In vivo responses of neural progenitor cells to extracellular matrix signaling under pathological conditions

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To my family

In vivo responses of neural progenitor cells to extracellular matrix signaling under pathological conditions

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ABSTRACT

The adult brain has an inherently low capacity for tissue regeneration, and neurons lost to disease or injuries are normally not replaced. This becomes especially apparent when stroke or brain trauma occurs. The discovery of neurogenesis and neural stem cells in the adult brain has opened up new avenues for treatment of the injured brain. In the adult brain, neural stem cells are present in two distinct neurogenic areas, the subventricular zone and the hippocampus. These neural stem cells are capable of generating new neurons throughout life. As these cells have been shown to respond to signals from the surrounding extracellular matrix, we were interested in how we can utilize these for the purpose of regenerating lost neurons. In **paper I**, we use an injectable self-assembling peptide amphiphile coupled to a migration-inducing peptide sequence derived from Tenascin-C, a glycoprotein natural occurring in the extracellular matrix of the neurogenic areas. In this paper, we re-directed cells from their normal path, the rostral migratory stream, to migrate into the cortex using the Ten-C-peptide amphiphile. Furthermore, this was done without causing an exacerbated glial response or glial scar. In **paper II** we used a similar biomaterial with another naturally occurring sequence, RGDS, derived from fibronectin. In this study, we were interested in the potential of the RGDS-peptide amphiphile as a possible cell scaffold for neural stem cell transplants. The introduction of a foreign material into the CNS can lead to a strong reactive gliosis response from endogenous astrocytes and microglia. Surprisingly, not only did the RGDS-peptide amphiphile not elicit a stronger glial response than the control needle wound injury, but it rather suppressed the reactivity of astrocytes and microglia. These results indicate great potential of the biomaterial for future use an artificial ECM for cell transplants in the CNS. In **paper III,** we were interested in the role of RHAMM, the receptor for hyaluronan, one of the most abundantly expressed glycosaminoglycans in the brain extracellular matrix, in stroke-induced neurogenesis. We observed RHAMM in both unlesioned and unlesioned animals being important for cell proliferation and neurogenesis in both the SVZ and hippocampus of the adult brain.

Keywords: Peptide Amphiphiles, Ten-C, RGDS, RHAMM, Reactive Gliosis, ECM

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SAMMANFATTNING PÅ SVENSKA

Stroke och traumatiska hjärnskador har ofta ödesdigra konsekvenser för den drabbade individen. Konsekvenser som kan innebära både nedsatt kognitiv och fysisk förmåga, som i sin tur leder till genomgripande förändringar i livet. Det har länge varit trott att hjärnans väldigt bristfälliga förmåga för återhämtning efter skada är en effekt av att den vuxna hjärnan saknar stamceller. Hjärnan sågs under en väldigt lång tid som ett statiskt organ. Dock ändrades detta i slutet av 60-talet i samband med att man upptäckte neuronala stamceller även i den vuxna hjärnan. Man lyckades visa att nybildningen av nerver i hjärnan är ett fenomen som inte slutar efter utvecklingen, utan att det snarare är en livslång process, även hos människor. Trots att antalet neuronala stamceller avtar med åldern, och i den vuxna hjärnan är antalet stamceller betydligt färre än i fosterstadiet, har de fortfarande en väldigt viktig roll när det kommer till underhåll av hjärnan då de ersätter både åldrande och skadade celler. Neuronala stamceller är celler som kan ge upphov till astrocyter, oligodendrocyter (gliaceller) och neuron (nervceller). Tack vare detta kan de bidra till återskapandet av vävnad, främst nervceller, som skadats eller dött pga trauma. Dessa två upptäckter, stamceller samt nybildning av nerver i den vuxna människohjärnan, revolutionerande neurovetenskapsfältet och etablerade vad vi idag kallar för ”adult neurogenes”. Detta gav även hopp om att hitta nya behandlingsmetoder för att kunna hjälpa hjärnan återskapa förlorad vävnad och celler efter skada, främst nervceller och återfå förlorad funktionalitet. Hjärnan är otroligt komplex, och under de senaste decennierna har forskningen gjort väldigt stora framsteg inom fältet, men trots det är vi fortfarande långt ifrån att kunna ”bota” hjärnan, och vi har mycket kvar upptäcka.

I denna avhandling har vi studerat effekten av tre stycken naturligt förekommande molekyler i hjärnan. Dessa har studerats med syftet att förhoppningsvis kunna hjälpa hjärnan återhämta sig efter skada och återbilda nerver. Vi har här använt oss av ett vätskeliknande biomaterial, peptide amphiphiles (PA), som hårdnar och bildar en gele när det kommer i kontakt med fysiologiska halter av joner. Joner som förekommer i tex blod. När man injicerar ett biomaterial löper man alltid risken att kroppens egna immunförsvar stöter bort det främmande objektet. Ett idylliskt biomaterial är ett biomaterial som kan ha en biologisk påverkan utan att skapa en större inflammatorisk respons än nödvändigt. Här har vi med hjälp av detta biomaterial som bär på en signal för inducering av migrering hos stamceller, dirigerat om migrerande stamceller från deras naturliga rutt till en ny slutdestination. Det visade sig även att biomaterial som vi använde, E_2Ten-C PA, inte påverkade den inflammatoriska responsen. Detta skulle kunna

användas senare vid tex stroke eller andra hjärnskador för att öka antalet stamceller vid det drabbade området, och på så sätt öka nervbildningen. Vidare så var vi även intresserde av att se ifall vi kan potentiellt använda detta biomaterial för att även transplantera stamceller i den adulta hjärnan. För detta använde vi en annan naturligt förekommande sekvens, RGDS. Till våran förvåning såg vi här att RGDS sekvensen har en dämpande effekt på den inflammatoriska responsen. Djur som fick E_2RGDS PA peptiden transplanterad uppvisade betydligt lägre av de markörer som används för att bedömma den inflammatoriska responsen.

Stamceller, och celler generellt, regleras till hög grad av sin direkta omgivning. Signaler från deras direkta omgivning kan få dom att migrera, föröka sig eller bilda nya nerver. Forskning har också visat att diverse hjärnskador, som tex stroke, kan leda till en ökad nynervsbildning. Därför var vi även intresserade av att se hur RHAMM, som är cellreceptorn till av dom mest tillgängliga glukosaminoglykaner i hjärnan, har för en effekt när det kommer till nynervsbildnings efter stroke. Här använde vi möss som saknar den fullständiga genen för att producera RHAMM. Våra resultat visade sig att RHAMM är högst delaktig i båda cell prolifieringen i de neurogena zonerna (områden där nynervsbildnings), både före och efter stroke. Detta för oss ytterligare ett steg närmre att förstå hur vi kan använda hjärnans egna förmåga för återhämtning för att hjälpa till vid skada.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Motalleb R, Berns EJ, Patel P, Gold J, Stupp SI, Kuhn HG. In vivo migration of endogenous brain progenitor cells guided by an injectable peptide amphiphile biomaterial. J Tissue Eng and Reg Med 2018; 12: e2123-e2133.

II. Motalleb R, Berns EJ, Stupp SI, Kuhn HG. Glial Response to an injectable peptide amphiphile biomaterial. *Manuscript*

III. Motalleb R, Lindwall C, Kuhn HG. Neurogenesis after cortical stroke in the adult brain of hyaluronan receptor RHAMM knockout mice *Manuscript*

CONTENT

ABBREVIATIONS

BrdU	Bromodeoxyuridine
CNS	Central nervous system
DAB	3,3'-Diaminobenzidine
DCX	Doublecortin
ECM	Extracellular matrix
GFAP	Glial fibrillary acidic protein
HMMR	Hyaluronan mediated motility receptor (gene)
Iba1	Ionized calcium-binding adapter molecule 1
NSPC	Neural stem progenitor cell
OB	Olfactory Bulb
PA	Peptide amphiphile
PFA	Paraformaldehyde
RGDS	Arginine-Glycine-Aspartate-Serine
RHAMM	Receptor for hyaluronic acid mediated motility (protein)
RMS	Rostral migratory stream
SGZ	Subgranular zone
SVZ	Subventricular zone
TBI	Traumatic brain injury
TBS	Tris-buffered saline
Ten-C	Tenascin-C (protein)

1 INTRODUCTION

1.1 ADULT NEUROGENESIS

The inherent capacity of the adult brain to regenerate nerve cells is insufficient, which becomes evident when stroke or other brain trauma occurs, causing massive cell death and functional damage without any significant tissue regeneration. This was long assumed to be an effect of the adult mammalian brain lacking neural stem cells capable of replacing dead neurons. But in the late 1960s, Joseph Altman changed the field of neuroscience by demonstrating in a series of publications that proliferation of cells in the postnatal and adult rat brain led to what seemed to be newly generated neurons of the hippocampus and olfactory bulb (Altman, 1969). Following this, Fernando Nottebohm indirectly demonstrated neurogenesis in the subventricular zone of the songbird by showing an increase in neuron numbers during the singing season (Goldman & Nottebohm, 1983). But it was not until another decade later that the concept of adult neurogenesis became accepted for higher mammals and a shift in the dogma was made, due to two milestone studies, Reynolds and Weiss isolating and identifying adult neural stem cells from the adult mammalian SVZ (Reynolds & Weiss, 1992), followed by Fred Gage's discovery of neural stem cells in the hippocampus (Palmer *et al.,* 1995; Ray *et al.,* 1995). Another major discovery came in the late 1990's, when Peter Eriksson and colleagues demonstrated adult neurogenesis in the human hippocampus (Eriksson *et al.*, 1998). These discoveries established the field of adult neurogenesis, and opened up avenues for novel treatment possibilities of individuals affected by stroke or traumatic brain injury using endogenous neural stem cells. Much progress has been made in the field of adult neurogenesis, but many discoveries are left before we can unlock the full potential of neural stem cells.

Neurogenesis is the process in which in neural stem cells produce new functional neurons. Neural stem cells are multipotent cells capable of self-renewal, but to also give rise to daughter cells that can differentiate into neurons, astrocytes and oligodendrocytes. The neural stem cells in the SVZ are located in the lateral walls of the lateral ventricles, separated from the ventricle space and CSF by a layer of ependymal cells. The SVZ runs alongside the entire ventricle wall, and while cells proliferate throughout the entire structure, the pool of proliferating cells is much larger in the anterior regions. The stem cells in the SVZ that will eventually differentiate and generate new neurons in the olfactory bulb (OB) are known as B-cells. They are a population of slowly diving stem cells originating from radial glial cells during early stages of brain development (Merkle *et al.,* 2004). In the rodent brain, these B-cells produce C-cells, i.e. rapidly dividing intermediate progenitor cells. The progenitor C-cells in turn give rise to A-cells which are the neuroblasts that migrate to the

olfactory bulb for differentiation into olfactory interneurons (Doetsch *et al.*, 1999; Mirzadeh *et al.*, 2008).

Kuhn and colleagues later reconfirmed adult hippocampal neurogenesis by double-immunolabeling. Using antibodies against BrdU injections for labelling dividing cells the polysialylated form of neural cell adhesion molecule (PSA-NCAM) for neural progenitor cells, and NeuN for mature neurons, they could demonstrated proliferation and production of new neurons in the dentate gyrus of adult rats (Kuhn *et al.*, 1996). Eriksson et al demonstrated the existence of adult neurogenesis in the human hippocampus (Eriksson *et al.*, 1998) using the same methodology. We now know that in the adult hippocampus, neurogenesis is restricted to the subgranular zone (SGZ) of the dentate gyrus, located at the border between the hilus and the granular cell layer. Neurons in the SGZ extend dendrites into the molecular layer and elongate axons into the CA3 regions for synaptic connections. By retrospectively birth dating neurons in the hippocampus, Spalding estimated that approximately 1400 new neurons are added daily to the adult human hippocampi (Spalding *et al.*, 2013), evidence of quite a substantial neurogenesis in human adults. This was done using a very ingenious method (Spalding *et al.*, 2005) that took advantage of the global increase in atmospheric levels of ^{14}C due to nuclear weapons testing during the cold war, and the 1963 international test ban causing a decline in ^{14}C levels. Atmospheric ^{14}C forms CO_2 by reacting with O2, and enters the biotope by way of photosynthesis. Humans in turn integrate ^{14}C into our genomic DNA by consuming plants and animals with elevated levels of ^{14}C.

The existence of postnatal and adult neurogenesis has proven to be necessary for maintaining memory and learning throughout life (Aimone *et al.*, 2014), but it also brought forth the idea of the adult brain being capable of regenerating lost function after injury. Studies have shown that ischemic insults to the brain can induce cell proliferation and neurogenesis in both neurogenic zones of the adult brain, the SVZ and SGZ (Ohira, 2011). When stroke occurs, the NSCs proliferate and expand their pool, differentiate into glial cells and neuronal progenitors for the subsequent migration to the lesion site (Arvidsson *et al.*, 2002).

1.2 NEUROBLAST MIGRATION

The transiently amplifying C-cells of the SVZ give rise to migratory A-cells, known as neuroblasts. Neuroblasts migrate from the SVZ to the most rostral part of the rodent brain, the olfactory bulb, within a structure known as the rostral migratory stream (RMS).

The RMS is a continuation of the SVZ reaching the OB. During development, the lateral ventricle of SVZ, that reached into the olfactory bulb, collapses and its wall eventually form the RMS. In the adult brain, moving rostral in the SVZ, the structure narrows gradually to become the RMS. Even though the RMS in rats is several millimeters in length, which is a considerable distance to migrate for a cell, neuroblasts travel the entire length of this well-defined path in a very strict and ordered fashion without leaving the RMS. This migration is known as tangential migration, and occurs as chain migration. Chain migration is a quite unique feature of neuroblasts using both extracellular cues and each other for migratory guidance. As the name implies, the neuroblasts form chain-like aggregates while migrating, the leading cell will extend its growth cone and pull itself forward, leaving behind a tail for the other cells to adhere to and follow. This allows for the neuroblasts to slide past each other, forming chains of migrating neuroblasts. This mode of travel results in considerably higher speeds of migration compared to other types of migration. Furthermore, the tangential (rostral) migration is also supported by stationary cells within the RMS. In the rodent brain, astrocytes ensheathe both the RMS but also smaller aggregates of chain migrating cells (Cleary *et al.*, 2006), guiding the cells to their final destination, the OB. Neuroblasts have also been shown to use the dense network of blood vessels running alongside the RMS as support for their migration (Snapyan *et al.*, 2009). There are also several important signaling cues and support from the ECM guiding the tangential migration, including laminins and tenascins (Franco & Muller, 2011). Regions in the brain besides the SVZ and RMS can also regulate neuroblast migration. The septum and the choroid plexus have been shown to produce the chemorepellent Slit, which binds to the Robo receptor expressed by neuroblasts and progenitor cells in the SVZ (Hu, 1999; Wu *et al.*, 1999).

Although there are several similarities between the RMS found in rodents and humans, such as the tangential migration exhibiting closely associated chains of DCX/PSA-NCAM positive cells, there are also quite a few differences. While in the rodent, the RMS is an uninterrupted stream of migrating neuroblasts with the OB as final destination, in humans this is not as clear. There seems to be features of SVZ neuroblast migration in humans, i.e. cluster of neuroblasts but no continuous RMS, and it remains open if the cells actually

reach the OB and give rise to neurons in the adult human brain. Instead, the Frisén group proposed, based on their ^{14}C data, that migration takes place radially rather than tangentially, meaning SVZ neuroblasts migrate in the adult human brain into the striatum instead (Ernst *et al.*, 2014).

Under pathological conditions neuroblasts are able to migrate into the striatum, septum, frontal cortex, corpus callosum instead of their normal migratory path described above. Injuries, such as stroke and other pathologies, can induce alternative migratory responses for neuroblasts including migration into the cortex (Arvidsson *et al.*, 2002; Goings *et al.*, 2004; Ohab & Carmichael, 2008; Osman *et al.*, 2011; Faiz *et al.*, 2015). While neuroblast migration in the RMS and its regulation have been studied quite extensively, the molecular cues that could induce a change of migration destination are less well described. One known chemokine is SDF1 alpha (CXCL12). SDF1 alpha is secreted at lesion sites and can attract neural progenitor cells from the SVZ/RMS via the CXCR4 receptor (Saha *et al.*, 2013; Merino *et al.*, 2015). The mechanism for neuroblast migration outside the RMS has also been studied. Cells migrating towards the lesioned area can form clusters or aggregates, indicating that migration of neuroblasts outside the RMS still related to chain migration. Migration of SVZ neuronal precursors to the lesioned area can also be astrocyte- and blood vessel-mediated (Stoll *et al.*, 1998; Mason *et al.*, 2001; Arvidsson *et al.*, 2002; Saha *et al.*, 2013).

1.3 THE NEURAL EXTRACELLULAR MATRIX

Tissues are comprised of a combination of cells and non-cellular components. The non-cellular environment is known as the extracellular matrix (ECM) which consists of an insoluble network of proteins and carbohydrates produced by cells, filling most of the intercellular spaces. It is a highly dynamic, heterogeneous mixture of tissue-specific molecules, composed of a very diverse and complex network of proteins, such as collagens, elastin, laminins and fibronectin, as well as proteoglycans and glycosaminoglycans (Theocharis *et al.*, 2016).

The ECM functions as a 3-dimensional structural support for the embedded cells, but it also provides the embedded cells with biochemical and biomechanical signals (Bonnans *et al.*, 2014). The physical properties of the surroundings can have modulatory effects on cells; characteristics such as topography, elasticity and stiffness have all been shown to affect cell behavior (Bellamkonda & Aebischer, 1994; Saha *et al.*, 2008; Chen *et al.*, 2012; Kim *et al.*, 2012a; Kim *et al.*, 2012b; Stukel & Willits, 2016). Further, the ECM has also been shown to regulate many cellular processes such as cell migration, growth, survival and differentiation via biochemical signals. Examples of such signaling involve molecules such as Ten-C, RGDS (fibronectin), hyaluronan etc. The extracellular matrix is also important in the mediation of cell-cell signaling.

The brain ECM is not homogenous, but varies due to the absence or presence of specific ECM molecules, although more often due to differences in the concentrations of ECM molecules. Specific compartments or niches are created and maintained by locally residing cells in the region secreting niche-specific ECM molecules, thereby creating their own microenvironment. The role of the neurogenic stem cell niches is to provide signals for stem cell survival and proliferation, but also for stem cells to keep their stemness (i.e. self-renewal) as they replicate. Other essential components contributing to the neurogenic niches are growth factors and cytokines, which can be associated with the ECM.

In this thesis we have focused on three ECM-associated molecules; Tenascin-C, fibronectin (specifically the peptide sequence RGDS derived from fibronectin), and RHAMM, the cell receptor for the ECM glycosaminoglycan hyaluronan.

1.3.1 TENASCIN-C

The tenascin family of ECM glycoproteins consist of five members; tenascin-C, tenascin-R, tenascin-W, tenascin-X and tenascin-Y (Joester & Faissner, 2001). The five members of the tenascin family all have a multimodular structure, sharing overall structure and several structural motifs. The tenascin glycoproteins are characterized by a cysteine-rich N-terminus, followed by one or more epidermal growth factor-like (EGF-like) repeats, several sequences of fibronectin type III (FNIII)-like modules, and sequence homologies to fibrinogen-β/γ chains at the carboxy-terminus (Pas *et al.*, 2006). Tenascins have a wide range of functions due to their multimodular structure, enabling interactions with a large number of ligands. Tenascin-C (Ten-C) is the founding member of the tenascin family and by far the most extensively studied member. The Ten-C molecule was originally discovered in the 1980s by several independently working laboratories. As each group reported on the glycoprotein based on its discovered function, Ten-C has historically been known under several names such as; myotendinous antigen, hexabrachion, cytotactin, neuronectin and J1220/200. Although the Ten-C protein has been associated with a multitude of cellular processes, as evident by its many names, our focus in this thesis will be on neural functions of Ten-C, specifically in the adult brain. Tenascin-C is a large approximately 190 kDA to 320 kDA glycoprotein assembled from six monomers forming a hexameric protein (Erickson & Inglesias, 1984) located extracellularly in the ECM (Erickson & Bourdon, 1989).

In healthy adult tissue, the expression pattern of Ten-C is very distinct and regulated, in contrast to the early stages of CNS development where Ten-C is transiently expressed in high abundance (Crossin *et al.*, 1989; Bartsch *et al.*, 1992; Bartsch *et al.*, 1994; Mitrovic *et al.*, 1994; Kawano *et al.*, 1995; Crossin, 1996; Gotz *et al.*, 1997; Joester & Faissner, 1999). In the adult CNS, Ten-C expression is limited to reactive astrocytes, certain populations of glial cells and the neural stem cell niches (Brodkey *et al.*, 1995). Ten-C expression can be seen in specific cell populations associated with, or adjacent to, regions with active neurogenesis, such as the SVZ and the hippocampus which that are densely populated by neural progenitor stem cells (NSPCs) (Miragall *et al.*, 1990; Gates *et al.*, 1996; Jankovski & Sotelo, 1996). This is not surprising as Ten-C has an important role in the modulation of neural cell development and function (Sekeljic & Andjus, 2012). Tenascin-C is also highly expressed in the rostral migratory stream (RMS), the migratory path of neuroblasts migrating from the SVZ to the olfactory bulb (Thomas *et al.*, 1996).

Tenascin-C has numerous functions, and has been implied in modulating NSPC behavior in various ways. Its role as a regulator of cell migration has been clearly established in several studies that have shown Ten-C promoting neurite outgrowth of neurons in both the PNS and the CNS (Crossin *et al.*, 1990; Wehrle & Chiquet, 1990; Lochter *et al.*, 1991; Lochter & Schachner, 1993; Taylor *et al.*, 1993; Gotz *et al.*, 1996). Tenascin-C regulates neural progenitor cell migration and proliferation (Garcion *et al.*, 2001). Andrews and colleagues demonstrated Ten-C being a potent promoter of neurite outgrowth via the extracellular α9 integrin domain (Andrews *et al.*, 2009). The Ten-C amino acid sequence VFDNFVLK from the alternatively spliced FNIII fnD domain has also been shown to promote neurite outgrowth, mediated by the integrin subunits α7β1 (Mercado *et al.*, 2004). Additional Ten-C FN domain sequences, besides the VFDNFVLK, promote neurite outgrowth and extension but via other integrin subunits, such as the fn6-8 and integrin α8β1 chains (Varnum-Finney *et al.*, 1995) or via other receptors such as the F3/contactin and fnB-D (Rigato *et al.*, 2002). As stated above, CNS neurons have affinity for several recognition sites in the Ten-C FNIII domains. While some FNIII domains support neurite extension, growth and differentiation of CNS neurons, other domains can produce inhibitory or repulsive effects on neurons (Gotz *et al.*, 1996).

While expression of tenascin-C outside of the neurogenic niches is restricted in the adult CNS, Ten-C expression has been shown to be upregulated during pathological conditions such as inflammation after injuries (Laywell *et al.*, 1992; Crossin, 1996; Gates *et al.*, 1996; Deller *et al.*, 1997) and during infections or tumor growth (Chiquet-Ehrismann & Chiquet, 2003; Orend & Chiquet-Ehrismann, 2006). Astrocytes appear to upregulate Ten-C expression in response to injury (Laywell *et al.*, 1992; Crossin, 1996; Gates *et al.*, 1996; Deller *et al.*, 1997).

Unravelling the many functions of the Ten-C glycoproteins is a complicated task due to their modular repeats. The modular structure allows for the combination of various splice variants of the FNIII repeats, resulting in a large number of Ten-C isoforms. In human Ten-C, the FNIII repeats 1-8 are always present and "consistent" (not spliced, but can be modified by glycosylation), but there are nine additional FNIII repeats (FNIII A-D) located between the FNIII 5 and 6 where alternative splicing is possible. This can result in potentially 511 variants of the human Ten-C protein alone.

Mapping of tenascin functions is further complicated as the tenascin core proteins can be modified post-translationally by glycosylation (Woodworth *et al.*, 2004) and proteolytic cleavage by matrix metalloproteinase-2. Also, not

only is Ten-C variant expression and subsequent function context-specific, but factors such pathology or even pH can also affect which splice variant are produced (Borsi *et al.*, 1996). Ten-C has also been demonstrated to have distinctive effects on different cell types, which complicates issues even further. For example, Ten-C is an adhesion molecule for some cells, while for others it has anti-adhesive properties (Erickson, 1993), and while in some cases considered a migration-inducing molecule, other isoforms of Ten-C function as a growth cone repellant (Meiners *et al.*, 1999).

1.3.2 RGDS

The RGDS amino acid sequence (arginine-glycine-aspartic acid-serine) is a cell adhesion sequence derived from fibronectin. RGDS was originally discovered in 1984 by Pierschbacher & Ruoslahti (Pierschbacher & Ruoslahti, 1984b; a). They discovered that antibodies binding to specific amino acid sequences on the fibronectin protein could effectively block its function, and thereby inhibit cell attachment. Fibronectin is a high-molecular weight (~440kDa) integrin binding glycoprotein located in the extracellular matrix of cells. The RGDS sequence is located on the FN-III10 module of the cell-binding domain of fibronectin (Dickinson *et al.*, 1994). With fibronectin being widely expressed in the brain ECM, subsequently so is RGDS. While fibronectin has been shown to have a major role in various cellular processes such as adhesion, proliferation, migration and differentiation, the RGDS functional motif, and the shorter RGD sequence, have been identified as the essential cell-attachment domain in fibronectin (Pierschbacher *et al.*, 1982; Pierschbacher & Ruoslahti, 1984a; Yamada & Kennedy, 1984; Ruoslahti & Pierschbacher, 1986). The RGD tripeptide is not exclusive to fibronectin as it has also been found within other proteins such as vitronectin, fibrinogen, von Willebrand factor (Hirano *et al.*, 1991). The endogenous RGDS sequence allows mainly for cellular interactions with the ECM through binding with several cell membrane integrins. RGDS is recognized by the two non-covalently associated subunits, α and β, of integrins. This allows for the RGDS sequence to be widely recognizable by several different integrins. The following cell membrane integrins; α3ß1, αvß5, α5ß1, αVβ3, αIIbß3, α8ß1, αvβ1 and αVß6, have all been identified as recognizing and binding to the RGD(S) sequence (Buck & Horwitz, 1987; Plow *et al.*, 2000).

According to the original RGDS studies by Pierschbacher & Ruoslahti, the RGD(S) sequence is the minimum signal required for the attachment of cells to fibronectin. The serine residue can be replaced by another amino acid

chemically similar to serine, such as cysteine, although including the serine amino acid seemingly results in optimal activity (Pierschbacher & Ruoslahti, 1984b). Each of the individual amino acids contributes to the binding of cells to fibronectin, as substituting individual amino acids in the sequence modified significantly the binding activity. The study also experimented with various other amino acids with corresponding charge relationship as the RGDS sequence, and again they did not observe the same cell adhesion promoting effect as with the RGDS sequence in their model (Pierschbacher & Ruoslahti, 1984b). The functional RGD(S) sequence is among the most effective sequences for promoting cell adhesion on material surfaces. This has prompted the incorporation of the RGDS motif, and the shorter RGD sequence, into and onto many polymers and biomaterials.

1.3.3 RHAMM-RECEPTOR FOR HYALURONAN-MEDIATED MOTILITY

The glycosaminoglycan hyaluronan (Hyaluronic Acid, HA) is major ECM component and is expressed abundantly throughout the brain ECM. Hyaluronan is an important glycosaminoglycan in the ECM, capable of regulating cell processes such as proliferation, migration, differentiation and angiogenesis (see (Su *et al.*, 2019) for review). The Receptor for Hyaluronan-Mediated Motility (RHAMM, also known as CD168/HMMR/IHABP, gene name HMMR) is one of two main receptors for hyaluronan. In the early 1990s, the RHAMM receptor was discovered and located at the cell surface of ras-transformed fibrosarcoma cells (Hardwick *et al.*, 1992). RHAMM, as evident by its name, was originally characterized as a pro-migratory molecule for its ability to mediate cell motility responses to hyaluronan as its expression was found to be elevated in motile fibroblasts, tumor cells, smooth muscle cells but also in migratory macrophages after injury. Since then, RHAMM has been recognized as a multifunctional protein associated with a much wider range of functions. It has been identified as a prognostic factor for cancer and appears to have an active role in tumorigenesis as it modulates cell migration and invasion in various forms of cancer (Shigeishi *et al.*, 2014; Misra *et al.*, 2015; Mele *et al.*, 2017).

While Hardwick et al identified the RHAMM protein as a cell surface receptor on tumor cells, further studies have recognized RHAMM to be distributed to a large extent intracellularly (Hardwick *et al.*, 1992; Zhang *et al.*, 1998; Assmann *et al.*, 1999; Evanko & Wight, 1999; Hascall *et al.*, 2004). Intracellular RHAMM has been located at the cell nucleus and cytoplasm, but

also with the cytoskeleton and mitochondria. The different functions of intracellular RHAMM have not been completely elucidated yet, but the protein has been associated with actin filaments, dynein, mitotic spindles, microtubules and the centrosomes, indicating intracellular RHAMM protein as a major component in many cellular processes such as differentiation, cell signaling and maintaining stem cell pluripotency (Maxwell *et al.*, 2003). Further, the interaction between hyaluronan and RHAMM triggers a number of signaling pathways. Both cell surface and intracellular RHAMM has been shown to mediate and regulate cell signaling pathways and kinases involving MAP kinases, RAS GTPases, focal adhesion kinases, protein kinase C and phosphatidylinositol kinase (Wang *et al.*, 1998).

Interestingly, although studies have shown RHAMM to be important for several biological process, both during development and in the adult, the deletion of HMMR is not lethal and does not seem to cause any major deleterious effects (Tolg *et al.*, 2003).

1.4 STROKE

Stroke is one of the leading causes of death and disability worldwide, with extreme cost and consequences for the society. Stroke occurs when the normal blood flow to the brain is interrupted or lost, either by blockage (ischemia) or rupture of a brain artery (hemorrhagic). The loss of blood flow causes loss of nutrients and oxygen to the affected area, which in turn causes sudden neuronal cell death, and often severe tissue loss to the brain. The severity of functional impairment after stroke is dependent on both the size of the lesion and the location of the affected brain area. Stroke can lead to devastating life-altering changes for the individual, with mainly sensory, motor and cognitive disabilities (Johnston *et al.*, 2009).

The stroke affected area in the brain can be divided into two parts, the ischemic core and the penumbra. The ischemic core is the region most severely affected by the stroke. Within minutes from the occurrence of occlusion, cell death can be detected, turning healthy tissues into a necrotic region with irreversible cellular damage and lost functionality. Following an intense inflammatory response to the lesion, the ischemic core becomes isolated by a glial scar, hindering the spread of apoptotic signals from the necrotic region, but also hindering the re-population and regeneration of the dead/dying core. The necrotic core is considered to be "lost" with no chance of rescue. Adjacent to the ischemic core, and at later stages separated by the glial scar, is the penumbra. The penumbra also suffers from impaired blood flow, hypoperfusion, but to a lower extent. Cell death in the penumbra is not as prominent as in the core, but rather delayed neuronal cell death occurs over the following days. While still compromised, tissue within the penumbra can be potentially salvaged with therapeutic interventions such as reperfusion, hypothermia, neuroprotective pharmacological treatments (Moustafa & Baron, 2008). While stroke causes massive cell death and neuroinflammation, it can also trigger neural stem cell responses. An increase in proliferation of stem cells can be observed in both the SVZ and the SGZ after ischemia (see chapter 1.2).

1.5 REACTIVE GLIOSIS

Injury to the CNS, such as stroke or traumatic brain injury, can trigger a strong neuroinflammatory response (Stoll *et al.*, 1998). The response can also be triggered by the introduction of a foreign exogenous substance (Anderson *et al.*, 2008; Yanez *et al.*, 2017). During neuroinflammation, cytokines, chemokines and reactive oxygen species are produced (Kempuraj *et al.*, 2016). They advance the inflammatory process by recruiting circulating leukocytes, but also by "activating" local glial cells, in a process known as reactive gliosis. We have in this thesis focused on the local aspect of the neuroinflammatory response, namely the gliotic response from the endogenous glial cells. Two of the main cell components of this gliotic response are the local microglia and the astrocytes (Ridet *et al.*, 1997; Buffo *et al.*, 2010).

Glial cells constitute the largest cell population in the adult brain. The glial cell population of the CNS is comprised of microglia, astrocytes and oligodendrocytes. Microglia were originally considered only as the resident immune cells of the adult CNS (Kim & de Vellis, 2005), but they are very much essential in supporting the normal functioning of the healthy CNS with numerous contributions beyond their immune role (Hanisch & Kettenmann, 2007). In the normal healthy adult brain, both astrocytes and microglia are considered to be in a "resting" state, with their main functions being that of maintaining the CNS homeostasis by various housekeeping functions and general surveillance of the surrounding tissue for irregularities (Barres, 2008; Sofroniew & Vinters, 2010).

Astrocytes are the most abundant cell type in the brain and account for approximately 50% of the glial cell population, with microglia corresponding to 5-20% of the total cellular composition of the brain. The cell body of "resting" ramified astrocytes is 10-20 µm with their processes extending another 20-30 µm. Astrocytes are an extremely heterogenous population of cells. Amongst their actions are numerous processes, such as providing energy metabolites to neurons, regulating blood flow, regulating synaptic functioning and plasticity, but also maintaining a fluid balance as well as ion and transmitter homeostasis (Sofroniew, 2009). Microglia are the resident macrophage population of the CNS. Microglial are slightly smaller than astrocytes, having a 5-10 µm cell body with numerous small diameter processes radiating from the soma (Davis *et al.*, 1994). These processes can extend several times the size of the microglial soma, and are highly branched. Although long considered dormant in the healthy tissue, they are indeed very active as they are constantly surveying the brain parenchyma for inflammatory signals, infections, and other disturbances.

Reactive gliosis (astrogliosis and microgliosis) is not an all-or-none process, but rather a spectrum of both qualitative and quantitative changes in astrocytes and microglia. Further, these changes are highly context-driven and depend on both the microenvironment of the injury and the type of injury/pathology (Cragnolini *et al.*, 2018). The extent of the glial cell response is determined by the type and severity of the pathological event. Both microglia and astrocytes have their characteristically ramified morphology in their "resting" state. When injury occurs or during pathological conditions, reactive astrocytes and microglia can be observed transitioning to a more amoeboid morphology (Pekny & Nilsson, 2005; Block *et al.*, 2007). Characteristic changes in reactive astrocytes include morphological changes such as cellular hypertrophy, emission of long and arborized processes, and upregulation of the intermediate filament components. While astrocytes can be identified using various markers, none are exclusive for reactive astrocytes. Ramified and reactive astrocytes are most often identified using GFAP, Vimentin or S-100β as markers, with their elevated expression levels being the sign of astrogliosis (Pekny & Nilsson, 2005; Sofroniew & Vinters, 2010). Astrogliosis is also accompanied by an increase in astrocyte cell proliferation and migration of astrocytes towards the site of injury. Microglia are considered the macrophages of the CNS, with phagocytosis as one of their main immune functions (Davalos *et al.*, 2005; Walter & Neumann, 2009). And just as with astrocytes, several changes can be observed in reactive microglia compared to their "resting" state. Microglia become motile when reactive, showing migration towards lesions or foreign material using chemotactic gradients (Davalos *et al.*, 2005; Dibaj *et al.*, 2010). Typical markers for microglia include Iba1 and the systemic macrophage-specific marker ED1 (CD68). During microgliosis, higher expression levels of both markers can be detected.

A consequence of the reactive gliosis response to injury, pathology or the presence of foreign material is the formation of a glial scar (Wanner *et al.*, 2013). The glial scar tissue consists primarily of reactive astrocytes, but also microglia, NG2-glia, oligodendrocyte precursor cells, and non-neuronal cells such as pericytes, ependymal and in cases where the meninges are compromised even meningeal cells (Goritz *et al.*, 2011; Cregg *et al.*, 2014). Moreover, the glial scar is enriched in ECM molecules such as collagen IV, vimentin and proteoglycans (Bradbury *et al.*, 2002). Immune cells will further contribute to activation of glial cells and scar formation by producing chemokines, cytokines and enzymes promoting activation (Cregg *et al.*, 2014). During scar formation, mature astrocytes can re-enter the cell cycle and proliferate, producing more scar-forming astrocytes (Bush *et al.*, 1999; Buffo *et al.*, 2008; Gadea *et al.*, 2008). As with all scars, the glial scars in the CNS and the structural changes to the ECM that are associated with it are long

lasting. Several studies have highlighted the importance and beneficial function of glial scar formation. Complete ablation of all reactive astrocytes and microglia resulted in increased tissue damage, lesion size and neuronal loss in various injury models (Lalancette-Hebert *et al.*, 2007; Hines *et al.*, 2009; Sofroniew, 2009). The lack of a glial scar not only exacerbated the pathology, but also impaired recovery of function. Although the glial scar is initially important and beneficial for the integrity of the CNS parenchyma, separating the affected tissue from the healthy, its long-term effect can arguably be detrimental, since glial scars will persist even after the triggering injury has been resolved. Since the glial scar functions as a dense physical barrier, it also inhibits cellular re-population of the damaged area. The glial scar negatively affects axon regeneration (Silver & Miller, 2004), not only mechanically, but also biochemically via the secretion of inhibitory axonal outgrowth molecules such as chondroitin sulphate proteoglycans (CSPGs), myelin-associated proteins and others (Silver & Miller, 2004). The glial scar is one of the biggest challenges for spinal cord injuries, as the scar limits functional recovery by hindering axonal re-growth across the scar. Further, astrocytes after lesion have been shown to modulate neurite extension by secretion of proteases and protease inhibitors (Ridet *et al.*, 1997), and evidence shows overactivated microglia can be neurotoxic by releasing molecules that can damage neurons and the ECM (Kim & de Vellis, 2005; Block *et al.*, 2007).

1.6 PEPTIDE AMPHIPHILES

1.6.1 STRUCTURE AND DESIGN

The biomaterial used in this thesis is a highly versatile peptide amphiphile which, due to its design, self-assembles into PA nanofibers under physiological conditions. The polymerized biomaterial forms aligned, micrometer-long cylindrical nanofibers with the surface of the PA displaying the biological peptide signal of choice (Zhang *et al.*, 2010). Peptide amphiphiles are a class of peptide-based molecules that can self-assembly into various supramolecular structures, such as vesicles or high aspect ratio nanofibers, thanks to their amphiphilic (hydrophilic and lipophilic) nature. The original PA was developed in 1995 by Matthew Tirrell and Greg Fields (Berndt *et al.*, 1995). The PA used in this thesis was developed and synthesized in 2001 by the Stupp laboratory (Northwestern University, Evanston, IL, USA., (Hartgerink *et al.*, 2001)). The supramolecular design of the PA in our studies consists of four key structural regions: (1) a hydrophobic alkyl tail, (2) a short peptide sequence, (3) charged amino acids, and (4) an optional bioactive signal sequence (see Fig. 1 for the PA structure). The unique design of the PA allows it to mimic the fibrous aspect of the ECM of cells, efficiently acting as a scaffold for cells.

The first region of the PA monomer, the alkyl tail, is a long hydrophobic palmitic acid tail consisting of 16 carbon atoms coupled to the second region. The strong amphiphilic nature of the PA, given primarily by the hydrophobic tail, allows for the self-assembly of the PA by hydrophobic collapse of the tails into the core of the nanostructure. The hydrophobic tail can be further tuned by varying the lengths of the alkyl tail or using other hydrophobic components to modify the PA structure.

Immediately adjacent to the alkyl tail is the second region. This peptide region is most often comprised of a short sequence of hydrophobic amino acids, for example a charged VVAA peptide sequence, that promotes hydrogen bonding and allows the PA to form intermolecular β-sheets. This interaction, combined with the hydrophobic interaction of region 1, is what causes the 1D structural growth of the PA nanofibers. By altering this sequence, the geometry of the nanostructure can also be altered. Using molecular modelling, Stupp and colleagues showed the “significance” of proper interplay between region 1 and 2 for the assembly of PA into cylindrical nanofibers. Excluding the formation of β-sheets in region 2, and having the hydrophobic forces as the only driving force for self-assembly, the PA formed micelles. While, if the hydrophobic

forces are neglected and only β-sheets formation is allowed to occur, the PA molecules will instead form aggregates.

The third region is added to ensure sufficient hydrophilicity and aid solubility in aqueous solutions. This domain is also the region that triggers the self-assembly of the PA when in contact with salts and/or changes in pH (Cui *et al.*, 2010; Webber *et al.*, 2013). Self-assembly is triggered by the charged amino acids exposed to divalent cations, such as $Ca2^{+}$ found in the extracellular spaces or in blood.

The fourth region, the epitope region, is by design located at the outermost end of the self-assembled PA, opposite to the hydrophobic tail (1st region), to ensure that during self-assembly the hydrophobic collapse of the alkyl tail into the central core of the cylindrical PA places the epitope region on the surface of the assembled PA. This allows for the presentation of a desired bioactive signal on the surface of the biomaterial, making the delivered bioactive signal easily accessible for cells. Previous studies from the Stupp lab have featured various mimetic biological epitopes such as glucagon-like peptide 1-mimetic, Maspin, IKVAV, RGDS and Ten-C (Khan *et al.*, 2012; Berns *et al.*, 2014; Li *et al.*, 2014; Zha *et al.*, 2015; Berns *et al.*, 2016).

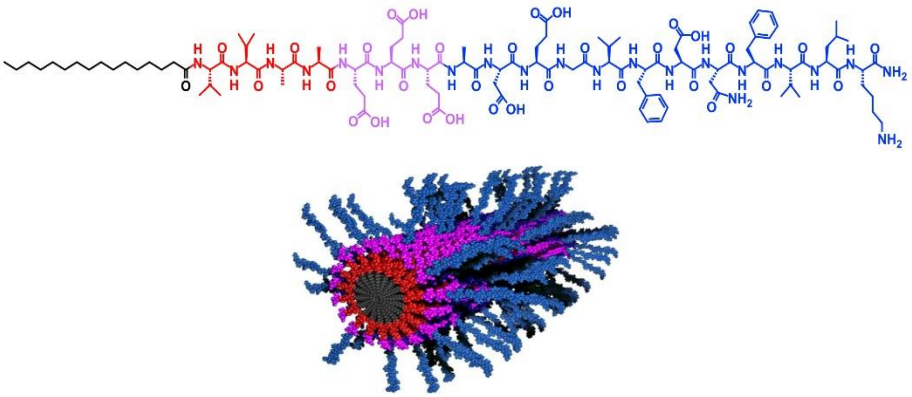

Figure 1. Representative images of peptide amphiphile design. Including a hydrophobic alkyl tail (black – region 1), β-sheet forming sequence (red – region 2), charged amino acids (purple – region 3), and a bioactive epitope (blue – region 4).

1.6.2 SELF-ASSEMBLY MECHANISM

One unique property of the PAs is their ability for self-assembly and gelling. The biomaterials change state from a low-viscosity water-like solution to a more gel-like state under physiological conditions, as in the presence of blood and extracellular fluids. This *in situ*-gelling makes it ideal for intracranial injections, compared to solid transplants of preassembled biomaterials. The formation of monodomain fibrous gels takes place by thermal pathways where isotropic solutions of peptide-containing molecules are converted to liquid crystals. These molecules can then group into long filaments of bundled nanofibers (Zhang *et al.*, 2010).

PAs dissolved in solution are at the nanoscale size, and in an aqueous liquid state. Heating PA molecules in an aqueous solution to 80°C for 30 minutes and then allowed to slowly cool to room temperature increases the viscosity of the solution threefold. The viscosity is increased further when divalent cations are added, leading to the formation of a gel. The increase in viscosity and gel formation is an effect of several interactions; the hydrophobic collapse of the C16 alkyl tail segment accompanied by entropic and enthalpic effects, hydrogen bonding promoted by the second segment, interactions of the middle peptide segments side-chains, and the electrostatic repulsion among the charged amino acids in the third segment. These interactions during the heating/cooling process produce a solution now consisting of aligned filaments, bundles of cylindrical nanofibers, while not affecting the supramolecular structures. The shear from injecting/drawing this solution across a salt containing medium produces a gel with longitudinal macroscopic alignment of the filaments, which is observed only in preheated solutions. The viscosity of the PA can be modified by changing the concentration of the dissolved PA. For a more detailed description of the physical properties and the polymerization process, see (Zhang *et al.*, 2010; Webber *et al.*, 2013).

By changing the chemical properties of the various PA regions, one can produce a variety of different structures with added biological signals if desired. The PAs used throughout this thesis have a cylindrical shape, but by changing the charged region other structures can be achieved. A VEVE peptide segment for example produces a flat sheet-like structure, but when replaced with the VVAA peptide segment, cylindrical vesicle-like nanofibers are formed instead.

1.6.3 APPLICATIONS OF PEPTIDE AMPHIPHILES

The peptide amphiphiles used in this thesis have previously been studied using various bioactive sequences as epitopes in tissues, although not in the brain. The Stupp Lab demonstrated that the peptide amphiphiles support a wide range of cellular functions. One important function is their ability to support cell viability. Berns and colleagues not only demonstrated that the E_2Ten-C PA (used in this thesis) supports neurosphere cell viability, but that it also promotes neurite outgrowth and cell migration in vitro (Berns *et al.*, 2014; Berns *et al.*, 2016). The PA also induces neuronal differentiation and survival of hESC-derived progenitor cells in vivo (Matsuoka *et al.*, 2017). Further, PA with the RGDS epitope has been demonstrated to support in vitro viability and proliferation of Schwann cells (Li *et al.*, 2014), as well as bone marrow-derived mononuclear cells (Webber *et al.*, 2010). Webber also demonstrated a higher survival rate of cells transplanted with the RGDS PA as cell scaffold (Webber *et al.*, 2010).

The ability to tightly control the density of the gelled PA, as well as the pattern and density of one bioactive epitope or a combination of several different signals, gives the PA biomaterial an enormous range of potential functions. Moreover, adding other molecules such as soluble bioactive peptides, or even live cells to the PA expands the possible uses of the peptide amphiphiles even further.

2 AIM

The overall aim of this thesis was to investigate how ECM-associated molecules can be utilized to help the adult brain regenerate lost tissue.

The specific aims of the three papers included in this thesis were to:

I. Investigate if a peptide amphiphile biomaterial carrying the migration-inducing tenascin-C sequence can stimulate neural progenitor cells to migrate towards a novel brain location.

II. Investigate the reactive gliotic response to a transplanted peptide amphiphile biomaterial with a conjugated RGDS sequence to evaluate its potential for cell transplants.

III. Investigate the role of the hyaluronan receptor RHAMM for neurogenesis in the post-ischemic brain

3 MATERIAL AND METHODS

The reader is referred to papers I-III for a more detailed description of materials and methods used in this thesis.

3.1.1 ANIMALS AND ANESTHESIA

All animal experiments were approved by the Gothenburg Committee of the Swedish Animal Welfare Agency (ethical applications nos. 101/2013, 103/2010, 99/12 and 147/12). All animals were housed in a barrier facility with ad libitum access to food and water with a 12 hr light/dark cycle.

Animals used in paper I and II were male Sprague-Dawley rats purchased from Charles River Germany. Animals in paper I were 6-7 weeks at date of surgery and 4-6 weeks in paper II. Animals were sedated by either way of intraperitoneal injection of ketamine cocktail (33 µg/ml Ketanol, Pfizer)/xylazine (6.6 µg/ml Rompun, Bayer Healthcare AG) at a dose of 2 ml/kg or with 5% isoflurane and maintained at 2.5% in a mixture of air and oxygen delivered via nose cone when animals were placed into a stereotaxic frame (Kopf Instruments, Germany).

In paper III, adult 2-3 months old C57Bl/6 mice (Charles River, Germany) and $HMMR^{-/-}$ mice on a C57Bl/6 background were used. The $HMMR^{-/-}$ were a kind gift from Dr. Eva A. Turley (Lawson research Institute, London Regional Cancer Centre, London, Ontario, Canada) and have been previously reported in Tolg et al (Tolg *et al.*, 2003). All animals were initially sedated with a 5% isoflurane and maintained at 2.5% in a mixture of air and oxygen delivered via nose cone when animals were placed into a stereotaxic frame (Kopf Instruments, Germany).

3.1.2 PEPTIDE AMPHIPHILE PREPARATION

The peptide amphiphiles used in this thesis were all synthesized and purified by Eric J. Berns (Samuel Stupp laboratory, Northwestern University, Chicago, USA) as previously described in (Goldberger *et al.*, 2011), delivered as lyophilized powder, and stored in -20 °C until use. Lyophilized PA in powder form can be stored at -20 °C for several years without losing its bioactivity. Peptide amphiphiles were re-constituted and prepared 12-36h before first day of surgery.

All PAs in this thesis were prepared as follows: PA powder was dissolved in sterile aqueous solution of 150 mM Nacl/3 mM KCl (referred hereafter as dissolving solution) to obtain a 1 wt% solution. pH was adjusted to 7-7.4 using NaOH and HCl, as it facilitates dissolving the PA powder. For bioactive PAs, equal amounts of backbone PA (E_2 PA) and backbone Ten-C or RGDS conjugated PA (E_2Ten-C or E_2RGDS) were mixed well before sonication for 15 min followed by heating in a water bath at 80 °C for 30 min, and left to slowly cool down overnight. For paper I, a Ten-C peptide lacking the entire backbone was also synthesized and prepared to serve as a nonpolymerizing, nonfibrous control PA. PAs were further diluted to 0.25 wt% in the solvent before injection.

Amino acid sequences of peptides used in thesis:

E_2 Base PA sequence: palmitoyl-VVAAEE-NH2

E_2Ten-C PA sequence: palmitoyl-VVAAEEEADEGVFDNFVLK-NH2

Ten-C peptide sequence without backbone: ADEGVFDNFVLK-NH2

E_2RGDS PA sequence: palmitoyl-VVAAEERGDS- NH_2

3.1.3 INDUCTION OF PHOTOTHROMBOTIC CORTICAL STROKE

In paper III, we opted to use the minimally invasive photothrombotic stroke model for the cortical stroke as it offers a more controlled ischemia compared to middle cerebral artery occlusion (MCAO). Animals were sedated (see "Animals"), and placed in stereotaxic frame with attached nose cone for isoflurane delivery. Sedation lowers body temperature, and a heating pad was therefore placed underneath the animals to maintain normal body temperature. The skull was exposed by a small incision, the skin on top of the head was retracted and the periosteum was removed. This was done to optimize laser penetration, and to make certain all stroke lesions affected the same area using the stereotaxic coordinate system with bregma as the zero point. Intraperitoneal (IP) injection of freshly prepared Rose Bengal dye (0.1 mL; 10mg/mL) was delivered 5 minutes before laser illumination. This was done as to allow for the Rose Bengal to properly circulate and reach the brain. Rose Bengal is a photosensitive dye, which, when exposed to illumination at specific wavelengths the dye, activates and induces endothelial damage and

thrombosis, resulting in local interruption of the normal blood flow. A laser source (Cobolt Jive, Stockholm, Sweden) was placed 3 cm above the region of interest and illuminated the underlying skull at 50mW power and wavelength 561 nm for 10 minutes. Photothrombotic stroke was induced at the following coordinates, relative to bregma; +2.7 mm lateral and +1 mm anterior. The skin was sutured and animals kept in a heated recovery box until awakening. Animals were sacrificed 24h, 3w and 6w post-lesion.

3.1.4 BRDU INJECTIONS

In paper III, BrdU (bromodeoxyuridine) injections were used for quantifying proliferating cells. Proliferating cells incorporate BrdU into their DNA by substituting thymidine for BrdU during the S-phase of DNA replication, effectively labeling proliferating cells with BrdU. All BrdU injections (50 mg/kg bodyweight) were single dose intraperitoneal injections approximately 24h before perfusion.

3.1.5 PEPTIDE AMPHIPHILES INJECTIONS

All surgeries and PA injections were done using stereotactic instruments (Kopf Instruments and Stoelting Co). A 10-µl syringe with 26G beveled needle tip attached to the stereotaxic frame was used for injections. All injections were done with bregma as the zero point.

In paper I, all animals received one injection per hemisphere (E_2Ten-C PA vs control), a total of two injections per animal, at the following coordinates: anteroposterior +2.7 mm and lateral ±1.2 mm targeting the ventral horn of the RMS.

In paper II, all animals received a total of four injections (E_2RGDS PA vs control), one anterior injection (AC, anterior coordinate) and one posterior injection (PC, posterior coordinate) in each hemisphere at the following coordinates; PC: anteroposterior +1 mm and lateral ±2.5 mm, and AC: anteroposterior +2.7 mm and lateral ±1.2 mm.

To ensure control of flow rate, an automated syringe pump was used. All injections were done at a flow rate of 1 µl/min, stopping after 5 minutes for 5 minutes to allow for PA polymerization and to minimize PA leakage. Needle

was retracted slowly during injection phase in order to create a tract of specific length. A total of 5 µl PA was injected in each tract.

3.1.6 TISSUE PROCESSING

Animals were sedated with an overdose of pentobarbital and perfused transcardially with sterile 0.9% saline followed by phosphate-buffered (0.1 M [pH 7.4]) 4% paraformaldehyde. Brains were removed following perfusion and post-fixed in 4% PFA for 24-48h at 4 °C. Post-fixed brains were prepared for cryosectioning by incubation in phosphate-buffered (0.1 M [pH 7.4]) 30% sucrose solution for at least 3 days. For paper I and paper II, brains were divided into left and right hemisphere (control vs bioactive biomaterial). All brains were cryosectioned coronally in 25 µm thick serial sections using a sliding microtome (Leica Microsystems) and stored at 4 °C in tissue cryoprotective solution (glycerol, ethylene glycol and 0.1 M PO4).

3.1.7 HISTOLOGY

For immunoperoxidase staining, free floating sections were washed in tris-buffered saline (TBS), pretreated with NaCl (10 mM, pH 6) at 80 °C for 30 min and let to cool down to room temperature for 10 min. Sections were then washed in TBS and incubated for 30 min in 0.6% H2O2, followed by incubation for 1h at room temperature in blocking solution (TBS with 0.1% Triton-X-100 and 3% normal donkey serum (Jackson ImmunoResearch, West Grove, PA). Sections were incubated with primary antibody diluted in blocking solution overnight at 4 °C. Sections were washed in TBS, incubated with secondary antibody diluted in blocking solution for 1h, followed by amplification with avidin-biotin complex (Vectastain ABC Elite, Vector Laboratories) and then visualized using a DAB/H2O2/NiCl2 in TBS solution for 5-10 min (0.25 mg/ml DAB, Saveen Biotech, 0.04% NiCl2). Sections were mounted on glass slides and coverslipped using NeoClear (Merck) and NeoMount (Merck).

For immunofluorescent staining, sections were washed in TBS before blocking (same as above) for 30 min. Sections were incubated with primary antibodies overnight at 4 °C. Sections were then washed in TBS and incubated for 2h at room temperature with secondary antibodies coupled to fluorophores for fluorescent detection. Sections were washed with TBS before mounting on

glass slides and coverslipped with Prolong Gold antifade reagent (Molecular Probes, Oregon, USA).

For detailed description of antibodies and histology, see papers I-III.

3.1.8 QUANTIFICATION, IMAGE ACQUISITION AND DATA ANALYSIS

All image acquisitions and quantifications were done using Stereo Investigator 10 Software (MicroBrightField Inc., USA) coupled to a Leica DM6000B microscope (Leica Microsystems, Germany).

In paper I, location of the PA or needle injection tract was detected by changes in the tissue texture visible under the differential interference contrast filter in brightfield microscopy. Only sections showing an injection tract were included, all sections without detectable PA or with a track not reaching the RMS were omitted from the study. The border of injection tract was traced and all DCX^+ cells quantified, DCX^+ cells with migratory morphology alongside the trace were quantified and included as "parenchymal tissue" migration. Distance migrated by DCX^+ cells was quantified using the X/Y-coordinates for each individual cell traced with the border of the RMS as origin (0,0).

For reactive gliosis analysis in papers I and II, cell density was quantified using Stereo Investigator 10 fractionator function. Counting frame size was set at 100*100 µm with a grid size of 500*500 µm. GFAP+ and Iba1+ cells were quantified up to 250 µm from the border of the lesion. For fluorescence intensity analysis, approximately eight evenly spaced 150 µm long lines were drawn per coronal image from either border or midline of injection tract. Fluorescence intensity for each individual underlying pixel was determined using ImageJ Version 1.49v (NIH, USA). Fluorescent intensity data were compiled across all lines and sections for each injection and averaged for three distance intervals from the injection tract, 1-50 µm, 51-100 µm and 101-150 µm. All image acquisition and analysis settings were kept constant throughout the study to ensure proper intensity acquisition.

In paper III, all lesioned animals without visible stroke lesions, or stroke lesions reaching into the corpus callosum were omitted from the study. Data for unlesioned baseline animals were counted and averaged from both hemispheres. For analysis of the dentate gyrus granule cell layer and

subgranular zone, cells located more than two cells into the hilus were omitted from analysis.

3.1.9 STATISTICS

All statistical analyses were performed using Prism 6 software (Graphpad Inc.). In paper I, density analysis for doublecortin-positive cells was performed using one-way rank ANOVA (Kruskal-Wallis test) with Dunn's multiple comparison test. In paper I and II, $GFAP^+$, $Iba1^+$ and $ED1^+$ cell density was analyzed using unpaired Students t-test. Two-way ANOVA with Sidak's multiple comparisons post-hoc test was used for immunofluorescent intensity analysis. In paper III, statistical analysis of difference between two groups was performed using Students t-test for unpaired data, or 2-way ANOVA with Sidak's multiple comparisons post-hoc test. Change over time analysis was performed using 2-way ANOVA. p values and adjusted p values $< .05$ were considered statistically significant (*). All error bars represent standard error of the mean (SEM).

4 RESULTS AND DISCUSSION

4.1 PAPER I

Neural progenitor cells in the adult SVZ migrate primarily to the OB, via the RMS. Studies have shown that after injury or during pathological conditions of the brain, these migratory neuroblasts in the SVZ and RMS can change their migratory path, and instead migrate into various other brain regions. In the case of ischemic insult, progenitor cells can migrate towards the stroke region (Thored *et al.*, 2007; Osman *et al.*, 2011). Some believe this to occur as an endogenous attempt to replace the dying neurons and tissue, while others speculate on a trophic support role of progenitors by releasing neuronal survival- and plasticity enhancing factors. This spontaneous migration is observed in the rodent brain, where distances are relatively small compared to the human brain and when lesions were located relatively close to the SVZ/RMS, such as the overlying motor cortex or the striatum (Arvidsson *et al.*, 2002; Thored *et al.*, 2007; Osman *et al.*, 2011).

Due to these limitations, we hypothesized in this paper, if it would be possible to re-route endogenous neuroblasts away from their normal migratory path inside the RMS of the healthy adult brain towards the cortex through the creation of an artificial migratory path using a biomaterial. For this, we utilized a self-assembling peptide amphiphile displaying the migration-inducing sequence of the Tenascin-C glycoprotein as a bioactive epitope (E_2Ten-C PA).

4.1.1 PA POLYMERIZE AND FORM ALIGNED NANOFIBERS *IN VIVO*

In the previous studies that have utilized this type of PA in vivo, none had been performed in the brain. In order to confirm that the PA polymerizes in brain tissue and forms a gel containing aligned nanofibers, transmission electron microscopy was performed. A solution of 1 wt% E_2PA was injected dorso-ventrally from the cerebral cortical surface of excised brains while slowly retracting the syringe. Examination of the brain tissue with PA gel was done on TEM micrographs of ultrathin sections cut in the direction of the PA track. This revealed dense regions of PA nanofibers, making it easy to identify the gel domains in higher resolution. The TEM results indicate that the interstitial fluid in the brain tissue contained a high enough concentration of divalent

cations for inducing polymerization and gelling of PA upon injection. We also observed that throughout the needle track, the injected PA formed nanofibers aligned in the direction of the injection track, with higher abundance near the border of the gel-tissue interface (fig 1d of paper I).

4.1.2 INVESTIGATING *IN VIVO* BEHAVIOR OF THE PA IN BRAIN TISSUE

To study if peptide amphiphiles carrying the migratory sequence derived from the Ten-C glycoprotein can stimulate in vivo migration of neuroblasts in the RMS, we injected E_2Ten-C PA targeting the ventral horn of the RMS. These coordinates were chosen for creating the alternative path for the neuroblasts, which are already in a migratory state at this location of the RMS. Cell migration is not only regulated by signaling molecules and, in the case of chain migration, other cells, but also by the permissiveness of the tissue/medium for migration. We had concluded from previous in vitro studies that higher wt% of dissolved PA corresponds to a higher-density gel (unpublished work). In order to find an optimal density of the gel for cell migration within the PA, three different PA concentrations were studied for neuroblast migration inside and alongside the PA. The 1 wt% PA resulted in a very dense gel, not allowing for sufficient infiltration by doublecortin-positive cells. Rather, all cells were observed bordering the biomaterial, indicating that the neuroblasts were attracted to the PA material; but, subsequently, no migration was observed inside the PA at 1 wt%. We also injected PA at 0.5 wt%, but due to the higher incidence of the PA gelling inside the syringe and clogging, just like with the 1 wt%, we opted to continue with the even lower 0.25 wt% concentration. In animals injected with 0.25 wt% E_2Ten-C PA we could observe DCX^+ cells entering and migrating within the PA. The cells within the PA displayed elongated morphology typical of migrating neuroblasts. Due to the fact that the PA depolymerized over time, we could detect little to no trace of the injected PA from two weeks post-injection onwards, indicating full degradation of the biomaterial.

To investigate if the bioactive epitope of the E_2Ten-C PA had a stimulatory effect on RMS neuroblasts, we injected as a control a Ten-C peptide without the nanofiber-forming regions, i.e. the alkyl tail, β-sheet region and charged region. While in the E_2Ten-C injected animals, we observed directed migration, the Ten-C peptide without the backbone induced a more random

and dispersed presence of neuroblasts at the interface between the RMS and the injection site, with no neuroblasts showing any long-distance migration within the injection track.

Further, as the PA is injected as liquid which polymerized in vivo to form a gel, we were also interested in the diffusion of the PA into the surrounding brain tissue. To this end, we used an E_2 PA with a covalently attached fluorescent TAMRA-derived molecule (E_2TAMRA PA). Injecting E_2TAMRA PA at 0.25 wt% we could observe a gradually declining fluorescent signal from the border of the injection track up to one week after injection (see fig. 2g of paper I), indicating that the PA could induce effects even in the vicinity of the gel track.

4.1.3 E_2TEN-C PA STIMULATES MIGRATION OF NEUROBLASTS IN THE RMS

For the migration analysis, biomaterial was injected and left in place for one week prior to analysis. Assuming the normal in vivo migratory speed of 5-7 mm per week for RMS neuroblasts (Lois & Alvarez-Buylla, 1994; Mejia-Gervacio *et al.*, 2011), the cells should reach the cortex from the ventral horn of the RMS during the 7-day interval. Doublecortin, a marker for migrating neuroblasts, was used to quantify cell migration associated with the PA injections. DCX^+ cell density was quantified for cells migrating both inside and alongside the injection tracks. For the PA injected animals, we observed the PA having a clear migratory effect. Significantly more cells were inclined to migrate out of the RMS and re-route towards the cortex when coming in contact with the PA biomaterial compared to tracks injected with saline control. We observed a 24-fold increase in total number of re-directed neuroblasts in the E_2Ten-C PA group compared to the saline-injected controls. While we observed a migratory effect for both PAs, with and without the bioactive Ten-C epitope, only the increase in density of migrating cells for the E_2Ten-C PA was statistically significant compared to controls. Further, as we had previously demonstrated, that the PA partially diffuses into the surrounding brain tissue before gelling, we also quantified DCX^+ cells migrating alongside the injection tracks ($\leq$250 μm). While the density of migrating cells was approximately twice as high inside the E_2Ten-C track compared to migration in the parenchymal tissue, we did observe a

substantially larger migration of DCX^+ cells in the parenchyma of E_2Ten-C PA injected tracks compared to the parenchyma of saline injected controls.

With our data showing a substantial migration in the biomaterial injected tracks, we were also interested in investigating the extent of this migration, in terms of distance travelled. For this we compared the E_2Ten-C PA with the epitope-free PA. Saline control injections were not included in this analysis as those tracks exhibited very low rates of migration. While only the E_2Ten-C PA carries the migration-stimulating Ten-C sequence, both PAs gel and form aligned nanofibers, and both were able to stimulate migration biomechanically through the formation of a scaffold. To further characterize PA effect on neuroblast migration, we plotted cells in migration against distance (fig 2D). Here it became apparent that a larger number of cells migrated further distances inside the E_2Ten-C PA compared to the E_2 PA lacking the migratory Ten-C signal. We detected about 40% of the migrating cells in both injection groups migrating less than 700 μm. Past the 700 μm point, the difference between the two groups became more obvious. In the E_2 Ten-C PA, approximately 14% of the cells migrated distances longer than 2,000 μm, while in the E_2 PA control group less than 2% reached the longer distances.

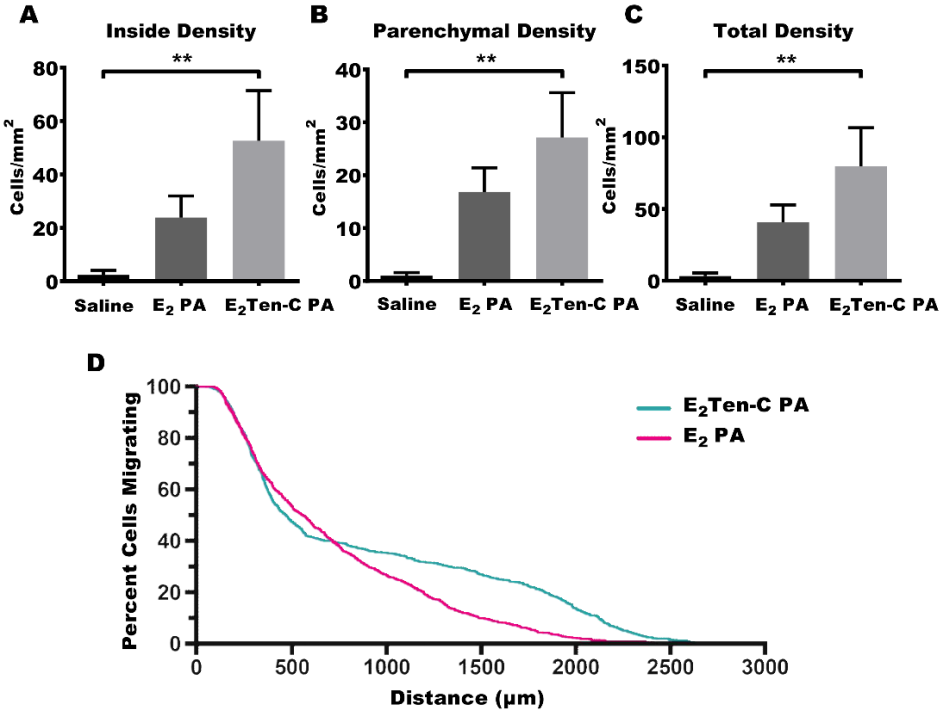

*Figure 2 Quantification of DCX⁺-cells redirected from the rostral migratory stream (RMS) using saline (n = 4 animals), E_2 PA (n = 8 animals), and E_2Ten-C PA (n = 8 animals). Substantial migration was seen for the E_2Ten-C PA both inside the track (A) and in the surrounding parenchyma (B). Twenty-four–fold increase in total number (C) of redirected doublecortin positive cells was seen for the E_2Ten-C PA–injected animals compared to saline–injected animals. All data presented as mean ± SEM. **p < .01. (D) Proportion of cells in migration as a function of distance migrated. All cells from eight animals per group were combined in distribution curves sorted according to their distance from the RMS*

4.1.4 E_2TEN-C PA INJECTION AND REACTIVE GLIOSIS

The introduction of a foreign substance into the brain, and the injury to the CNS caused by the injection, trigger a cellular response from local astrocytes and microglia. Astrocytes and microglia respond by becoming reactive, proliferative and by migration towards the lesion site and/or foreign substance, in this case the PA biomaterial. The astrocytes and microglia accumulate and form a glial scar. As the injection causes damage to the tissue, and a foreign substance was introduced, we were interested in the extent of the glial response from astrocytes and microglia to the E_2Ten-C PA gel, compared to what would be expected from a needle stab wound alone, when no additional space is occupied post-injection and no foreign material is introduced.

To investigate the extent of the reactive gliosis response to E_2Ten-C PA transplants, $GFAP^+$ astrocytes and $Iba1^+$ microglia were quantified with saline vehicle as control injections (sterile 150 mM NaCl, 3 mM KCl saline solution). Reactive astrocytes and microglia surrounding the injection sites were quantified up to 250µm from the border of the injection track and analyzed as cell densities. Interestingly, we did not observe an increase in reactive gliosis for E_2Ten-C PA injections compared to the vehicle control injections for both cell types. Further, as reactive gliosis also causes an upregulation of the expression of their respective markers, we also quantified fluorescent intensity for GFAP- and Iba1-expressing cells. Fluorescent intensity was measured along 8 lines drawn perpendicular to the border of the track into the tissue, and pixel intensities were determined along these lines up to 150 µm surrounding the track. To get a proper estimation for the fluorescent intensity of the GFAP- and Iba1-positive cells, knowing reactive gliosis decreases with increasing distance from the lesion site, we opted to divide the measured distance into three intervals; 0-50 µm, 51-100 µm and 101-150 µm. Analyzing the fluorescent intensity for the expression of GFAP and Iba-1, we observed that E_2Ten-C PA did not elicit a stronger response from astrocytes and microglia, in any of the intervals measured, compared to control injections. We conclude that the E_2Ten-C PA itself does not aggravate astrogliosis or microgliosis any further than the initial needle stab lesion would cause during the injection procedure.

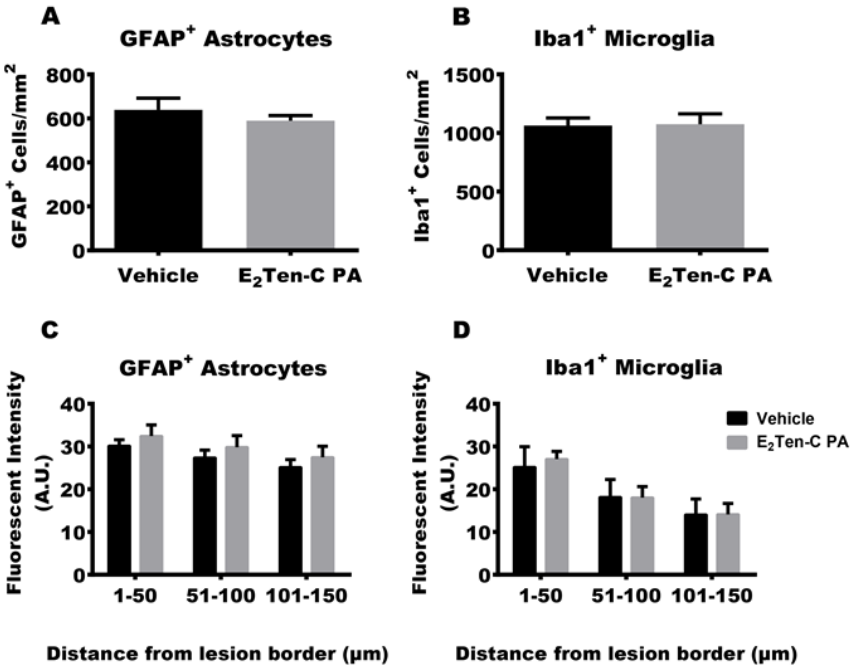

Figure 3 Glial response to PA injection. Cell density was quantified in the parenchymal tissue up to 250 µm from the border of the track. E_2Ten-C PA injection had no effect on astrogliosis (A) determined by GFAP$^+$ cell counts or microgliosis (B) determined by Iba1$^+$ cell counts compared to the vehicle control (n = 6 animals per group). Fluorescent intensity analysis of (C) GFAP$^+$ and (D) Iba1$^+$ cells up to 150 µm bordering the lesion. No difference observed in upregulation of GFAP (C) or Iba1 (D) immunofluorescence in E_2Ten-C PA compared to vehicle group (n = 6 animals per group). All data presented as mean ± SEM. p value <.05 considered significant

4.1.5 CONCLUSIONS

In this work, we showed that neuroblasts migrating in the rostral migratory stream of the healthy adult brain can be influenced to migrate to a novel location without stroke or lesion being the cause for migration. We demonstrated here that the self-assembling peptide amphiphile E_2Ten-C can be used as a migratory scaffold for targeted neuroblasts migration. A large number of DCX^+ neuroblasts were re-routed from their normal migratory path in the RMS to the cortex instead of the OB, following the aligned nanofibers provided by the PA gel. We also demonstrated the effectiveness of the E_2Ten-C PA as a migratory complex as cells migrating within the E_2Ten-C PA migrated significantly longer distances than saline vehicle control or injections lacking either the backbone of the PA or the bioactive Ten-C epitope.

We hypothesize that the observed migratory effect of the E_2Ten-C PA on neuroblasts is two-fold. Neural progenitor cell migration is directed by several types of signals such as the mechanical cues provided by the cell environment, and biochemical cues such as chemoattractants or adhesion molecules in the ECM. The importance of these signals becomes evident from the composition of the RMS, where migrating cells are guided from the SVZ to the OB across a distance of several millimeters. The RMS provides neuroblasts with a migratory scaffold with mechanical cues in form of glial tubing and blood vessels running alongside the structure. This is complimented by expression of ECM molecules such as laminins, fibronectin and tenascins. Our biomaterial mimics some aspects of the RMS structure by providing the cells with aligned nanofibers which the neuroblasts can adhere to and migrate along, similar to what can be observed in neuroblasts chain migration. Also, as we demonstrated, the Ten-C peptide can induce motility without the E_2 backbone, although in a non-directed and more random pattern. The superior migration observed in the E_2Ten-C PA injections, compared to E_2 PA, leads us to believe that the long-distance migration observed in this work is also an effect of the biochemical signals delivered by the biomaterial. This is consistent with studies showing Ten-C promoting neurite outgrowth and axon elongation in neural progenitor cells, two processes with similarities to migration. Our theory is further strengthened by the fact that E_2Ten-C used in vitro can stimulate cell migration and neurite outgrowth (Berns *et al.*, 2014; Berns *et al.*, 2016), and others showing Ten-C increasing neurite length (Meiners *et al.*, 2001).

Furthermore, as our biomaterial injections did not cause an aggravated reactive gliosis, beyond what was observed from vehicle injections, we conclude that self-assembling peptide amphiphiles carrying a biological signal can be used in vivo to modulate cell behavior without eliciting any significant glial response from the local astrocytes and microglia. That neither the PA, nor the Ten-C signal were cause for the reactive gliosis observed is a very important, and arguably one of the most important qualities for any biomaterial aiming for in vivo use. While tenascin-C has been previously reported to have pro-inflammatory properties, we did not see any evidence for this in our model. Due to the multimodular structure of Tenascin-C, the protein can exist in various isoforms, and we can now assume that the specific tenascin-c sequence used in this work does not act as a pro-inflammatory stimulus.

4.2 PAPER II

In this work, we showed that neuroblasts migrating in the rostral migratory stream of the healthy adult brain can be influenced to migrate to a novel location without stroke or lesion being the cause for migration. We demonstrated here that the self-assembling peptide amphiphile E_2Ten-C can be used as a migratory scaffold for targeted neuroblasts migration. A large number of DCX^+ neuroblasts were re-routed from their normal migratory path in the RMS to the cortex instead of the OB, following the aligned nanofibers provided by the PA gel. We also demonstrated the effectiveness of the E_2Ten-C PA as a migratory complex as cells migrating within the E_2Ten-C PA migrated significantly longer distances than saline vehicle control or injections lacking either the backbone of the PA or the bioactive Ten-C epitope.

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4.2.1 E_2RGDS PA LOWERS GLIAL CELL DENSITY SURROUNDING BRAIN LESION

To assess the extent of the reactive gliosis caused by E_2RGDS PA transplants, we investigated the local astrocyte and microglial reaction to the biomaterial compared to control injections of vehicle saline solution (sterile 150 mM NaCl, 3mM KCl). The reactive gliosis and formation of glial scarring was evaluated by quantifying $GFAP^+$ astrocytes and $Iba1^+$ cells associated with the injections. Each animal received two injections of biomaterial in one brain hemisphere and two control injections in the opposite hemisphere. Gliosis was evaluated at five days post lesion, as reactive gliosis peaks at around 5 days after lesion (Fawcett & Asher, 1999).

Interestingly, when analyzing the $GFAP^+$ and $Iba1^+$ cells surrounding the lesions, we noted that the anterior and posterior injection sites showed different responses to the injections. Evaluating cell density of $GFAP^+$ astrocytes at the anterior coordinates, no difference was observed when comparing the E_2RGDS PA vs control injections. While the E_2RGDS PA did not elicit a stronger astrocytic immune response than the needle stab wound with vehicle solution at the anterior position, in the posterior position we could observe a difference between the two groups. At the posterior coordinates, a significantly lower density of $GFAP^+$ cells were registered for the E_2RGDS PA injected track compared to controls. E_2RGDS PA injections resulted in a remarkable 42% lower density of GFAP positive cells.

Similar results were observed for microgliosis. No difference in cell density for Iba1$^+$ cells were observed between the two groups at the anterior coordinates, similar to our astrocyte findings. But at the posterior coordinates, we again observed significantly lower density of microglia with E_2RGDS PA injections. For microglia, we registered a 17% lower density of Iba1$^+$ cells in the parenchymal tissue of E_2RGDS PA injected tracks compared to vehicle controls.

The combination of lower cell densities for both astrocytes and microglia at the posterior injection site indicates that E_2RGDS PA has a significant suppressive effect on the reactive gliosis, which could also result in a smaller glial scar. We therefore analyzed the extent of glial response further in terms of distribution and intensity of glial response markers.

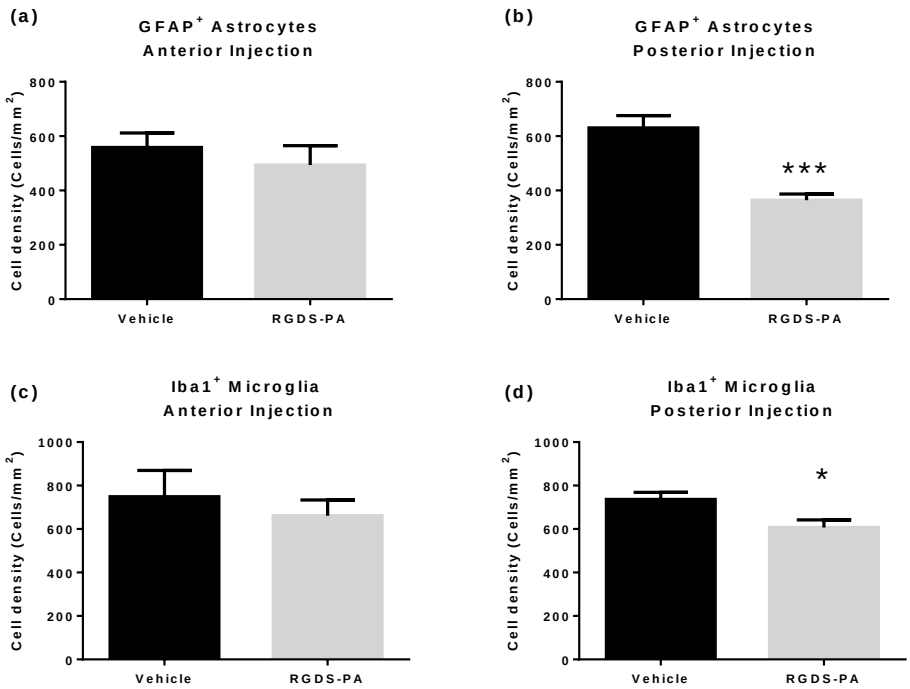

Figure 4 Glial cell response to E_2RGDS PA injections. Graphs showing cell density of GFAP+ cells and Iba1+ in both the anterior and posterior injection sites. E_2RGDS PA did not have an effect on astrogliosis in the anterior injection site (a) determined by quantifying GFAP+ cells up to 250μm surrounding injection. In the posterior injection site, GFAP+ cell density was significantly lower in the E_2RGDS PA injected hemisphere than control injected (b). E_2RGDS PA did have an effect on microgliosis in the anterior injection (c) while in the posterior injection site (d) a significant reduction in Iba1+ cell density could be observed. All data presented as mean ± SEM. p value < .05 considered significant. n = 5 animals per groups.

4.2.2 E_2RGDS PA SUPPRESSES LESION INDUCED REACTIVE GLIOSIS

During reactive gliosis, astrocytes and microglia have been shown to upregulate their respective markers, GFAP and Iba1. The expression level of these markers corresponds to the severity of the lesion and reactive gliosis. We therefore performed fluorescent intensity analysis of these markers to better characterize the glial response to the E_2RGDS PA, to further compliment the cell density analysis. We also included the microglia/macrophage marker ED1 (CD68), as it, in contrast to Iba1 which labels all microglia, does not label resting microglia, and can be used as marker for severity of lesion.

As we had previously shown that the PA diffusion into the surrounding tissue occurs before complete gelling of the biomaterial, we were interested if the E_2RGDS PA can lower gliosis at distances further into the parenchyma of the lesion. Fluorescent intensity analysis was therefore performed across the three segments, 1-50 µm, 51-100 µm and 101-150 µm, from the track border. We observed the E_2RGDS PA having a general alleviating effect on reactive gliosis. At the anterior injection site, E_2RGDS PA injections showed a significant 22.4% lower fluorescent intensity for the astrocyte GFAP marker at the interval closest to the lesion, 1-50 µm. We did not observe a difference between the two groups in the more distal 51-100 µm and 101-150 µm intervals. At the posterior injection site, we also observed significantly lower GFAP immunofluorescence intensity compared to vehicle controls. For the Iba1 labelled cells, transplanting E_2RGDS PA resulted in a significantly lower fluorescence intensity compared to control injections without the biomaterial. In the anterior injection sites, we observed a significant 14% lower intensity of Iba1 fluorescence E_2RGDS PA at the 1-50 µm interval, while no difference was observed for the more distant intervals. In the posterior injection sites, we measured a 21% lower fluorescent intensity of $Iba1^+$ microglia at the 1-50 µm interval for the E_2RGDS PA injected tracks. At this location, the suppressive effect of the PA was observed across all distances measured. For the 51-100 µm and 101-150 µm intervals, E_2RGDS PA transplants resulted in 22% and 24% lower fluorescent intensity, respectively.

We also included the reactive/activated microglia marker ED1 in our analysis. We observed results similar to the Iba1 analysis. For the anterior E_2RGDS PA injections, fluorescent intensity at the 1-50 µm interval was 35% lower than in controls, with no difference between the two groups for the other intervals. In the posterior injection sites, we could again observe the E_2RGDS PA having

an effect across all distances analyzed. We measured 44% lower intensity in the 1-50 µm interval for E_2RGDS PA injection, and 56% and 61% lower at the 51-100 µm and 101-150 µm respectively.

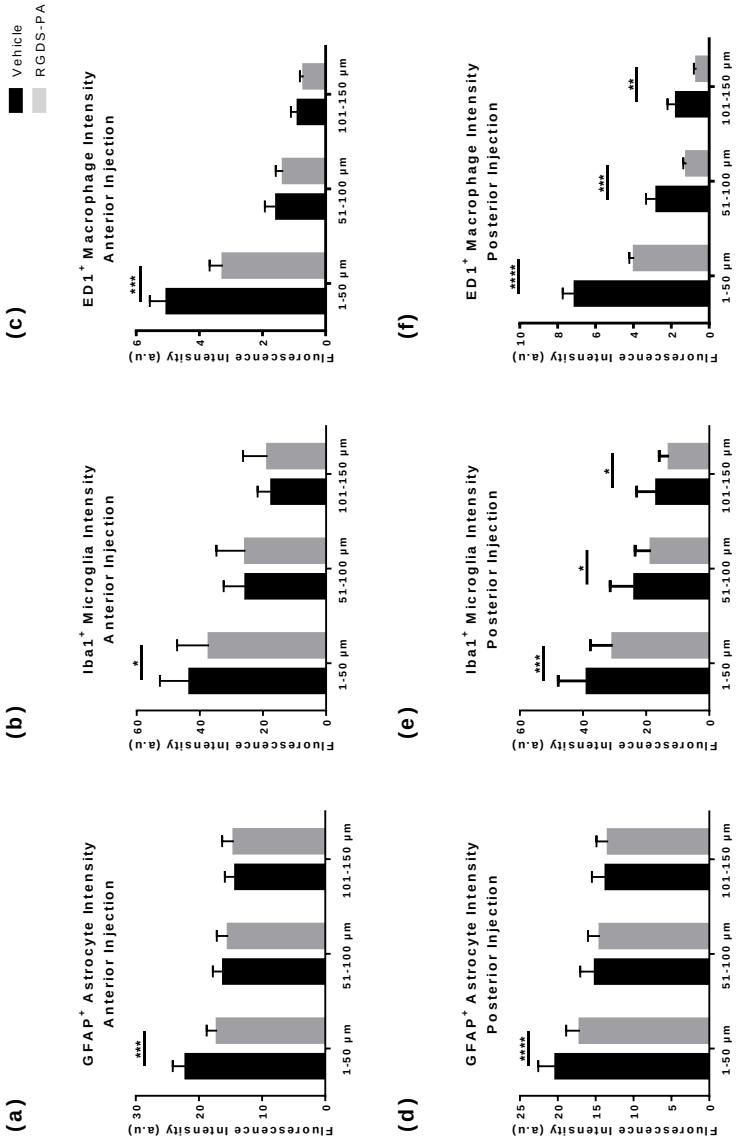

Figure 5 Graphs showing reactive gliosis response after E_2RGDS PA injection represented by fluorescent intensity measurement of astrocyte and microglia markers, GFAP, Iba1 and ED1. Intensity measurement was divided into three segments. Fluorescent intensity of GFAP labeled cells in the anterior injection was significantly lower in the range closes to the injection for E_2RGDS-PA injections in both the anterior (a) and the posterior lesion site (b). Iba1+ microglia intensity in the anterior coordinate was also lower for E_2RGDS-PA injections at 1-50 µm (b), while in the posterior injections we observed significant reduction in Iba1 expression of microglia for E_2RGDS PA across all distances measured. ED1+ microglia/macrophage measurements showed E_2RGDS PA lowering ED1 expression in the anterior 1-50 µm range (c), while in the posterior site a lower intensity of ED1 expression could be observed at 1-150 µm from the border of the lesion for E_2RGDS PA injections. All data presented as mean ± SEM. p value < .05 considered significant. n = 5 animals per groups.

4.2.3 CONCLUSIONS

Studies from the Stupp lab and colleagues have shown the PA biomaterial with various epitopes supporting cell viability when used as extracellular scaffold in vitro (Sur *et al.*, 2012; Newcomb *et al.*, 2014; Berns *et al.*, 2016). In this work we wanted to investigate if the PA can potentially be used in vivo as a scaffold, for future use as cell carrier in cell transplants in the brain. For this, we decided to utilize peptide amphiphiles with a conjugated bioactive RGDS amino acid sequence derived from the cell binding domain of the ECM protein fibronectin (Dickinson *et al.*, 1994). With reactive gliosis and immune responses being one of the major obstacles for cell transplants, our main focus in this study was to investigate if the E_2RGDS PA triggers and elevates the reactive gliosis more than would be expected from a needle stab wound injury. Surprisingly, our results indicated rather the opposite. The E_2RGDS PA has a suppressive effect on the reactive gliosis response from both astrocytes and microglia.

In reactive gliosis, cell proliferation of local glial cells, and cell migration into the affected region can be observed, causing an increase in astrocytes and microglia cell numbers surrounding the lesion and/or transplant. Our cell density analysis for $GFAP^+$ and $Iba1^+$ cells demonstrated that injecting the E_2RGDS PA does not elicit a stronger glial response than a saline injection, but actually resulted in reduced density of astrocyte and microglia cell numbers in the posterior site. With cell marker expression levels being a measurement of how intense reactive gliotic response is, the E_2RGDS PA injections also resulted in a smaller gliotic response at both, an anterior (prefrontal cortex/nucleus accumbens) and a posterior (parietal cortex/striatum) injection site as demonstrated in our fluorescent intensity analysis. This effectively shows the E_2RGDS PA itself is not causing a glial response, making it suitable for transplantation studies. The effect on gliotic response observed here also opens up new therapeutic avenues for the E_2RGDS PA beyond cell transplantations, such as injections into CNS lesion or ischemic regions to help reduce gliosis and glial scarring, possibly allowing for axonal regrowth.

The Stupp lab demonstrated, using an alternative backbone sequence for the RGDS PA, that subcutaneous injections of the biomaterial did not results in a large-scale inflammatory response. They speculated this could be due to the RGDS sequence being too short to elicit an immune response (Guler *et al.*, 2006). Further, they have also shown PA displaying an IKVAV epitope limited astrogliosis and scar tissue formation via interaction with β1-integrin (Pan *et*

al., 2014), by possibly suppressing astrocytic differentiation. Interestingly, both the Ten-C sequence used in **paper I** and the RGDS sequence interact with the β1-integrin (Meiners *et al.*, 2001; Mercado *et al.*, 2004). Our results are also consistent with transplantation studies utilizing other biomaterials which included the RGD(S) sequence. Cui et al showed transplanting a hyaluronic acid-based hydrogel immobilized with RGD peptides did not results in a glial scar at the interface of the hydrogel and the host tissue (Cui *et al.*, 2006). There are also several other studies showing RGD(S) incorporated biomaterials demonstrating axonal growth. While not all of them studied glial activation and reactive gliosis, we can assume that axonal regrowth would most likely not have occurred following glial scarring and the expression of inhibitory proteoglycans (Rudge & Silver, 1990; McKeon *et al.*, 1991; McKeon *et al.*, 1995; Hejcl *et al.*, 2010).

In **paper I**, we injected PA with a conjugated tenascin-C bioactive epitope. There, we did not observe a reduced glial response compared to control (Motalleb *et al.*, 2018), indicating that the RGDS peptide sequence itself might have a possible gliosis suppressing role when coupled to peptide amphiphiles. While it remains unclear exactly how the E_2RGDS PA exerts this effect, we speculate that the suppression effect seen here could be mediated by the interaction of the RGDS domain with the glial cell integrins. Although elucidating which specific α and β subunit combination(s) of the integrin is active in this case is not straightforward as (1) the RGD(S) sequence has been demonstrated to interact with a wide array of integrins (Buck & Horwitz, 1987; Plow *et al.*, 2000), and (2) astrocytes and microglia respectively are not homogenous populations, demonstrating regionalization in several studies (Cragnolini *et al.*, 2018).

The composition of the ECM is not identical between the two injection areas, which in turn could affect cell response to stimuli (Johnson *et al.*, 2015), in this case the E_2RGDS PA and the needle tract lesion. But even more important is the heterogeneity of astrocyte and microglia populations due to temporal and spatial variations. Microglia in the healthy brain have specific region-dependent transcriptional identities and phenotypes (Grabert *et al.*, 2016). Using several methods for single-cell analysis of CNS tissue, Masuda and colleagues showed both spatial and temporal heterogeneity for the rodent brain, but also for the healthy and pathological human brain (Masuda *et al.*, 2019). Böttcher et al. also profiled human microglia and could demonstrate regional heterogeneity at the single-cell level (Bottcher *et al.*, 2019). In a previous publication from our lab we demonstrated microglia in the SVZ and hippocampus differ in their mRNA expression response to brain irradiation (Hellstrom *et al.*, 2011). Human astrocyte populations differ in their

microRNA expression patterns in relation to anatomical location and age (Rao *et al.*, 2016), and Cragnolini demonstrated how regional response to mechanical injury (such as our lesion) can differ, with astrocytes derived from the cortex, hippocampus and striatum using a scratch injury assay (Schitine *et al.*, 2015; Cragnolini *et al.*, 2018). These studies indicate that several differences between glial populations in the brain exist, which could explain the differential response to the biomaterial transplant at the two locations in our study, but further studies are required to address this in more detail.

Any form of biomaterial transplant in the brain will unavoidably trigger some form of reactive gliosis, either by tissue damage or simply due to its mere presence as foreign material. A biomaterial, such as E_2RGDS PA, which does not elicit a stronger glial response, but rather suppresses the glial cell response in a lesion, shows that peptide amphiphiles have great potential for future use as cell scaffolding for therapeutic transplants in the CNS.

4.3 PAPER III

Stroke is one of the leading causes of death and disability in the elderly. Understanding how the cellular milieu, more specifically the extracellular molecules in the adult brain, can affect neural stem/progenitor cells (NSPCs) could provide insight into stimulating the regenerative responses of the adult brain and ultimately lead to establishing new and more effective therapeutic strategies for the functional and structural repair of the brain after stroke. Stroke has been shown to stimulate neurogenesis in two of the neurogenic niches of the adult brain, the hippocampus and the subventricular zone (Takagi *et al.*, 1999).

Hyaluronan is one of the most abundantly expressed glycosaminoglycan in the brain ECM. It has the property of keeping tissue in a soft-solid, viscoelastic state, while creating a diffusion barrier to control the transport of soluble molecules through the intercellular space (Laurent *et al.*, 1996). In the bone marrow stem cell niche, Hyaluronan appears to play an important role in stem cell homing, proliferation and differentiation (Nilsson *et al.*, 2003). Similar roles have been proposed for the developing and adult CNS (Preston & Sherman, 2011). We were interested in the significance of one of hyaluronan's main receptors, RHAMM (receptor for hyaluronic acid mediated motility). In previous work from our lab, Lindwall et al showed selective expression of RHAMM in the adult mouse subventricular zone and the RMS (Lindwall *et al.*, 2013). In the current work, we focused our interest on the significance of RHAMM in stroke-induced neurogenesis. This was evaluated by quantifying DCX^+ and $BrdU^+$ cells in the two neurogenic niches, the SVZ and the hippocampal dentate gyrus (DG) of cortically lesioned adult $HMMR^{-/-}$ mice.

4.3.1 UNLESIONED HMMR KNOCKOUT MICE

Before investigating if RHAMM, or rather the absence of RHAMM, affects stroke-induced cell proliferation and DCX expression in the neurogenic zones of the adult mouse brain, we first established if there was a difference between $HMMR^{-/-}$ mice and to controls at baseline. Cell proliferation was evaluated by BrdU labelling of dividing cells 24h before sacrifice and quantifying $BrdU^+$ cells in the two neurogenic zones, the SVZ and the DG. As we were interested in neurogenesis, we also quantified cells expressing the neuronal progenitor

cell marker doublecortin (DCX) in the SVZ and DG as a measurement of ongoing neurogenesis.

In unlesioned adult HMMR$^{-/-}$ mice, we observed significantly lower cell proliferation in the DG and the SVZ. Cell density of BrdU labelled cells in the DG was 30% lower than in control animals (fig 6a). In the SVZ, we noted a 22% decrease in cell proliferation in the absence of RHAMM (fig 6c). While we measured a 47% higher density of DCX^{+} cells in the dentate gyrus of HMMR$^{-/-}$ animals, we did not detect a difference in the SVZ (fig 6b & 6d).

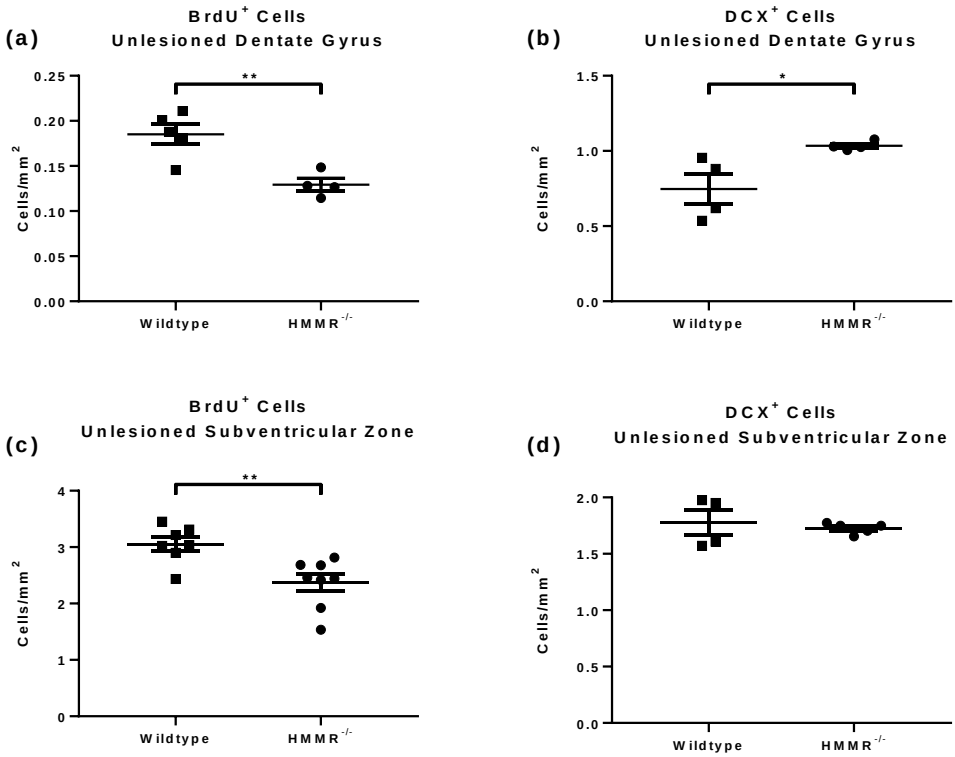

Figure 6 Quantification of BrdU labelled proliferating cells and DCX+ neural progenitor cells in the unlesioned brain of HMMR$^{-/-}$ mice and wildtype controls. Significantly lower proliferation was observed in the dentate gyrus (a) and subventricular zone (c) of HMMR$^{-/-}$ mice compared to controls. HMMR$^{-/-}$ mice had a significantly higher cell density of DCX labelled cells in the dentate gyrus (b) but not in the subventricular zone (d). All data presented as mean ± SEM. p value < .05 considered significant.

4.3.2 STROKE INDUCED PROLIFERATION IN HMMR$^{-/-}$ MICE

While in the unlesioned animals we observed wildtype mice having significantly higher density of proliferating cells, in the acute phase after stroke no difference was detected in proliferation between the ipsilateral DG of HMMR$^{-/-}$ mice and wildtypes. At the later timepoints, 3 weeks and 6 weeks post stroke, we could again see HMMR$^{-/-}$ having significantly lower density of BrdU^{+} cells in the ipsilateral DG by 50% and 43% respectively (fig 7a).

In the contralateral DG of HMMR$^{-/-}$ mice, cell proliferation in the acute phase after stroke (fig. 7b) showed similarities with the data from unlesioned animals. Cell density of BrdU^{+} cells in lesioned HMMR$^{-/-}$ was 39% lower than in wildtypes 24h after stroke. This difference between the two genotypes carried over to the 3-week timepoint. We detected 45% lower cell proliferation in HMMR$^{-/-}$ compared to wildtype. Interestingly, six weeks after stroke, the difference between the two animal groups was not detectable due to an increase in BrdU labelled cells in the HMMR$^{-/-}$ mice.

As stroke can also induce cell proliferation in the SVZ, we also quantified BrdU^{+} cells in the SVZ (fig. 7c-d). The difference observed in the unlesioned animals, HMMR$^{-/-}$ animals, having a significantly lower proliferating population in the SVZ, disappeared post-lesion. Our data did not show any statistically significant differences in the SVZ between the two groups in any of the timepoints following stroke, on neither the contralateral nor the ipsilateral side. The only difference observed was in the contralateral SVZ at six weeks after stroke, when proliferation was 79% percent higher in HMMR$^{-/-}$ mice than in controls. We noted that proliferation in wildtype animals dropped by a significant 32% in the ipsilateral SVZ and 54% in the contralateral SVZ in the six-week group compared to the three-week group, while the HMMR$^{-/-}$ proliferation did not differ between the two timepoints.

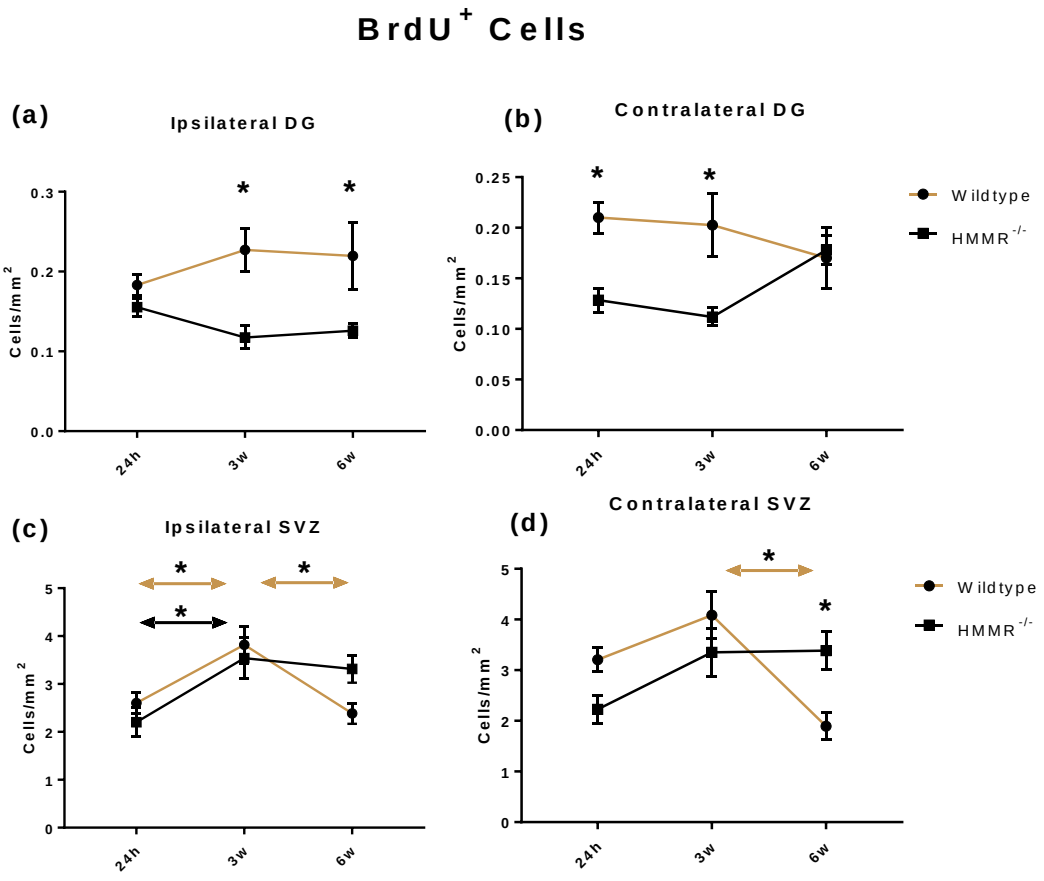

Figure 7 2-way ANOVAs of quantified BrdU+ cells in the ipsilateral dentate gyrus (a), contralateral dentate gyrus (b), ipsilateral subventricular zone (c) and contralateral subventricular zone (d) at 24h, 3w and 6w after stroke lesion. All data presented as mean ± SEM. p value <.05 considered significant. n ≥ for each group and timepoint.

4.3.3 NEUROGENESIS IN HMMR$^{-/-}$ MICE AFTER CORTICAL STROKE

As cell proliferation is not a sufficient indicator for stroke-induced neurogenesis, we also quantified neuronal progenitor cells by immunolabelling cells for the immature neuron marker doublecortin. DCX is used as a marker for neurogenesis since it is highly expressed in newly produced neural progenitor cells of the adult neurogenic zones (Brown *et al.*, 2003).

Evaluating neurogenesis in the acute phase after lesion, we detected 33% lower density of DCX-expressing cells the ipsilateral DG of HMMR$^{-/-}$ mice compared to wildtypes controls. Similar results could be seen in the contralateral DG where our data shows a 23% reduction in neurogenesis. In contrast, unlesioned HMMR$^{-/-}$ actually had a larger population of DCX-expressing cells than controls. When analyzing the DCX expression for the later timepoints after stroke, our data showed a downward trend of neurogenesis in both animal groups. In the ipsilateral DG of HMMR$^{-/-}$ mice three weeks after stroke, neurogenesis did not differ from wildtypes. There was a clear decrease in DCX-expressing cells in the wildtype animals while the HMMR$^{-/-}$ mice stayed at approximately the same levels. A similar response was observed at the six-week timepoint where we also did not detect a difference between the two animal groups in the ipsilateral DG. Data for the contralateral DG were consistent with our ipsilateral results. We did not observe a difference between the two animal groups in the density of DCX-expressing cells at either the three-week or six-week timepoints.

In contrast to the DG, analysis of the subventricular zone revealed that RHAMM does not seem to play a significant role in the post-stroke SVZ neurogenesis. Cell density for doublecortin labelled cells in HMMR$^{-/-}$ animals did not differ from wildtypes mice in the ipsilateral or contralateral SVZ across all timepoints measured, including the unlesioned baseline.

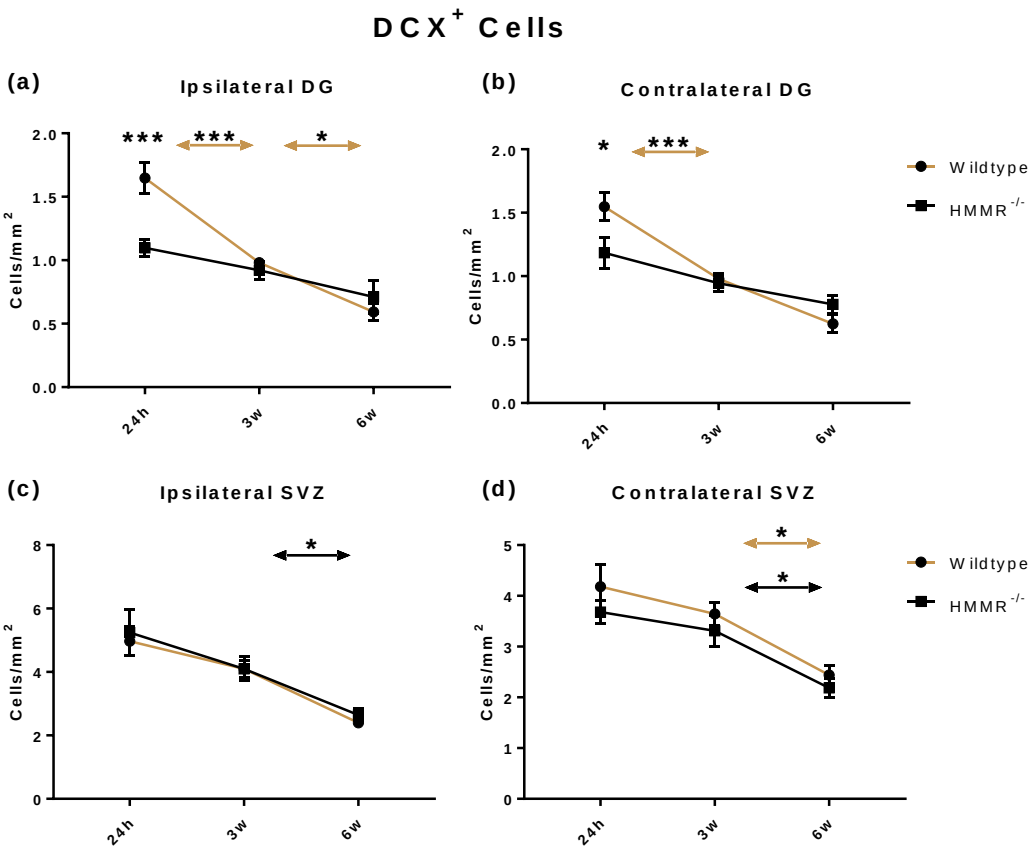

Figure 8 2-way ANOVAs of quantified DCX+ cells in the ipsilateral dentate gyrus (a), contralateral dentate gyrus (b), ipsilateral subventricular zone (c) and contralateral subventricular zone (d) at 24h, 3w and 6w after stroke lesion. All data presented as mean ± SEM. p value <.05 considered significant. n ≥ 4 per group and timepoint.

4.3.4 CONCLUSION

Although RHAMM has been studied extensively, mostly for its role in cancer biology or cell nucleus-associated processes, very little is known on the role of RHAMM in the context of adult neurogenesis. In this work, we used BrdU as marker for proliferating cells and DCX as a marker for neuronally determined precursor cells and early post-mitotic neurons to assess neurogenesis in an effort to investigate if RHAMM has a functional role in adult neurogenesis after ischemia. Our observations clearly indicate that RHAMM plays a role regulating neurogenesis in the dentate gyrus and subventricular zone. Although we see an effect in regard to both cell proliferation and DCX expressing cells, its function does not seem to be essential for neurogenesis, as we observe neurogenesis occurring in the neurogenic zones of adult brain of $HMMR^{-/-}$ mice. Surprisingly, our data also show RHAMM possibly acting region-specific, having a greater functional role in the DG neurogenesis than the SVZ.

We speculate that the differences we observed in this work might be related to the intracellular RHAMM functions, more specifically its role for spindle integrity during mitosis. Both during CNS development establishing the DCX^{+} cell population in the neurogenic zones and after lesion in the stroke induced neurogenesis phase.

In the CNS, RHAMM expression is reported to be higher in areas with higher cell proliferation rates (Casini *et al.*, 2010; Lindwall *et al.*, 2013; Li *et al.*, 2017). Studies have shown RHAMM expression to be cell cycle-regulated, with mRNA expression being higher in the G_2/M phase of the cell cycle, peaking in the G_2 phase, while the protein reaches peak levels at the end of the S phase (Sohr & Engeland, 2008). Intracellular RHAMM has also been demonstrated to act as an important cell cycle regulator during mitosis. The centrosome-targeting c-terminus of the RHAMM protein localizes at centrosome and microtubules of the mitotic spindle during interphase (Assmann *et al.*, 1999; Zhou *et al.*, 2002; Maxwell *et al.*, 2003; Li *et al.*, 2015). This is important for spindle integrity. The orientation of the spindle during division in mitosis can influence how the cell divides, symmetric vs asymmetric, and subsequently also the fate of the progeny. Maxwell and colleagues showed that injection of anti-RHAMM antibodies disrupted mitotic RHAMM, which produced symmetrical and asymmetrical tripolar and tetrapolar spindles, thereby affecting the spindle integrity (Maxwell et al., 2003). This effectively shows that RHAMM is important for maintaining spindle integrity during mitosis. In another study, RHAMM was shown to regulate the spacing and stability of microtubules via interaction with intracellular hyaluronan (Evanko *et al.*, 2004). While RHAMM is important

for mitosis, its role in cell division is not critical as homozygous mutant $HMMR^{-/-}$ mice are viable and appear normal in their gross morphology. This is further substantiated by only a quarter of the cells injected with anti-RHAMM antibodies during mitosis showing an irregular spindle phenotype (Maxwell *et al.*, 2003). Although centrosomes are the dominant microtubule-organizing centers in many cell types, they are not necessarily essential for cell division, but rather contribute to efficient assembly of the mitotic spindle (Heald *et al.*, 1997; Khodjakov *et al.*, 2000; Sir *et al.*, 2013).

During embryonic development, neural epithelial cell division is strictly symmetrical, creating the neural progenitor pool. In the rodent development of the CNS, neurogenesis starts at embryonic day 10-12 (Gotz & Huttner, 2005; Matsuzaki & Shitamukai, 2015). We propose that the absence of RHAMM could cause centrosome instability and modify the angle of the spindle during cell division for some, but not all cells, shifting the division of progenitor cells both during development and in the adult towards a more asymmetric division. This shift in division would produce a smaller pool of proliferating neural progenitor cells as well as a larger number of progenitor cells, which continue to develop along the neuronal lineage. This model would explain why we observed a smaller population of proliferating $BrdU^{+}$ cells in the SVZ and DG while registering a higher number of DCX^{+} cells in the unlesioned $HMMR^{-/-}$ compared to controls. More drastic effects have been observed using a mutant HMMR mouse model expressing a truncated form of RHAMM that lacks the centrosome-targeting domain (Li *et al.*, 2017). Li and colleagues were able to show that the truncated RHAMM induced a shift in the differentiation of the affected cells, due to spindle misorientation, causing premature differentiation and an increase in the generation of differentiating neurons, which led to a megalencephaly phenotype.

In conclusion, RHAMM has a modest, but not a vital, function and contributes to the optimization of mitosis mechanisms. Further, we must also consider some type of compensatory mechanism as not all cells were equally affected in Maxwell's anti-RHAMM experiments. One candidate worth investigating is the other major receptor for hyaluronan, CD44. Hyaluronan has been shown to shown to regulate quiescence and differentiation in adult hippocampal neural stem cells via the CD44 receptor (Su *et al.*, 2017). CD44 has also been shown to be expressed in undifferentiated neural progenitor cells at the embryonic stage (Naruse *et al.*, 2013). This work contributes to an already complex image for RHAMM function, but further investigations are needed to elucidate the role of RHAMM in normal neurogenesis and reactive neurogenesis after tissue damage.

5 CONCLUDING REMARKS

Stroke and traumatic brain injury are major problems worldwide, causing a severe long-term burden for the patient, the family and society. While the field of neural stem cells and adult neurogenesis has grown at a fast rate over the last decades, and great progress is being made, we are still far from "curing" the brain after injury or disease. As the field is growing, so have the different approaches emerged. Ever since the discovery of neural stem cells, neuroscientists have been trying to use these stem cells to regenerate lost cells and tissue, by either boosting the brains endogenous capability for neurogenesis or via stem cell transplants. And although some success has been seen, the road to unlocking the potential of neural stem cells is long and filled with obstacles. The work in this thesis contributes to the field in two parts.

All transplants, be it cells, biomaterials or the combination face similar problems. How will the host tissue react and how can we control the fate of the transplant? There are several aspects to consider when designing a biomaterial for in vivo use. An ideal biomaterial should be able to serve its purpose delivering cells or appropriate biological signals, while causing little to no damage or neuroinflammatory response from the host. It should be minimally invasive as to cause little damage to the tissue when transplanted, preferably the material should be injectable. It should be biocompatible, so that the host immune system does not reject it, and it should also be biodegradable to lower the need for further interventions. In **paper I** and **paper II**, we demonstrated that injectable peptide amphiphiles (PA) fulfill all of the abovementioned criteria, making them ideal for CNS transplantation. In **paper I** we demonstrated that a PA carrying the bioactive Ten-C sequence as epitope can be used for re-directing migrating neuroblasts into a novel brain area. This was done as a first step towards using the endogenous cells for regenerating tissue lost due to stroke or traumatic brain injury.

While we in **paper I** wanted to make use of the endogenous progenitor cells, **paper II** had a different approach. One major issue facing all stem cell transplants in the CNS is the host glial cell response. This leads to low cell survival rates, and also creates a sub-optimal environment for differentiation into neurons. In **paper II** we wanted to test if the PA had potential for future use as cell carrying scaffold for transplants. For this we chose the E_2RGDS PA and we could show a reduction in the reactive gliosis response to this biomaterial. In conclusions, both PAs satisfy the criteria for a biomaterial well suited for CNS in vivo applications. While our results were very positive, they were only the first proof of concept studies and tested feasibility, while more

functional tests regarding possible improvement in recovery in lesion models would be the next step. Considering the many ways the PAs can be modified and combined, the difficulty for future experiments will rather be to limit experimental variables in order to produce testable hypotheses.

In **paper III** we show a new role for the well-known hyaluronan receptor RHAMM. Although RHAMM has been studied extensively, its role in adult neurogenesis is quite unexplored. Our data indicate that RHAMM has a significant role in the neurogenic niches affecting both cell proliferation and DCX^+ expressing progenitor cells. We can only speculate as to why this is, and further investigation is needed.

In summary, this thesis work highlights the important role of the extracellular matrix in the effort to unlock the regenerative potential of the CNS. Not only the presence or absence of stem cells will have an impact on the success of repair processes but also the structural and biochemical surrounding that the extracellular matrix provides. The existing CNS neurogenic niches have unique features with regard to the ECM, and brain regions that are not privilege to neurogenesis may benefit from introduction of such signals to make these regions more repair-permissible. There is still much to be discovered.

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