zone and rostral migratory stream and in ischemic cortex. *Brain research* **1503**:62-77.

III. Burguillos M.A., Svensson M., Schulte T., Boza-Serrano A., Garcia-Quintanilla A., Kavanagh E., Santiago M., Viceconte N., Oliva-Martin M.J., **Osman A.M.**, Salomonsson E., Amar L., Persson A., Blomgren K., Achour A., Englund E., Leffler H., Venero J.L., Joseph B., Deierborg T. 2015. Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation. *Cell reports* 10: 1626-1638.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNF	Brain derived neurotophic factor
BLBP	Brain lipid binding protein
BMPs	Bone mphogenic proteins
BrdU	Bromodeoxyuridine
CA3	Cornus ammonis 3
Caspase	Cycteine-appartate protease
CCL2	Chemokine (C-C motif) ligand 2
CD	Cluster of differentiation
ChAT	Choline acetyltransferase
CldU	Chlorodeoxyuridine
CM-DiI	Chloromethyl-Dioctadecyl-tetramethyl indocarbocyanine
CNS	Central nervous system
CSF	Cerebrospinal fluid
DCX	Doublecortin
E	Embryonic day
ENS	Enteric nervous system
ENSPCs	Enteric neural stem/progenitor cells
FGF	Fibroblast growth factor
GABA	γ-aminobutyric acid
GCL	Granule cell layer
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
Iba-1	Ionized calcium binding adaptor molecule 1
ICE	Interleukin 1β converting enzyme

IdU	Iododeoxyuridine
IGF-1	Inulin-like growth factor 1
IL	Interleukin
LPS	Lipopolysaccharide
NGF	Nerve growth factor
NSCs	Neural stem cells
NT-3	Neurotrophin-3
OB	Olfactory bulb
PEDF	Pigment epithelium-derived factor
PRRs	Pattern recognition receptors
PSA-NCAM	Polysialic acid neural cell adhesion molecule
Q-VD-OPh	QuinolineValAsp(Ome)-CH2-O-phenoxy
RGL	Radial glia-like cells
RMS	Rostral migratory stream
ROS	Reactive oxygen species
SDF-1α	Stromal cell-derived factor 1alpha
SGZ	Subgranular zone
SHH	Sonic hedgehog
SOX	Sex determining region Y)-box
SVZ	Subventricular zone
TAPs	Transient amplifying precursors
TGFβ	Transforming growth factor beta
TH	Tyrosine hydroxylase
TLR	Toll- like receptors
V-SVZ	Ventricular-subventircular
VEGF	Vascular endothelial growth factor
Wk	Week
Wnt	Wingless
αSMA	Alpha smooth muscle actin
βIII	Beta III tubulin

1. Introduction

For long, the mammalian brain was considered a static organ incapable of producing new neurons after birth, and that this process was restricted to embryonic development. As a consequence, if brain damage occurred, the neuronal loss will not be replaced. This dogma was strongly supported by Santiago Ramon y Cajal, one of the fathers of the neurobiology, when he scripted "… in adult centres the nerve path is something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for science in the future to change, if possible, this harsh decree" (Cajal and May, 1959), the statement adopted from (Fuchs and Gould, 2000). However, after brain damage occurs, gradual functional and behavioral recovery could be noticed (Benjamin and Thompson, 1959). Accordingly, this partial functional improvement was attributed to occur as a result of dynamic events within the brain, where ongoing neuronal reorganization is happening through reactive synaptogenesis, re-routing of axons, the phenomenon later known as neural plasticity (Finger and Almli, 1985).

In early 1960s, the introduction of labeling DNA precursors, such as with thymidine ^{3}H and the subsequent tracing in radiographs, made it possible to detect the dividing cells (Messier and Leblond, 1960). This thereafter, facilitated breaking the dogma and brought the first evidence of ongoing proliferation and neurogenesis in some areas in adult mammalian brain (Altman, 1962). Some years later, generation of new neurons was reported in species other than rodents, when Nottebohm and coworkers showed that the number of neurons increases in canaries during the singing season (Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984). However, back then neurogenesis was not detectable in primates (Rakic, 1985).

In the early 1990s, isolation and expansion of cells from adult mouse brain, in the presence of mitogen, was reported (Reynolds and Weiss, 1992). These cells were expressing markers of neural epithelium and capable of generating neurons and astrocytes. Since then, and also because of usage of another thymidine analogue, Bromodeoxyuridine (BrdU), that allows birthdating of cells, and the combination with other phenotypic markers, that allows fate mapping of the newborn (Kuhn and Cooper-Kuhn, 2007), extensive research

data has confirmed that life-long sustained adult neurogenesis in the olfactory bulb and the hippocampus from neural stem cells (NSCs) reside in two main regions in the brain, the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) in the dentate gyrus, respectively (Gage, 2000; Gould et al., 1999b; Lois and Alvarez-Buylla, 1994).

In humans, neurogenesis has been proven in the hippocampus, as approximately 1,400 new neurons are added daily to both hippocampi (Eriksson et al., 1998; Spalding et al., 2013). However whether or not life-long addition of neurons to the olfactory bulb exists is still a matter of debate, but nevertheless it occurs during infancy (Curtis et al., 2007; Sanai et al., 2011).

Studies have shown the necessity of postnatal neurogenesis in maintaining learning and memory throughout life in the intact brain (Aimone et al., 2014), and also brought evidence that the brain is holding the capacity of healing itself after injury. When an injury occurs, NSCs expand their pool, differentiate into glial and neuronal progenitors, and subsequently routing the damaged region (Arvidsson et al., 2002; Benner et al., 2013). Moreover, strategies aimed for grafting exogenous cells for the sake of brain repair has also been applied (Boucherie and Hermans, 2009; Trueman et al., 2013).

The purpose of this thesis is to study the role of the NSCs in brain repair after injury caused by irradiation, an effective tool for treating cancer patients, or after cortical ischemia, as the case in stroke patients; either by activation the endogenous pool, or by cellular engraftment.

1.1 Adult neurogenesis

1.1.1 Neural stem cells and the neurogenic niches

Stem cells are those cells that are capable of self-renewing and differentiating into specific cell lineage (s) (Gage, 2000; Weissman et al., 2001). Hence, it has been a fundamental concern whether NSCs do exist in the adult brain or if those cells involved in adult neurogenesis are rather restricted progenitors. Accordingly, NSCs should fulfill the criteria of renewing themselves as well as to be able of producing neural cells, such as neurons, astrocytes, and oligodendrocytes (Temple, 2001). Taking advantage of genetic labeling of

cells, it has been recently proven that radial glia-like cells (RGL) in adult brain have properties of NSCs (Bonaguidi et al., 2011).

In postnatal brain, NSCs are found primarily in two neurogenic niches, the ventricular-subventircular zone (V-SVZ) of the lateral ventricle, and in the SGZ in the dentate gyrus in the hippocampus (Zhao et al., 2008). NSCs display radial morphology and retain ultra-structure and molecular features of astrocytes (Silva-Vargas et al., 2013). They express the intermediate filament glial fibrillary acidic protein (GFAP), glutamate aspartate transporter (GLAST), brain lipid-binding protein (BLBP), and the transcription factor SRY (sex determining region Y)-box2 (SOX2) (Braun and Jessberger, 2014; Lim and Alvarez-Buylla, 2014). When NSCs are activated and begin exiting the quiescence state, they express the intermediate filament nestin (Lim and Alvarez-Buylla, 2014). Recent data showed that NSCs have lipid metabolic activity (Knobloch et al., 2013), and frequently express a protein associated with lipogenesis called Spot 14 (also known as thyroid hormone-responsive protein), and accordingly it has been proposed as a novel marker to identify NSCs (Knobloch et al., 2014).

Different terms are used to describe NSCs and their progeny. In the V-SVZ, NSCs are identified as type-B cells, which have basal processes contacting the blood vessels and apical endings intermingle between the ependymal cells and extend into the ventricle, and therefore, establishing direct contact with the cerebrospinal fluid (CSF). This unique cellular organization confers the ventricle surface an architecture resembling a pinwheel appearance (Mirzadeh et al., 2008). When exiting quiescence, B-cells give rise to rapidly dividing transient amplifying precursors (TAPs), identified as type-C cells. C-cells undergo approximately three symmetrical divisions, and then turn into migratory neuroblasts, identified as type A cells. Type-A cells are often characterized by their expression of polysialic acid neural cell adhesion molecule (PSA-NCAM) and doublecortin (DCX). They tangentially migrate in chains along the rostral migratory stream (RMS) towards the olfactory bulb (OB). While migrating toward the OB, they also divide one to two times (Brown et al., 2003; Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996; Ponti et al., 2013). Once reaching the OB, neuroblasts migrate radially and

differentiate into interneurons (Lim and Alvarez-Buylla, 2014; Lois and Alvarez-Buylla, 1994; Ponti et al., 2013).

In the SGZ, NSCs are called type-1 cells. When type-1 exist the radial state, they give rise to TAPs, called type-2 cells that also rapidly divide. Type-2 cells then give rise to neuroblasts, called type-3 cells, which migrate a short distance within the granule cell layer (GCL) generating new excitatory granule neurons (Aimone et al., 2014).

Signaling between various cell types within the neurogenic microenvironment is the central regulator for NSCs. This is mediated either by secretion of soluble factors or through a direct cell-cell contact. Cellular components in the neurogenic compartments primarily include ependymal cells (in the SVZ), vasculature, astrocytes, neurons, and microglia (Bjornsson et al., 2015).

The ependymal cells lining the lateral ventricle wall and directly contact the CSF. They possess microvilli and motile cilia that contribute to the CSF hydrodynamic flow, and therefore, act at as important regulators for accessibility of the CSF components to the neurogenic zone (Spassky et al., 2005). Moreover, they secrete factors that regulate NSCs, such as noggin (Lim et al., 2000).

The blood vessels in the brain are formed by specialized tightly interconnected endothelial cells, and enwrapped by pericytes. Endothelial cells and pericytes are embedded in the same basal lamina that has connection with astrocyte end-feet, resulting in what is called the blood-brain barrier (BBB). In both neurogenic regions, vasculature is a key regulator of NSCs. NSCs and clusters of TAPs are often found adjacent to blood vessels (Palmer et al., 2000; Shen et al., 2008; Tavazoie et al., 2008). A special feature in the V-SVZ, is that NSCs and TAPs are contacting blood vessels in areas lacking astrocyte end-feet and pericytes coverage, and it also seems that the BBB allows extravasation of small compounds from the blood circulation into the brain tissue (Tavazoie et al., 2008). Factors secreted by endothelial cells and pericytes, such as pigment epithelium-derived factor (PEDF) and transforming growth factor beta (TGFβ) act as key regulators for the NSC pool (Bjornsson et al., 2015) .

Neurons are also a key component in the neurogenic niches. Neuronal subtypes and their local circuit activity have been shown to influence the NSC

pool through neurotransmitters (Aimone et al., 2014; Lim and Alvarez-Buylla, 2014).

Astrocytes are the most abundant cell type in the brain and accomplish a wide range of functions (Pekny and Pekna, 2014). They have a direct contact with blood vessels, and are therefore important in the communication between the systemic environment and brain parenchyma. Astrocytes can influence NSCs in a number of ways via secretion of factors, depending on their gene expressions and metabolic state (Aimone et al., 2014; Cao et al., 2013). They are also considered a key determinant of NSC lineage commitment (Oh et al., 2010; Song et al., 2002; Wilhelmsson et al., 2012).

Microglia are the resident immune cells in the brain. The microglial regulation of neurogenesis will be detailed later (see **1.4.1.1**).

1.1.2 Morphological and physiological maturation of new neurons

In SVZ-OB axis, once neuroblasts reach the OB, they migrate radially and differentiate into interneurons. Numerous newborn cells reach the OB every day, however, only half of them survive and integrate into the pre-existing neuronal circuit (Lledo et al., 2006). By two to four weeks after birth, immature neurons establish synapses with the preexisting neurons. The successful circuit integration, will predominately contribute to the decision of whether the new neurons survive or undergo cell death (Yokoyama et al., 2011). The position and neuronal specification is largely depending on where the NSCs resided within the SVZ. NSCs residing in the dorsal part of the SVZ generate superficial granule neurons and anterior tyrosine hydroxylase- (TH) positive periglomerular neurons, and very rarely produce calbindin-positive neurons. In contrast, NSCs from the ventral SVZ generate deep granule calbindin-expressing neurons, but not TH-positive periglomerular neurons (Ihrie et al., 2011; Merkle et al., 2007).

The integration of new neurons in the hippocampus has been extensively studied. One week after differentiation, newborn neurons extend apical dendrites toward the molecular layer. By day 10-11, they project axon mossy fibers towards the *cornu ammonis* (CA3) region, and when they are approximately two weeks old, dendritic spines start to appear (Zhao et al.,

2006). Newborn granule neurons reach maturity in approximately two months, where they receive input form the entorhinal cortex and establish functional synapses with the hilar interneurons, mossy cells, and CA3 pyramidal cells. (Aimone et al., 2014; Toni et al., 2008).

Upon their physiological maturation, γ-aminobutyric acid (GABA) and glutamate receptors are observed in 3-day-old immature neurons (Esposito et al., 2005). As in the developing brain, GABA induces neural depolarization (excitation) instead of hyperpolarization (inhibition) in newborn neurons. The GABAergic activity is detected at one week, and this effect is owed to the presence of high-level chloride ions through the expression of Na^+ K^+ $2Cl^-$ (NKCC1), a Cl^- importer channel, (Ge et al., 2006). By reduction of chloride ions later, the activation of glutamatergic synapses occurs (Walker and Semyanov, 2008) and subsequently, newborn cells release glutamate as their main neurotransmitter (Toni et al., 2008).

1.1.3 Regulation of adult neurogenesis

Neurogenesis is regulated by multiple factors, including intrinsic (local) and extrinsic (systemic) factors. Intrinsic factors include: Signaling by the developmental pathways such as wingless (Wnts), sonic hedgehog (SHH), Notch, bone morphogenic proteins (BMPs), fibroblast growth factor (FGF), and ephrins (Fuentealba et al., 2012). Neuronal activity via neurotransmitters, such as GABA, 5-hydroxytryptamine (5-HT; Serotonin), dopamine, choline acetyltransferase (ChAT) (Alfonso et al., 2012; Banasr et al., 2004; Hoglinger et al., 2004; Liu et al., 2005; Paez-Gonzalez et al., 2014; Song et al., 2013). Exclusively in the hippocampus, a recent study has shown that NSCs themselves shape their niche and regulate neurogenesis via secretion of their own vascular endothelial growth factor (VEGF) (Kirby et al., 2015).

The extrinsic factors are either positive or negative regulators. Factors known to increase neurogenesis include: physical activity, environmental enrichment, olfactory or hippocampal dependent learning (Alonso et al., 2006; Gould et al., 1999a; Kempermann et al., 1997; van Praag et al., 1999). Factors known to downregulate neurogenesis include: Stress, aging, inflammation, circadian

disruption and sleep deprivation (Gibson et al., 2010; Mueller et al., 2008), and alcohol abuse (Herrera et al., 2003; Kuhn et al., 1996; Monje et al., 2003). Diet is also regulating neurogenesis, and its effect could either be positive or negative depending mainly on its compositions. For instance, a diet lacking essential vitamins and minerals decreases neurogenesis, and by contrast, a diet supplemented with polyphenols and omega-3 fatty acid increases neurogenesis (Stangl and Thuret, 2009).

1.1.4 Functional implications of adult neurogenesis

Under physiological conditions, newborn neurons functionally integrate in preexisting neuronal circuitry in the hippocampus and OB (Toni et al., 2008; Yokoyama et al., 2011), and therefore it is believed that they contribute to hippocampal and olfactory dependent learning and memory (Aimone et al., 2014; Sakamoto et al., 2014). Moreover, adult hippocampal neurogenesis is essential for pattern separation, the process by which keeping similar episodes different when recalling previous event based on spatial cues (Clelland et al., 2009; Sahay et al., 2011). Recently, hippocampal neurogenesis has been shown to regulate forgetting, as increased neurogenesis after memory formation has induced forgetting when assessed in mice (Akers et al., 2014).

Under injury circumstances, NSCs have been shown to respond to a variety of injuries. The NSC pool expands after the injury occurs, and their progeny migrate to the injured region. The NSC response has been proposed, at least partly, to contribute to the spontaneous recovery that happens after the brain injury, which can either be through cell replacement or secretion of trophic factors (Gage and Temple, 2013; Yu et al., 2014). The NSC response after the brain injury varies depending on the nature of the injury. In this thesis, the focus will be on the NSC response after injuries caused by irradiation and stroke.

1.2 Irradiation brain injury

Ionizing irradiation has a cytocidal effect either by causing direct double-stranded DNA breaks, or indirectly via formation of free radicals. The free

radical formation occurs when a photon strikes the atom leading to excitation and emission of electrons. As water is abundant in the body, free radicals can thereafter generate reactive oxygen species (ROS), which can result in secondary damage and mutation in the DNA. Moreover, ROS can destroy lipids and proteins, leading to damage of supporting cellular structures and organelles, and eventually to apoptosis. Mitotically active cells, such as cancer cells are more susceptible radiation damage (Robbins and Zhao, 2004).

Ionizing radiation has been successfully used to treat primary and metastatic brain tumors. Although the number of cancer survivors is greatly increased worldwide (Crossen et al., 1994; Shah et al., 2015), irradiation is however, has neurotoxic effects and leads to long-term sequelae in cancer survivors. It induces pathological changes such as acute and sub-acute encephalopathy, leukoencephalopathy, cerebral atrophy, vascular changes, demyelination, and white matter necrosis. At the behavioral level, brain irradiation leads to neurocognitive impairment, and dementia (DeAngelis et al., 1989; Perry and Schmidt, 2006). The cognitive impairment is the most progressive side effect, especially in pediatric survivors. The cognitive dysfunction is attributed to, for example, deficits in short-term memory, visual motor processing, and attention (Abayomi, 1996). Classically, the irradiation-induced cognitive impairment was thought to be due to induced abnormalities in vasculature, demyelination, and white matter necrosis (Calvo et al., 1988; Schultheiss and Stephens, 1992; van der Maazen et al., 1993). However, after the discovery of adult neurogenesis, and the emerging evidence of contribution of newborn neurons to learning and memory (Aimone et al., 2014), ablation of neurogenesis caused by irradiation is proposed to be, at least in part, the cause of the cognitive decline (Barlind et al., 2010; Kalm et al., 2013; Karlsson et al., 2011; Marazziti et al., 2012; Monje, 2008).

1.2.1 The effects of irradiation on neurogenesis

The NSCs are mitotically active, and accordingly they are prone to irradiation damage. Irradiation inhibits neurogenesis both in developing and adult brain, including humans (Fukuda et al., 2004; Monje et al., 2002; Monje et al., 2007;

Parent et al., 1999). Proliferative NSCs are sensitive to irradiation even at very low doses (Kempf et al., 2014), resulting in long-term irreversible failure in hippocampal neurogenesis (Kalm et al., 2013). The severity of depleted neurogenesis seems to differ according to age, gender, and the delivered dose, as often the young subjects, female, and the higher irradiation dose has worse outcome (de Guzman et al., 2015; Fukuda et al., 2005; Roughton et al., 2012). Impairment of the microenvironment of NSCs by irradiation is thought to be a reason of the hindered neurogenesis (Monje et al., 2002). There is consensus that the inflammatory milieu created after radiation injury is a major impeder of neurogenesis, both in young and adult brains, albeit that inflammation in the developing brain is rather transient (Dong et al., 2015; Kalm et al., 2009; Monje et al., 2003). Moreover, irradiation-induced impaired neurogenesis was exacerbated in animals with systemic inflammation (Roughton et al., 2013), and the blockade of inflammation can restore neurogenesis (Monje et al., 2003). Besides the inflammation, irradiation perturbs the association between the proliferating NSCs and their progeny with the vasculature (Bostrom et al., 2014; Monje et al., 2002). As support to the notion that irradiation alters the neurogenic microenvironment, a recent report showed that the number of quiescent NSCs remains unaffected by irradiation, and factors secreted by endothelial cells, such as TGFβ, block neurogenesis (Pineda et al., 2013). Moreover, microglia (an important regulator of neurogenesis) in irradiated brains gain aging–like changes in their transcriptome, which led to the speculation of possible shared mechanisms between aging- and irradiation-induced cognitive decline (Li et al., 2015).
Besides blocking inflammation, restorative interventions to rescue irradiation-induced loss of neurogenesis have also included physiological stimulation by voluntary running, and NSC transplantation (Acharya et al., 2009; Naylor et al., 2008; Sato et al., 2013).

1.3 Stroke

The World Health Organization defines stroke as a clinical syndrome that has rapid onset of focal or global cerebral deficit lasting for more than 24 hours (unless interrupted by surgery or death). It affects about 15 million individuals

each year worldwide (Moustafa and Baron, 2008). It is ranked as the third most common cause of death, after coronary heart disease and cancer. The mortality rate is about one-third among the affected patients, and about two-thirds suffer from severe disabilities (Johnston et al., 2009).

Depending on the cause, stroke can either be ischemic or hemorrhagic. The ischemic is more prevalent as about 80% of the stroke patients had ischemia, while 20% of the patients had hemorrhagic stroke caused by primary intra-cerebral or subarachnoid hemorrhage.

Ischemic stroke occurs due to interruption of blood flow resulting from thrombosis, embolism, or systemic hypo-perfusion, which lead to insufficient supplementation of oxygen and glucose that are needed to maintain the cellular homeostasis, as well as removal of toxic metabolites, leading to breakdown of the metabolic process, and eventually causes cell death. This process leads to excitotoxicity, ionic imbalance, oxidative and nitric stress, and inflammation, which all together exacerbate the injury (Doyle et al., 2008).

The injured area is divided into two main regions. The first region is the ischemic core, where irreversible cellular damage and electrical silence have occurred, and it therefore is beyond therapeutic rescue. The second region is the penumbra, which is the region adjacent to the infarct core that is hypo-perfused, and therefore considered as a region at risk (Moustafa and Baron, 2008).

The current available treatments for stroke are mainly thrombolysis, generally aimed to restore the blood circulation and make the hypoxic tissue salvageable. These drugs have a very narrow time window, as they are only efficient within 3-4.5 hours after the onset of the symptoms. Moreover, they can also increase the risk of initiating intracranial hemorrhage, and therefore need urgent attention (Brott and Bogousslavsky, 2000; Carpenter et al., 2011).

1.3.1 Neural stem cell responses after stroke

Both stroke types, the ischemic and the hemorrhagic, trigger NSC responses. After ischemia, NSCs in the SVZ and SGZ increase their proliferation, which often peaks at 1-2 weeks after the onset of the insult (Arvidsson et al., 2002;

Liu et al., 1998; Parent et al., 2002; Sgubin et al., 2007). Concomitantly, neural and glial progenitor cells migrate along the blood vessels form the SVZ toward the injured area (Benner et al., 2013; Thored et al., 2007). The presence of immature neural progenitors in the injured site has also been reported in human brain (Jin et al., 2006). This migratory response is regulated by several mechanisms, including inflammatory chemokines, such as stromal cell-derived factor 1 α (SDF-1α) and the chemokine (C-C motif) ligand 2 (CCL2) (Young et al., 2011). Despite that stroke injury triggers neural progenitor migration for at least one year after the occurrence of the insult (Kokaia et al., 2006), about 80% of migrating progenitors undergo death and only a small fraction survive and functionally integrate in the circuitry in the injured striatum (Arvidsson et al., 2002; Hou et al., 2008; Parent et al., 2002). Whether generation of new neurons in the cortex after ischemia is happening is still a matter of debate, when examined in animals (Arvidsson et al., 2002; Gu et al., 2000; Leker et al., 2007). In humans, a recent report has denied generation of new cortical neurons after stroke based on a retrospective birthdating approach (Huttner et al., 2014). Interestingly, stroke can trigger stemness outside the neurogenic niches as it can induce reprogramming of cells, such as astrocytes, to generate neural progenitor cells (Magnusson et al., 2014; Ohira et al., 2010).

1.4 Neuroinflammation

The presence of the BBB and the absence of lymphatic vessels, make the brain isolated from the peripheral immune system, under physiological conditions, and therefore becomes an immune privileged site (Engelhardt, 2008). However, emerging concepts perceive the brain as only a partially immune privileged site, since the communication between the CNS and the immune system is bidirectional and they influence each other (Galea et al., 2007; Perry and Teeling, 2013), especially after the very recent discovery of functional lymphatic vessels lining the dural sinuses that able to carry both fluid and immune cells from the CSF and connected to the cervical lymph nodes (Louveau et al., 2015).

Neuroinflammation represents the inflammatory process occurring in the CNS as a result of pathological conditions caused by pathogens, brain damage, or neurodegeneration (Glass et al., 2010). The process is generally characterized by activation of microglia, as the first line of defense, however other cells types are also involved such as astrocytes and endothelial cells. The peripheral immune cells can also infiltrate and contribute to such a process, as the BBB becomes permeable, and even damaged in some pathological conditions (Glass et al., 2010; Gonzalez et al., 2014; Goverman, 2009; Hagberg et al., 2015; Lucin and Wyss-Coray, 2009). Neuroinflammation is initiated by stimulation of recognition receptors on the cell membrane known as pattern recognition receptors (PRRs), such as toll-like receptors (TLR). These receptors are sensitive to molecules such as lipopolysaccharide (LPS), flagellin, double-stranded DNA, and endogenous oxidized aggregates (Gonzalez et al., 2014; Kim et al., 2013). Beside the PRRs, the inflammatory response is also triggered through purinergic receptors that are capable of responding to ATP released after cell death and damage (Di Virgilio et al., 2009). In the CNS, both microglia and astrocytes express these receptors (TLR and purinergic) (Glass et al., 2010). Activation of PRRs and the puringergic receptors leads to several signal transduction pathways that regulate multiple transcriptional processes and later results in production of inflammatory mediators such as cyto/chemokines, nitric oxide, and ROS (Glass et al., 2010; Gonzalez et al., 2014).

1.4.1 Microglia

Microglia are the resident mononuclear phagocytes of the CNS and constitute 10-15% of the total number of CNS cells. Microglia have attracted substantial attention during the last past years. As of the first week of August 2015, a PubMed search for the world “microglia” revealed 20,834 search results in which 9,400 were published after the year 2009.

Historically, microglia were first described by Franz Nissl in the late 19th century as rod cell elements that have migratory and phagocytic potential. Soon after, these cells were called as mesoglia by W. Ford Robertson to indicate their mesodermal origin. Later, Santiago Ramon y Cajal called them

the third element of the nervous system, eventually termed microglia by his student del Rio-Hortega (Ginhoux et al., 2013).

The origin of microglia has long been a central debate as to whether they originate from the neuroectroderm or the mesoderm. Today, there is a common agreement that microglia have mesodermal/mesenchymal origin, and that these cells have migrated into the brain during development (Chan et al., 2007; Ginhoux et al., 2010). Microglia are derived form the yolk sac during embryogenesis. The yolk sac-derived primitive macrophages spread in the blood around the embryonic day (E) 8 to 10 (McGrath et al., 2003), and their entrance to the brain is assumed to be around E8.5-E9 (Alliot et al., 1991). In humans, microglial cells enter the brain in about 4.5 weeks of gestation (Monier et al., 2007; Rezaie et al., 2005). During early postnatal life, the microglial population expands through proliferation (Harry, 2013), nevertheless, a second wave of the bone marrow-derived cells has also been suggested to contribute to this expansion (Beers et al., 2006). Microglia enter the brain in an amoeboid state, and after series of transitional forms, microglia adopt a ramified morphology (Ginhoux et al., 2013), and by postnatal day 15, the entire parenchyma is populated with ramified microglia (Harry, 2013).

For long, the ramified phenotype in the intact brain has been referred to as resting microglia (Gonzalez et al., 2014). However, recent time-lapse imaging experiments revealed that microglia are dynamic and continuously surveying the brain parenchyma (Nimmerjahn et al., 2005). Microglia are maintained in a non-activated state via the ligand/receptor interaction with neurons. Neurons express ligands such as the chemokine CX3CL1 (fractalkine), CD200 (OX2), CD47, and CD22; and microglia express their receptors CX3CR1, CD200R, and signal regulatory protein alpha (SIRPα), and CD45.

Upon activation microglia undergo morphological changes and acquire macrophage characteristics. Depending on the stimulus microglia can either become pro-inflammatory (the classically activated, M1; neurotoxic) or anti-inflammatory (the alternatively activated, M2; neuroprotective) (Eggen et al., 2013).

Beyond the immune role, evidence is accumulating that microglia are essential to maintain the brain function by shaping the neuronal circuit

structure, release of soluble factors, and regulation of postnatal neurogenesis (Paolicelli et al., 2011; Salter and Beggs, 2014; Sierra et al., 2010; Tremblay et al., 2011).

1.4.1.1 The role of microglia in adult neurogenesis

Microglia are one of the cell types that present in the neurogenic compartments (Bjornsson et al., 2015). Under physiological conditions, surveying microglia are involved in removal of apoptotic newborn cells that failed to integrate in the existing circuitry, in an immune-silent manner (Sierra et al., 2010). Moreover, microglia secrete trophic factors such as nerve growth factor (NGF), FGF2, glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), inulin-like growth factor 1 (IGF-1), brain-derived neurotophic factor (BDNF) that influence NSC proliferation, neuronal differentiation, and survival (Bjornsson et al., 2015; Ziv and Schwartz, 2008). As physical exercise and the environmental enrichment are potent inducers of neurogenesis, studies are suggesting that microglia might mediate such effects (Ehninger et al., 2011; Gebara et al., 2013; Vukovic et al., 2012). Signaling through the CX3CL1/CX3CR1 axis has been shown to be involved in microglial regulation of neurogenesis and cognitive function (Gemma and Bachstetter, 2013).

In the injured brain, activated microglia regulate neurogenesis in a number of ways, and the NSC response is largely depending on the nature of the injury as well as the activation state. Findings in this regard are detailed in paper IV.

1.5 The enteric nervous system

The enteric nervous system (ENS) is part of the peripheral nervous system. It controls the gut motility, blood flow, and the enzymatic secretion. It contains a vast number of neurons (approximately 100 millions), and glial cells that assemble in interconnected ganglia. The enteric ganglia are arranged into two plexi, the outer myenteric plexus, embedded between muscle layers, and the deeper submuscosal plexus (Lake and Heuckeroth, 2013).

1.5.1 The enteric neural stem cells and ENS development

During embryogenesis, the enteric neural stem/progenitor cells (ENSPCs) are derived from vagal and the sacral segments of the neural crest. The vagal neural crest is the major source of the ENSPCs, as they colonize the entire gut wall, while the sacral neural crest ENSPCs colonize only the hindgut (Heanue and Pachnis, 2007). The neural crest cells begin to migrate to the gut wall around the E9.5 in mice (about 4 weeks in humans), and they have fully colonized the gut by E15 in mice (week 7 in humans) (Lake and Heuckeroth, 2013; Schafer et al., 2009).

Several studies have shown the possibility to isolate self-renewing and multi-potent ENSPCs from the ENS of rodents, as well as from humans (Bondurand et al., 2003; Metzger et al., 2009; Suarez-Rodriguez and Belkind-Gerson, 2004). These cells express the neural epithelial marker nestin, the low-affinity nerve growth factor receptor (P75), and Sox10. When differentiated *in vitro*, ENSPCs generate neurons, astrocytes, and myofibroblasts (Heanue and Pachnis, 2007), and when transplanted into the gut, they generate functional neurons (Hotta et al., 2013). Due to the fact that the ENSPCs are easily accessible, for example through laparoscopy, and do not require reprogramming, they therefore have been suggested for therapeutic purposes for the CNS (Schafer et al., 2009).

1.6 Caspases

Caspases (cysteine-aspartate proteases) are a group 18 intracellular proteolytic enzymes known to play a role in programmed cell death (apoptosis) and inflammation. However, they are also involved in other physiological process, such as cell proliferation, migration, differentiation, tumor suppression, and neuronal development and plasticity (Shalini et al., 2015; Venero et al., 2011). Seven caspases are involved in the apoptosis process, in which 4 are classified as initiator caspases (caspase-2, -8, -9, and -10), and the other three referred to as executioner caspases (capase-3, -6, and -7).

The inflammation-related caspases are caspase-1, -4, -5, -11, and -12. Caspase-1 was first referred to as interleukin-1β- (IL-1β) converting enzyme

(ICE), where it´s catalytic activity is essential for maturation of the cytokines IL-1β and IL-18. Caspase-1 activity is regulated by multi-protein complexes called inflammasomes (Schroder and Tschopp, 2010).

Recently, the apoptotic caspases, particularly caspase-3, -7, and -8, have been implicated in neuroinflammation, as they are required for microglia activation. Inhibition of these caspases halted the microglial activity and the subsequent neurotoxicity, and the pathological changes *in vitro* and in animal models (Burguillos et al., 2011; Fricker et al., 2013; Rohn et al., 2009).

2. Aims of the thesis

General aim

The main aim of our studies was to address the possibilities for brain restoration after injuries caused by irradiation or ischemia, either by activation of the endogenous neural stem cells pool or by cell engraftment, and the implication of microglia in such processes.

Specific aims

- We intended to study whether the ENSPCs can be used as a novel cell source for brain repair after injury caused by radiotherapy.
- We intended to study the temporal and spatial response of neural progenitors and cortical neurogenesis after stroke.
- We intended to study whether targeting the cell death, particularly caspase-mediated cell death, would promote cortical neurogenesis after stroke.
- We intended to study the impact of microglial subtypes on NSC survival, proliferation, migration, and differentiation.

3. Materials and methods

Refer to materials and methods section for each paper (paper I- paper IV).

4. Results and discussion

4.1 Paper I: Transplantation of enteric neural stem/progenitor cells into the irradiated young mouse hippocampus

Irradiation results in long-lasting depletion of hippocampal neurogenesis, which may result in cognitive impairments (Bostrom et al., 2013; Kalm et al., 2013). As a restorative intervention, transplantation of NSCs into the irradiated brain has been shown to generate neurons and astrocytes, and subsequently to improve cognitive performance (Acharya et al., 2011; Sato et al., 2013). However, such an approach requires a clinically relevant source of cells. As in the brain, the enteric nervous system harbors ENSPCs that have been proposed to be such a source of cells to treat CNS pathologies (Schafer et al., 2009). Therefore, in this paper we intended to investigate whether the ENSPCs could be utilized as a novel cell source to restore neurogenesis and the cognitive impairments following brain irradiation. We first isolated cells from the muscular layers/myenteric plexus of young mice, and expanded them *in vitro* for one week (wk) (Fig. 2A). Neurosphere-forming cells were immune-reactive for the neural epithelial marker nestin and the nerve growth factor receptor P75, indicating they were ENSPCs (Fig. 2B and 2C). When differentiated, ENSPCs were capable of generating neurons (beta III tubulin-positive; βIII+), astrocytes GFAP-positive; GFAP+), and myofibroblast (α smooth muscle actin-positive; αSMA+) (Fig. 2D–2F).

We next labeled the syngeneic ENSPCs with a reporter dye, called CM-DiI (DiI), and grafted them into an intact or irradiated young mouse hippocampus (Fig. 1A and 1B). Four weeks after transplantation, we detected 0.5% and 1% of the grafted ENSPCs in the dentate gyrus of the sham-irradiated (control) and irradiated animals, respectively (Fig. 3A). The majority of grafted ENSPCs homed to the dorsal part of the GCL, forming large clusters (Fig. 3B); however, ENSPCs were also seen in ventral part of the GCL, the hilus, and the molecular layer (Fig. 3C). When we assessed the ENSPC survival at 4 months after grafting, only 0.1% of the grafted ENSPCs were detected, indicating that the transplanted ENSPCs were dying over time. It is known that grafted cells have poor survival rates as a result from multiple causes such as

anoikis, the recipient´s immune response, and hypoxia (Sart et al., 2014). However, compared to brain-derived NSCs when transplanted into the irradiated brain (Acharya et al., 2011; Sato et al., 2013), the loss of the ENSPCs appears to be considerable.

We next examined the differentiation capacity of transplanted ENSPCs at 4 wks after grafting. Our results displayed that all grafted cells were expressing P75 and nestin, and no co-localization with NeuN (pan mature neuronal marker), peripherin (peripheral neuronal marker), GFAP, or α-SMA was seen, indicating they remained undifferentiated, both in sham and irradiated animals (Fig.3D-3I). Grafted ENSPCs were also neither expressing the immature neuronal marker DCX (Fig. 3J), nor the proliferation marker Ki67 (Fig. 3K), suggesting that they were not even under delayed differentiation or in a proliferative state. Combined, these data suggest that the brain is either lacking signals required for ENSPC differentiation, or that the microenvironment was not permissive to ENSPC differentiation.

Disruption of the GCL architecture is expected after intragyral injection (Hofferer et al., 1994; Sato et al., 2013). Here, we found disruption of the GCL structure in the hemisphere injected with ENSPCs, but not the vehicle. This was found to be either as thinning of the GCL or nearly total loss (Fig. 4A and 4B).

Inflammation is crucial for survival and death of transplanted stem cells (Darsalia et al., 2011; Zhou et al., 2011). Therefore, we next looked at the immune reaction at 4 wks and 4 months post grafting. At 4 wks, transplanted ENSPCs resulted in significantly increased numbers of microglia in sham animals, but not in the irradiated ones (Fig. 4C). However, elevated numbers of microglia in irradiated animals was seen at 4 months post grafting, suggesting that ENSPCs caused long-term microglia accumulation in the dentate gyrus (Fig. 5A). Moreover, activated and phagocytic microglia (Iba1+/CD68+) were frequently seen surrounding and infiltrating the ENSPC clusters (Fig. 4D and 4E). Moreover, we observed an upregulation of hypertrophic astrocytes (GFAP+) in the hemisphere injected with cells, and all ENSPC clusters were surrounded by pronounced astrogliosis (Fig. 4F). No CD3-positive cells (pan T-cell marker) were seen (Fig. 4G). All together, despite the use of syngeneic cells, and the absence of T-lymphocytes, these

data suggest that the grafted ENSPCs were likely rejected by the innate immune system.

Stem cells exert their beneficial effects either by cell replacement or secretion of trophic factors (paracrine effects) that either modulate the inflammatory response or stimulate the endogenous stem cells pool (Burdon et al., 2011; Dutta et al., 2013). Therefore, we examined the impact of ENSPC transplantation on hippocampal neurogenesis after irradiation at 4 months post grafting (Fig. 1B). We found that the grafted ENSPCs did neither restore the NSC proliferation nor neurogenesis in the dentate gyrus (Fig. 5 C-5F).

Last, we were interested in investigating the effect of ENSPC transplantation on the animals' learning behavior after irradiation. To test this, we used the IntelliCage platform, in which each animal was randomly allocated to drink from water bottles placed in certain corners during 3 testing periods, in which the corner was changed every 5 days (Karlsson et al., 2011). Our results displayed that irradiated animals which received ENSPCs had the poorest learning abilities (Fig. 6A-6D), which presumably resulted from the ongoing inflammatory reaction and subsequent loss of granule neurons.

In summary, no beneficial effects of ENSPC grafting could be demonstrated, at least not with the current culture and grafting protocols. However, recent studies have reported that ENSPCs can differentiate into neurons and glial cells when grafted into the brain slices *in vitro* (Hagl et al., 2013), and can improve neurocognitive performance in animals modeled for Parkinson´s disease (Parra-Cid et al., 2014).

4.2 Paper II: Long-term stimulation of neural progenitor cell migration after cortical ischemia in mice

In this study, we aimed first to investigate the temporal and spatial response of NSCs residing in the SVZ to cortical ischemia, and second to evaluate whether the NSCs can replenish the loss of cortical neuron after stroke.

We induced an ischemic lesion restricted to the neocortex using a photothrombotic stroke model (Watson et al., 1985), and we subsequently analyzed the number of neural progenitor cells (neuroblasts), as identified by their expression of DCX (Couillard-Despres et al., 2005), in the striatum, the corpus callosum, and the peri-infarct cortex, starting 2 wks after the injury and up to one year.

We found that the ischemic injury resulted in a significant increase in the number of DCX-expressing cells in the ipsilateral hemisphere compared to the contralateral hemisphere in all examined regions. In the striatum, this increase was seen for 6 wks after the injury, however in the corpus callosum and the peri-infarct cortex the increased number was observed for one year (Fig. 1). Interestingly, we found that most of the migrating neuroblasts were aligned with the fiber tract in the corpus callosum (Fig. 2), suggesting the importance of the white matter in the cell trafficking towards the damaged area.

Long-term migration of neuroblasts towards the ischemic striatum has been previously reported (Kokaia et al., 2006; Thored et al., 2007). In rodents, neural progenitor cells start expressing DCX shortly after exiting mitosis and lack it´s expression when they become fully mature neurons in a time window extending approximately 6 wks (Brown et al., 2003). In macaque primates, the expression of DCX in neural progenitors can last up to six months (Kohler et al., 2011). We therefore wondered whether the neuroblasts found in the injury site were recently born cells, or if time window of DCX expression was rather extended under injury circumstances. Taking the advantage of the possibility to label the dividing NSCs at different time points with thymidine analogues in the same animal (Vega and Peterson, 2005), we labeled neuroblasts born within the first 10 days after the injury with chlorodeoxyuridine (CldU), and those born at later time points after the injury, within 2 wks before sacrificing the animals, with iododeoxyuridine (IdU). Our results revealed that CldU/DCX

co-expression in the peri-infarct cortex was only seen up 6 wks after the injury, and IdU/DCX co-expression was detected in all studied time points up to one year (Fig. 3A and 3B), indicating that neuroblasts found in the peri-infarct cortex are newly born, and accordingly neural progenitor migration is sustained for at least one year.

Cortical neurogenesis after injury has been previously reported (Magavi et al., 2000), however, particularly after stroke, generation of new cortical neurons is controversial (Arvidsson et al., 2002; Gu et al., 2000). Here, we did not detect cortical neurogenesis from progenitors born early after injury (CldU+ cells), when they were analyzed for the expression of the mature neuronal marker NeuN (Fig. 3C). However, we have seen a cohort of neuroblasts expressing NeuN (Fig. 3D). Neuroblasts start to express NeuN around day 15 after their birth (Brown et al., 2003), however prediction of their survival and maturation is not certain, especially under pathological conditions (Zhou et al., 2011).

4.3 Paper III: Caspase inhibition impaired the neural stem/progenitor cell response after cortical ischemia in mice

In light of the findings from paper II, cortical neurogenesis from neural progenitor cells born within a few days after the injury, a period presumed to overlap with the peak of the inflammatory response (Zhou et al., 2011), was not detectable; and therefore we speculated that neural progenitors born at later time points after the injury might survive and fully differentiate into neurons. To aid the neural progenitor survival, we administered the broad-spectrum caspase inhibitor Q-VD-OPh (QuinolineValAsp(Ome)-CH2-O-phenoxy), a drug that has greater potency and specificity to irreversibly block apoptosis (Caserta et al., 2003; Chauvier et al., 2007).

Previous studies have shown that a lack of genes involved in apoptosis can disrupt NSC proliferation and migration (Kim et al., 2007; Osato et al., 2010), we thus first evaluated the effect of Q-VD-OPh on these parameters in the intact brain. Animals received daily injections of Q-VD-OPh or vehicle for 10 days, and the proliferating NSCs were labeled with BrdU (Fig. 1A). Results from this experiment displayed that Q-VD-OPh did neither altered NSC proliferation nor neuroblast migration (Fig. 1B-1E).

To assess cortical neurogenesis after stroke, cortical ischemia was induced using the photothrombotic stroke model, and neural progenitors born 2 months after the injury were labeled with BrdU. Twelve days after labeling of the last cohort of newborn progenitors, animals received daily injections of either Q-VD-OPh or vehicle for 2 weeks (Fig.2A). The co-expression of BrdU and NeuN was then analyzed one month after the last injection of Q-VD-OPh. Our results demonstrated that cortical neurogenesis was very rare and caspase inhibition did not promote cortical neurogenesis, as BrdU/NeuN co-expression was only seen in 2 animals out of 8 per each treatment group (Fig. 2B). Interestingly, we found that treatment with Q-VD-OPh selectively affected the injury-induced neuroblast migration as the number of DCX-expressing cells in the peri-infarct cortex was 60% reduced after treatment with Q-VD-OPh, while no alteration was seen in the SVZ or in the RMS (Fig. 2C and 2D). Since brain injury is often accompanied by inflammation, and signals through chemokines secreted by inflammatory cells, such as microglia, are major inducers for neural progenitor migration to the injured area (Young et al.,

2011; Zhou et al., 2011), we next analyzed the number of microglia and their activation state, as indicated by expression of CD68, in the peri-infarct cortex. We found that the number microglia was increased in the injured animals compared to sham control animals, however, we neither observed differences in the cell numbers nor in the activation between ischemic animals of the two treatment groups (Fig. 2E and 2F). Of note, the time that we analyzed microglia was about one month after cessation of the treatment with Q-VD-OPh. Hence, we performed an acute experiment, in which animals received either Q-VD-OPh or vehicle for 2 wks starting one day after induction of cortical ischemia, the time that neuronal death is expected to reach its maximum (Porritt et al., 2012), and therefore we avoided the neuroprotective effect of caspase inhibition (Fig. 3A). Brains were then harvested for histology and protein assays. We first analyzed the infarct size in the ischemic animals. As expected, no difference was seen between animals treated with Q-VD-OPh or vehicle (Fig. 3B). When we counted the number of DCX-expressing cells in the peri-infarct area, we again observed a reduction in numbers of migrating neuroblasts toward the injured cortex (Fig. 3C), confirming that inhibition of caspases with Q-VD-OPh influenced the injury-induced neural progenitor migration.

The increased NSC proliferation in response to stroke often peaks at 2 weeks after injury (Arvidsson et al., 2002; Parent et al., 2002), so we evaluated the number of proliferating NSCs in the SVZ as indicated by the expression of phospho-histone H3 (PHH3). Our result demonstrated that treatment with Q-VD-OPh also reduced the injury-induced increased NSC proliferation (Fig. 3D).

When we analyzed the number of microglia and their activation in this acute phase after treatment, we again did not detect differences between the ischemic animals of the treatment groups (Fig. 3E and 3F). Activated microglia can acquire a pro-inflammatory (M1) or anti-inflammatory (M2) polarization and both express the activation marker CD68 (Taylor and Sansing, 2013). Moreover, caspase-1 is known to be involved in inflammation (Martinon et al., 2002), and recently the apoptosis caspases have been shown to be required for the induction of the M1 microglial phenotype (Burguillos et al., 2011; Fricker et al., 2013). We therefore analyzed the levels

of interleukin-1β (IL-1β) and IL-10, cytokines categorized as pro-inflammatory and anti-inflammatory, respectively. Interestingly, we found that the levels of IL-1β was significantly increased in ischemic animals treated with vehicle compared to the sham controls, and treatment with Q-VD-OPh after ischemia prevented this increase. When we analyzed the levels IL-10, we did not detect differences between any of the four studied groups (sham controls or ischemic animals treated with Q-VD-OPh or vehicle) (Fig. 4A and 4B), suggesting that treatment with Q-VD-OPh reduced the pro-inflammatory response.

The chemokines CCL2 and its receptor CCR2, and SDF-1α and its receptor CXCR4 are involved in attraction of neural progenitor cells to injured area (Robin et al., 2006; Yan et al., 2007). When we analyzed the amount of these chemokines, we found that the levels CCL2 were highly increased in ischemic animals compared to sham controls, however, when we compared the levels of CCL2 in ischemic animals between the treatment groups, we observed significantly lower levels of CCL2 in animals treated with Q-VD-OPh (Fig. 4C). No changes of the levels of SDF-1α were seen (Fig. 4D). These data suggest that alteration in signaling through the CCl2/CCR2 axis might be involved in the reduced neural progenitors migration in response to stroke.

Overall, treatment with the caspase inhibitor Q-VD-OPh seems to reduce the pro-inflammatory profile, and also to interfere with the neural stem/progenitor cell response after stroke. Hence this warrants consideration when caspase inhibition is aimed for neuroprotection after brain insults.

4.4 Paper IV: The anti- inflammatory microglial secreteome promoted neural stem cell survival and migration, but reduced astrogliogenesis

Microglia are present in the neurogenic niches, and they play a role in regulating neurogenesis (Gemma and Bachstetter, 2013; Sierra et al., 2010). After injury, microglia become activated, proliferate and migrate to the injured area. When activated, microglia can become either neurotoxic (M1) or neuroprotective (M2) (Eggen et al., 2013). Therefore, in this paper, we intended to address the influence of different microglial subtypes on NSC survival, proliferation, migration, and differentiation.

NSCs expressing green fluorescent protein were grown *in vitro* in conditioned media collected from either non-stimulated microglia, or microglia stimulated with LPS (pro-inflammatory, M1) or with IL-4 (anti-inflammatory, M2).

As inflammation is known to create a hostile microenvironment that might impede the regenerative cellular response (Zhou et al., 2011), we accordingly first evaluated the survival of NSCs after being exposed to the different microglial conditioned media. As expected, our results displayed that factors released form LPS-stimulated microglia were markedly toxic to NSCs compared to factors secreted by non-activated microglia or microglia stimulated with IL-4 (Fig. 2A and 2B). When we analyzed the effect of different microglial conditioned media on NSC proliferation, we did not detect differences, as indicated by BrdU incorporation (Fig. 3).

Microglia, particularly the M2 subtype, are known to regulate cancer cell migration (Wesolowska et al., 2008; Ye et al., 2012). After brain injury, inflammatory chemokines are major recruiters for neural progenitors to an injured area (Imitola et al., 2004; Yan et al., 2007). Therefore, we next wanted to investigate the effect of microglia subtypes on NSC migration. Our results revealed that factors present in the conditioned medium from IL-4-stimulated microglia promoted NSC migration compared to factors in media collected form non-stimulated microglia or microglia stimulated with LPS (Fig. 4A and 4C).

Several studies have addressed the effects of microglia and inflammatory cytokines on NSC differentiation, however these data are somewhat contradictory, reviewed in (Borsini et al., 2015). Here, by comparing the effect of factors released form microglial subtypes on NSC differentiation, we found

that conditioned media from IL-4-stimulated microglia induced less astrocytic differentiation compared to conditioned media collected form LPS-stimulated ones, and no influence on neuronal or oligodendrocytic differentiation was seen among NSCs exposed to the different microglial media (Fig. 5).

In conclusion, it appears that microglial subtypes differently influence NSCs. Hence, an increased understanding of the molecular mechanism by which microglia regulate NSCs is important in order to design novel interventions.

5. Conclusions

- The enteric neural stem/progenitor cells did not differentiate when transplanted into the brain, triggered neuroinflammation, and did not restore irradiation-induced loss of hippocampal neurogenesis, at least not under the current protocol. Hence, modification of the experimental protocol is probably required.
- Cortical stroke induced long-term neuronal progenitor migration from the subventricular zone toward the injured site.
- Generation of cortical neurons after stroke was very rare, despite the continuous supply of neuroblasts. This raises the possibility that the migrating neural progenitors in the injured area might have biological function(s) other than cell replacement.
- Inhibition of caspases did not promote cortical neurogenesis after stroke, but it reduced the pro-inflammatory profile, and even diminished stroke-induced neural progenitor response. It therefore seems to be a double-edged sword when applied for neuroprotection after injury, as it might halt endogenous brain healing.
- Microglial subtypes differently regulated neural stem cells. The pro-inflammatory (M1 phenotype) augmented NSC death and promoted astrocytic differentiation, whereas the anti-inflammatiory (M2 phenotype) promoted NSC migration.

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