# INDUCED EXPRESSION OF THE CYTOSKELETON LINKER PROTEIN RADIXIN IN THE ADULT BRAIN

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2012

*Cover illustration: Radixin expressing neuroblasts migrating in the explant migration assay.*

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*Tryck: Ineko AB, Göteborg, 2012*
*ISBN:* 978-91-628-8566-3

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## Abstract

Neural stem and progenitor cells (NSPCs) proliferate throughout life in two regions of the brain, namely the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of dentate gyrus in the hippocampus. In the adult SVZ, NSPCs give rise to neuroblasts that leave the SVZ for long distance migration along the rostral migratory stream (RMS), on their way to the olfactory bulb where they mature and are integrated in the neural network. Understanding how adult neuronal migration is regulated is of importance for the development of new therapeutic interventions using endogenous stem or progenitor cells for brain repair strategies. Long distance migration of neuroblasts in the RMS requires a highly dynamic cytoskeleton with the ability to respond to surrounding stimuli. In this thesis, we hypothesized that cytoskeleton rearrangement in the RMS is mediated by the ERM (Ezrin/Radixin/Moesin) family of proteins. ERM proteins regulate actin polymerization through interaction with actin and transmembrane adhesion molecules in many different parts of the body, however, limited studies exist of ERM proteins in the adult brain. In the first paper, our studies demonstrate the specific expression of radixin in neuroblasts in both the ventricular and hippocampal neurogenic niches. We also demonstrate the presence of radixin in Olig2 expressing cells throughout the adult brain. In the second study, inhibition of radixin using a selective quinocarmycin analog interrupts the ability for radixin to link the actin cytoskeleton to the membrane. Inhibition of radixin in SVZ explant cultures selectively blocked the migration of neuroblasts, whereas glial migration remained unaltered, suggesting that these populations use different ERM proteins for actin polymerization. In addition, intracerebroventricular infusion of the radixin inhibitor resulted in aberrant neuroblast chain formation and decreased neuroblast proliferation in the RMS.

In the third paper, EGF treatment is known to greatly reduce the migratory population in the SVZ and RMS. Nevertheless, EGF infusion elevated the radixin expression twofold in the SVZ and RMS. Accordingly, a new radixin expressing population was present in the RMS after EGF treatment and these cells also expressed Olig2. Proliferation of the radixin/Olig2$^+$ population occurred already after 24h, even in parts of the RMS that are distal to the SVZ, suggesting local activation by EGF throughout the RMS rather than migration from the SVZ. The radixin/Olig2$^+$ cells in the RMS were arranged in chains and migrated in explants cultures *in vitro*. Being negative for NG2 and CNPase, these radixin/Olig2$^+$ cells are likely not oligodendrocyte progenitors.

In the fourth study, radixin expression was induced in the peri-infarct region after cortical stroke. Unexpectedly, the number of cortical radixin/Olig2$^+$ cells decreased after stroke and radixin was instead present in a subpopulation of activated microglia. In the healthy brain and in the contralateral cortex, microglia did not express radixin. A new dual concept of microglial activation suggests the presence of classically activated M1 microglia and an alternatively activated M2 microglia population, which has more beneficial effects for the survival of neurons under inflammation conditions. The expression profile of radixin after stroke implies similarities with the type M1 microglia and radixin might be useful as a new microglia activation marker.

Taken together, these data suggest a role for radixin in NSPC proliferation and migration in the adult brain, as well as in activation of microglia after stroke.

## Förökning och förflyttning av neurala stamceller i vuxen hjärna

Neurodegenerativa sjukdomar, som Parkinsons sjukdom eller Alzheimers sjukdom, eller skador efter till exempel stroke, orsakar celldöd i hjärnan. Denna förlust kan ge bestående funktionsnedsättningar som vi än idag inte kan bota. Omogna celler med potential att omvandlas till hjärnans tre huvudsakliga celltyper (neuron, astrocyter och oligodendrocyter) finns i särskilda områden i den vuxna hjärnan. De omogna cellerna kallas stamceller och ger genom celldelning upphov till blivande nervceller, neuroblaster, vilka förflyttar sig längs definierade stråk i hjärnan. När neuroblasterna når sin destination mognar de och blir till nya fullt funktionella neuron. Man vet att stamceller kan aktiveras av skador i hjärnan, men man vet inte om de bidrar till återbyggnad av de skadade områdena. En grundläggande kartläggning av hur stamcellspopulationen kan utökas och hur neuroblasterna förflyttar sig skulle därför kunna bidra till utveckling av nya behandlingsstrategier. Därmed skulle kroppens egna omogna celler kunna rekryteras till skadade områden och bidra till läkning.

I detta avhandlingsarbete har ett nytt protein, radixin, identifierats i hjärnans stamceller och neuroblaster hos vuxna råttor och möss. Det är känt att radixin reglerar förflyttning av celler i andra delar av kroppen och genom att hämma funktionen av radixin i hjärnan kunde vi visa att detta protein är särskilt viktigt för förflyttningen av neuroblaster. Det är sedan tidigare känt att stamcellspopulationen kan ökas markant genom att behandla hjärnan med tillväxtfaktorn epidermal growth factor (EGF). Våra studier visar att EGF även genererar en ny population av omogna celler i ett mycket större område än vad som tidigare visats. Den nya populationen kunde dessutom förflytta sig och cellerna uttryckte den aktiva formen av radixin. Att omogna celler finns i större utsträckning än tidigare visats ökar chanserna för att de ska kunna förflytta sig till skador i anslutning till det nya området.

Vid stroke aktiveras och förflyttar sig en liten population av omogna celler till det skadade området. För att ta reda på hur uttrycket av radixin påverkas i omogna celler efter stroke, använde vi en experimentellt inducerad stroke-modell på vuxna möss. Vi fann att neuroblaster som rörde sig mot strokeskadan bara uttrycker den inaktiva formen av radixin. Eventuellt saknas signaler för att aktivera radixin utanför stamcellsområdet och detta kan vara en orsak till att de omogna cellerna inte förflyttar sig särskilt effektivt utanför stamcellsområdet. Vidare upptäckte vi att hjärnans egen immuncell, mikroglia, uttrycker radixin i den inflammerade miljön kring strokeskadan. Aktivering av mikroglia kan både hjälpa eller förhindra återhämtningen efter stroke mer studier krävs innan dessa olika funktioner klargjorts, och om funktionen av radixin är positiv eller negativ för läkningsprocessen.

Sammanfattningsvis har vi identifierat ett nytt protein med en viktig funktion i förflyttningsprocessen av blivande nervceller. Resultaten tyder på att radixin-uttrycket ökar tillfälligt i celler med hög aktivitet i form av celldelning och förflyttning, och kan vara ett användbart mål för stimulering av blivande nervceller.

This thesis is based on the following papers;

I. **Åsa Persson**, Charlotta Lindvall, Maurice Curtis, Georg Kuhn. Expression of ERM proteins in the adult subventricular zone and the rostral migratory stream. Neuroscience. 2010 May 5;167(2) s312-22.

II. **Åsa Persson**, Olle Lindberg, Georg Kuhn. Radixin inhibiton reduces neuronal progenitor migration. In manuscript.

III. Olle Lindberg*, **Åsa Persson*,** Anke Brederlau, Aidin Shabro, Georg Kuhn. EGF responsive cell population resident in the RMS. PLoS One. 2012;7(9):e46380. Epub 2012 Sep 28.

IV. **Åsa Persson***, Ahmed Osman*, Hayde Bolouri, Carina Mallard, Georg Kuhn. Radixin expression in activated microglia after cortical stroke. Submitted.

** these authors contributed equally to this paper.*

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# Introduction

The discovery of new neurons formed in the adult brain greatly altered the old picture of the brain as a static organ with no means for repair. Today there is no cure for the loss of function due to neuronal death after, for example, neurodegenerative disease or stroke. However, there is evidence that the brain is plastic and the function of neural networks in the hippocampus can be stimulated by for example, running (van Praag et al., 1999). If new neurons can be added to existing circuits, there may be a way to stimulate endogenous neural stem/progenitor cells to repair damages in the brain. In the rodent brain, neural stem/progenitor cells migrate to the site of injury after stroke (Arvidsson et al., 2002). In the human brain, cells in the stem cell niche display increased proliferation after stroke, although limited migration of neural stem cells occurs (Macas et al., 2006).

Experimental modulation of neural stem/progenitor cells can reveal ways to stimulate repair actions. EGF treatment of the neural stem cell niche increases proliferation but decrease migration of immature neurons (Doetsch et al., 2002). Stroke increases both proliferation and migration of neural stem/progenitor cells (Arvidsson et al., 2002, Li et al., 2010). In this thesis, a new protein, radixin, involved in neural stem/progenitor cell migration and proliferation was discovered. EGF stimulation and a stroke model were used to study the regulation of radixin expression in the adult rodent brain.

# Background

## Neural stem/progenitor cells in the adult brain

New neurons are generated from **neural stem/progenitor cells (NSPCs)** throughout life in **the subgranular zone (SGZ)** in the hippocampus, **the subventricular zone (SVZ)** of the anterior lateral ventricles, and in the core of **the olfactory bulb (OB)**. Neurogenesis in these regions has been demonstrated in several species including rodents, monkeys and humans (Altman and Das, 1965b, Eriksson et al., 1998, Kornack and Rakic, 1999, Bedard and Parent, 2004). The presence of cell proliferation in the adult brain was first described in the 1960s (Altman and Das, 1965a, Altman, 1969), although these findings were long ignored and the potential of these cells was demonstrated much later. In the early 1990s, a significant breakthrough was the discovery of self-renewal and multipotency *in vitro* by cells derived from the tissue lining the lateral ventricle (Reynolds and Weiss, 1992, Vescovi et al., 1993, Morshead et al., 1994). However, the full potential of modulating and mobilizing NSPCs to achieve repair and regeneration at sites of brain injury and disease remains to be discovered.

### The concept of neural stem and progenitor cells

A neural stem cell is defined as a cell displaying both long-term **self-renewal** and **multipotency**, i.e. the ability to generate all mature cell types in the nervous system, including neurons, oligodendroglial and astroglial cells. Accordingly, dividing neural stem cells can give rise either to new neural stem cells or a more restricted progeny, often referred to as **progenitor cells** (Morshead and van der Kooy, 1992).

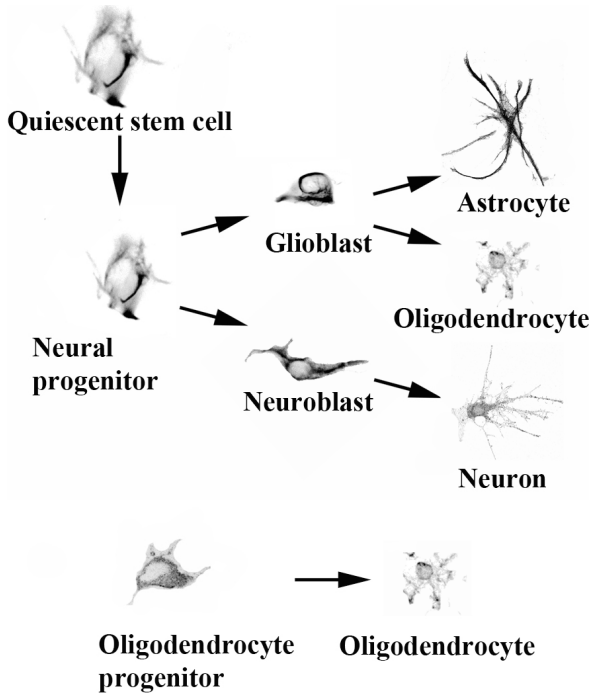


***Figure 1.*** *Schematic picture of the neuronal stem cell lineage in the adult brain.*

Differentiation restricts the self-renewal and multipotency potential of NSPCs during the course of becoming an integrated neuron. Progenitor cells are also able to proliferate for a period and then continue to differentiate into either the neuronal or the glial lineage. **Neurogenesis** is the generation of mature neurons from neural progenitor cells.

Glial progenitors give rise to astroglial progenitors or oligdodendroglial progenitors which differentiate into astrocytes or oligodendrocytes (Raff et al., 1983). Considering the difficulties in distinguishing between neural stem and progenitor cells in the adult neurogenic niches they are referred to as neural stem/progenitor cells (NSPCs) in this thesis.

Furthermore, **restricted oligodendrocyte progenitor cells (OPCs)** are present throughout the adult brain (Levine and Reynolds, 1999). But OPCs can also be present in the adult neurogenic niche (Ligon et al., 2006) which is of importance for some results in this thesis.

### Cell proliferation

Four phases are required to complete a cell division; G1-, S-, G2- and M-phase. As a cell enters the G1-phase it is ready for division and during S-phase a second copy of the DNA is synthesised (Goodrich et al., 1991). In the G2-phase, the cell is tetraploid and continues to grow. After passing a control point to ensure that the cell is ready for mitosis, the M-phase occurs, in which the chromosome pairs are separated and cell division is completed (Innocente et al., 1999). Non-dividing, quiescent or mature cells; are confined to a resting G0-phase. Cell division of stem cells can be symmetric or asymmetric, where symmetric division give rise to two new cells with preserved 'stem' cell properties, and asymmetric division instead give rise to one cell with 'stem' properties and another cell, which becomes more differentiated towards a certain lineage (Morrison and Kimble, 2006).

## Basic methodology for labelling and studying NSPCs

### Neural stem cell culture

NSPCs isolated from the SVZ or SGZ of the adult rodent brain have the capacity for self-renewal in culture during several passages in the presence of the growth factors **epidermal growth factor (EGF)** and **basic fibroblast growth factor (FGF-2)** (Reynolds and Weiss, 1992, Vescovi et al., 1993, Morshead et al., 1994). Furthermore, cultured NSPCs are multipotent with the potential to differentiate into both neuronal and glial lineages when plated on adherent substrates (Reynolds and Weiss, 1992, Vescovi et al., 1993, Morshead et al., 1994). NSPCs are grown in suspension cultures *in vitro* and proliferate in clusters generally called **neurospheres**. Neurosphere culturing is a valuable tool for the study of NSCPs and their progeny. However, due to the artificial conditions in cell cultures, comparison with the *in vivo* situation should be made with caution.

### *In vivo* labelling of NSPCs

**BrdU** is a thymidine analog which is integrated in the DNA during the synthesis phase (S-phase) of the cell cycle and is thereafter retained in the postmitotic cell (Dolbeare, 1996). To follow the progeny of cells dividing during BrdU injection, immuno-fluorescence labelling against BrdU and markers specific to different cell types are performed at certain time-points after cell division. The cell cycle of NSPCs in the SVZ was estimated to 12 h, and the S-phase to 4.2 h (Morshead and van der Kooy, 1992). Since BrdU is rapidly degraded and only biologically available for a maximum of 2

hours, a single injection labels only a subpopulation of all dividing cells in this region. Thus, repeated injections are needed to label larger portions of the progenitor population.

### NSPC migration assay

The progeny of NSPCs that differentiate into progenitors with commitment to the neuronal lineage, migrate a significant distance to their final destination where they become mature neurons. This migration can be studied *in vitro* through the culturing of neurospheres, or tissue pieces, so called explants of the SVZ in a gel derived from the extracellular matrix of mouse brain, **Matrigel®**. In this assay, sphere- or explant-derived cells migrate though the Matrigel and display many characteristics of *in vivo* NSPC migration (Wichterle et al., 1997, Mason et al., 2001, Persson et al., 2010).

## NSPCs in the subgranular zone of the hippocampus

NSPCs in the hippocampus reside along the border between the granular cell layer and the hilus of the dentate gyrus (white arrow in figure 2B), migrate into the granule cell layer, and are functionally integrated in the existing circuitry (Altman and Das, 1965a, Kempermann and Gage, 2002). The proliferation of progenitor cells in the hippocampus persists into adulthood, but declines with age in rodents (Kuhn et al., 1996). Differentiation restricts the self-renewal and multipotency potential of NSPCs during the course of becoming an integrated neuron. The proliferating cells in **the subgranular zone** give rise to mature neurons that migrate a short distance into the granule cell layer, where they extend dendrites and axons (Hastings and Gould, 1999). Hippocampal adult neurogenesis plays a central role for certain memory and learning tasks, and is important for cognitive function (Gould et al., 1999, Jessberger et al., 2009). Neurogenesis in the hippocampus is increased by exogenous stimuli such as exercise, enriched environment (Kempermann et al., 1997, van Praag et al., 1999) and hippocampus-dependent learning (Gould et al., 1999), while stress (Gould et al., 1997) and irradiation decrease the generation of new neurons (Monje et al., 2002). Considering the plasticity of adult neurogenesis it is tempting to suggest development of approaches recruiting NSPCs for the regeneration of damaged areas after stroke or neurodegenerative disease. However, the short migration distance and the isolated location of hippocampal NSPCs makes this population less useful in future repair approaches that aim at recruitment of endogenous stem cells to areas remote from the neurogenic niches. For this purpose, NSPCs in the SVZ of the anterior lateral ventricles are more suitable.

## NSPCs in the subventricular zone of the lateral ventricles

The largest pool of NSPCs in the adult brain resides in **the anterior wall of the lateral ventricles** and gives rise to new neurons far away from their place of birth (Altman, 1969). After **long distance cell migration** through the forebrain, neuroblasts arrive in the OB (Figure 2B). Neuroblasts mature into dopaminergic or GABAergic interneurons in the granular or periglomerular layers of the OB (Kosaka et al., 1995, Kosaka and Kosaka, 2008).

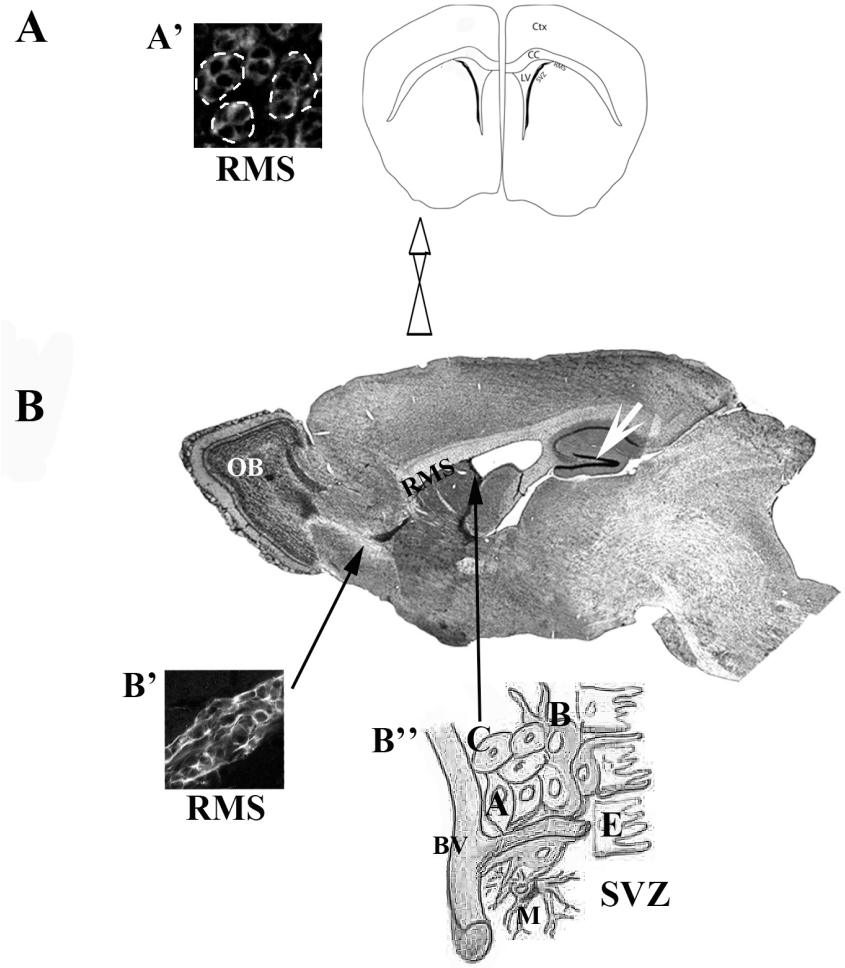


**Figure 2. Overview of the SVZ and RMS in the adult rodent brain.** (A) Coronal sections of the rodent brain. (A') In coronal sections the chains of RMS neuroblasts are visible as clusters of cells. (B) Sagittal section of the rodent brain. The RMS transverses the forebrain from the SVZ to the OB (B') In sagittal sections, chains of elongated neuroblasts are visualized in the RMS. (B'') The SVZ cell types are clustered close to the ependymal cells lining the ventricular wall (black arrow). The white arrow in (B) points at the dentate gyrus of the hippocampus. Abbreviations; A, A-cell or neuroblast, B, B-cell, C, C-cell, E, ependymal cell, M, microglia, RMS, rostral migratory stream, SVZ, subventricular zone.

## The NSPC lineage in the adult SVZ

### Type B-cells

A population of slowly dividing cells was identified in the SVZ, capable of self-renewal and multipotency (Morshead et al., 1994). Selective ablation of dividing cells expressing **the glial fibrillary acidic protein (GFAP)** in the adult brain revealed an almost complete loss of the ability to grow neurospheres *in vitro* (Morshead et al., 2003), indicating that the stem cell lineage in the SVZ starts with an astrocyte-like cell. Accordingly, the SVZ contains both regular astrocytes, **type B1 cells**, as well as a stem cell expressing the astrocyte protein GFAP, **type B2 cells** (Doetsch et al., 1997, Merkle et al., 2004). The identity of the SVZ stem cells is still debated and it was recently

suggested that B-cells could switch between a quiescent (the B1 cell) and activated state (the B2 cell), in which stem cells divide more frequently (Basak et al., 2012).

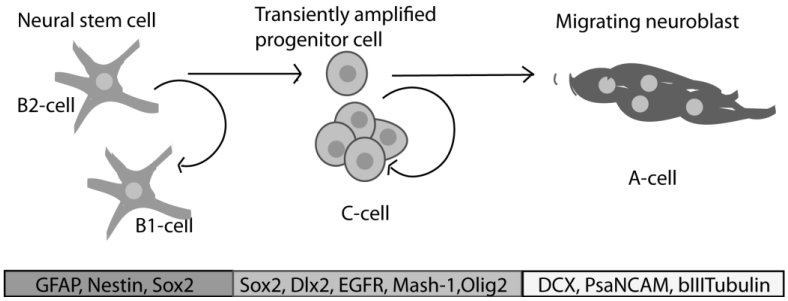


***Figure 3.*** Marker proteins of NSPCs in the adult SVZ.

To distinguish between cell types *in vivo* several cell-type specific protein markers and combinations of markers are commonly used (Figure 3). The type B-cell expresses **GFAP**, **nestin** as well as **high levels of Sox2 (Sox2$^{high}$)** (Doetsch et al., 1997, Doetsch et al., 1999, Ferri et al., 2004). However, even when using these markers, the distinction between type B1 and B2 cells remains difficult. Furthermore, it has been suggested that **ependymal cells** lining the ventricle wall may be the origin of stem cells in the adult forebrain (Chiasson et al., 1999, Johansson et al., 1999).

### Type C- and A-cells

B-cells give rise to a population of transiently amplifying **type C-cells** which also reside in the SVZ (Doetsch et al., 1997). The type C-cell is a progenitor cell and more restricted than a stem cell. It undergoes a short period of intense proliferation before differentiating into **the type A-cell**, which is a neuronally restricted progenitor cell, also called **neuroblast**. The more restricted population of rapidly dividing type C-cells expresses the transcription factors **Sox2**, **Dlx2**, **Mash-1**, **Olig2** and **the EGF receptor (EGFR)** (Doetsch et al., 2002, Aguirre and Gallo, 2004, Ferri et al., 2004, Parras et al., 2004, Menn et al., 2006), whereas neuroblasts are typically detected by intense expression of **Doublecortin** (DCX, Gleeson et al., 1999), **polysialylated neuronal cell adhesion molecule (PSA-NCAM)** (Hu et al., 1996) and **βIII-tubulin** (Mokry et al., 2004). Neuroblasts can also express Sox2, although at a low expression level **(Sox2$^{low}$)** (Ferri et al., 2004).

### Ependymal cells

Besides B1 and B2, C and A-cells, the stem cell niche also consists of **ependymal cells**, microglia, and endothelial cells, which influence the NSPCs. Whether stem cells or not, ependymal cells have an important role in the SVZ niche. Lining the ventricle wall, the ependymal cells function as a barrier between the cerebrospinal fluid (CSF) in the ventricles and the parenchymal tissue. These cells have multiple cilia projecting into the ventricles and regulate the CSF flow, which in turn can affect neuroblast migration (Sawamoto et al., 2006).

### Microglia

**Microglia** are the immune cells of the brain. There is evidence for regional differences in microglia (Hellstrom et al., 2011); and in the SVZ, microglia are constitutively semi-activated compared to other parts of the brain (Goings et al., 2006). It was shown *in vitro* that neurogenic stem cells forming multipotent neurospheres, progressively lose the ability to generate committed neuroblasts with continued culture, interestingly this feature could be rescued by co-culture with microglial cells or microglia-conditioned medium, further supporting a role for microglia in regulating neurogenesis. (Walton et al., 2006).

### Blood vessels in the SVZ

A dense network of blood vessels covers the SVZ and NSPCs are located in close proximity to capillaries, often making direct contact to endothelial cells. This interaction regulates the proliferation rate of NSPCs (Shen et al., 2008). Furthermore, neuroblast chains line up along the vessels, which are oriented towards the RMS, suggesting a guiding role for the vasculature in the SVZ (Shen et al., 2008).

### Olig2 expression in OPCs and SVZ C-cells

During development OPCs spread throughout the brain to reach their final destination where the majority differentiate into mature oligodendrocytes although a subset is maintained as progenitors (Bradl and Lassmann, 2010). **Adult OPCs** express high levels of **Olig2**, **the platelet derived growth factor alpha (PDGFα)** receptor and **the NG2 chondroitin sulphate proteoglycan** (Pringle et al., 1992, Ligon et al., 2006, Bradl and Lassmann, 2010). Low levels of Olig2 expression are maintained in differentiated oligodendrocytes. The proportion of $NG2^+$ cells within the $Olig2^+$ population is higher in the SVZ compared to the cortex (Ligon et al., 2006). In the SVZ, progenitors displaying an OPC-like immunophenotype (Olig2/NG2/nestin/PDGFα/$Mash\text{-}1^+$) are present (Aguirre and Gallo, 2004). Although $NG2^+$ cells are generally considered to be OPCs, a subpopulation of FACS sorted postnatal $NG2^+$ cells could generate neurospheres under EGF treatment and subsequently differentiate into neuronal cell types (Aguirre and Gallo, 2004), suggesting similarities to the SVZ type C-cell with respect to the induction of multipotency.

Fate determination of SVZ progenitors is partly regulated by Olig2 (Hack et al., 2005). Rapidly dividing **C-cells express Olig2** during proliferation and inhibiting Olig2 function results in depletion of the C-cell population (Hack et al., 2005). Furthermore, overexpression of Olig2 in the SVZ induces progenitor proliferation and migration to the corpus callosum and increased oligodendrocytic differentiation. When Olig2 is subsequently reduced in C-cells, neuronal differentiation continues (Hack et al., 2005).

### Plasticity of NSPCs in the SVZ

During aging, the rodent SVZ continues to generate NSPC's, however, at a reduced rate (Maslov et al., 2004). A fraction of NSPCs undergoes apoptosis in the naive SVZ (Bauer and Patterson 2005). Furthermore, an increased rate of SVZ proliferation was accompanied by increased apoptosis (Belvindrah et al., 2002), suggesting that increasing the SVZ turn-over may be adjusted to a base line level through apoptosis. Furthermore, olfactory input can modulate the degree to which new neurons are added to the OB by altering neuroblast apoptosis (Mandairon et al., 2003). This suggests an **inherent plasticity in the SVZ-OB niche** in keeping the degree of neurogenesis homeostatic. Furthermore, exogenous stimuli have the ability to modulate the proliferative activity in the SVZ, for example tissue damage after **traumatic brain injury** and **ischemia stimulate proliferation and increase migration towards damaged areas** (Arvidsson et al., 2002, Li et al., 2010). Modulation of the neurogenic niches may provide information about the usefulness of stem or progenitor cells in future brain repair strategies. However, it is yet not known whether the SVZ stem cells have unlimited capacity for self-renewal. Recent studies in the rodent brain propose the deforestation theory which suggests that increasing SVZ proliferation will lead to early exhaustion of the stem cell pool (Encinas and Sierra, 2012). This would be an explanation for the decline in neurogenesis during aging in rodents, and could be a reason to the limited physiological levels of neurogenesis in the adult human brain. Modulation of the NSPCs and their progeny in response to stroke and EGF stimulation respectively will be described in more detail below.

## Chain migration in the rostral migratory stream

NSCPs originating in the SVZ generate large numbers of neuroblasts which migrate along a well-defined pathway called **the rostral migratory stream (RMS)** towards the OB (see Figure 2B). RMS neuroblasts **migrate in chains** and, upon arrival in the OB; they detach from their migratory chains and continue migrating as single cells. The final destination of the neuroblasts is the granular and peri-glomerular layers of the OB where they become mature neurons and are integrated in the existing network of neurons.

### RMS neuroblasts migrate in chains

During embryonic stages neuronal progenitors migrate individually to the OB (Kishi et al., 1990). The RMS is then formed from the collapsed embryonic olfactory ventricle and during early postnatal weeks, neuroblasts start to migrate along each other in chains (Lois et al., 1996, Pencea and Luskin, 2003, Peretto et al., 2005). **Tangential chain migration is highly efficient**, reaching a velocity of 50-100μm/h, which is at least 20% faster than migration of individual cells (Bovetti et al., 2007, Kim et al., 2009). However, these cells do not move constantly at this speed, but advance in a saltatory manner with intermittent periods of inactivity (Davenne et al., 2005, Kim et al., 2009).

### Migration permissive environment in the RMS

The special environment surrounding the chains of neuroblasts contributes to the efficient migration of these cells. In part, this is achieved by a high content of migration permissive extracellular matrix components. Furthermore, a dense network of astrocytes develops into the **glial tubes** that are wrapped around the chains of neuroblasts, and provide support, directional cues and restrict the dispersal of neuroblasts (Doetsch and Alvarez-Buylla, 1996, Lois et al., 1996). Notably, chain migration was demonstrated to precede glial tube formation. In addition, the vasculature surrounding the RMS has an important role in stimulating and guiding the neuroblasts during migration. The **organization of blood vessels** is altered in the postnatal forebrain resulting in vessels horizontal to the RMS which physically outlines the stream (Bozoyan et al., 2012). Despite a clear role for attractive and repulsive forces present along the RMS, bulbectomy does not ablate anterior migration of neuroblasts (Jankovski et al., 1998, Kirschenbaum et al., 1999).

### Neuroblasts divide on their route to the OB

A vast number of cells are active in **dividing along the entire route of the RMS** and the majority of dividing cells are neuroblasts. Notably, dividing neuroblasts display leading and trailing processes immediately after incorporating BrdU during the S-phase of the mitotic cycle (Luskin, 1993), however, this methodology cannot reveal whether the cells had processes before cell division. Proliferation of neuroblasts was further examined by time-lapse imaging of NSPCs (Coskun et al., 2007). It was demonstrated that neuroblasts stop migrating, retract their processes and undergo several divisions before resuming migration. During process retraction the cell morphology changes from elongated to round, and the reappearance of an elongated morphology and process formation starts already 90 minutes after division (Coskun et al., 2007).

## Modulators of neuroblast migration in the RMS

Neuroblast migration in the RMS is regulated by several molecules mediating a range of different cellular functions including chemoattraction/repulsion, cell adhesion, motility and cytoskeleton dynamics, suggesting a complex system of regulation. Additionally, some factors influence both neuroblast migration and proliferation of NSPCs.

### ECM molecules

The ECM of the RMS resembles some features of the embryonic brain, creating a permissive structure for migration. One example are the increased levels of ECM molecules such as **tenascin C**, **laminin** and **chondroitin sulphate-containing proteoglycans**, which provide appropriate levels of adhesion and prevent differentiation, contributing to the pro-migratory environment (Jankovski and Sotelo, 1996, Thomas et al., 1996, Peretto et al., 1997). Laminin is a ligand for integrin receptors which mediate signals from the ECM into the cell. **β1-integrin** is present on RMS neuroblasts and blockage or deletion of endogenous integrins disrupt the characteristic chains of RMS neuroblasts

(Emsley and Hagg, 2003, Belvindrah et al., 2007). Neuroblasts dispersed into adjacent tissue although some still found their way to the OB (Emsley and Hagg, 2003, Belvindrah et al., 2007). Similar results were described for laminin mutant mice (Belvindrah et al., 2007). In addition, injection of a laminin tract close to the healthy RMS attracted neuroblasts from the stream (Emsley and Hagg, 2003), suggesting a role for laminin as a chemoattractant in RMS migration, in addition to regulating chain assembly.

### Cytoskeleton components

Cell migration involves the continuous rearrangement of the cytoskeletal apparatus. During migration, the leading edge of the cell is pushed forward by growing actin filaments (Theriot and Mitchison, 1991). Actin filaments are elongated by branching of existing filaments at the leading edge through activation of Arp2/3 complexes, creating short branches on a longer backbone. The filament elongation is constantly accompanied by disassembly of the actin polymer at the rear, regulated by cofilin complexes. A constant assembly and disassembly is necessary to not deplete the pool of unpolymerized actin (Pollard, 2003). In the mouse brain, deletion of the **serum response factor** lead to decreased β-actin expression and cytoskeletal actin fiber density, along with aberrant cofilin activity (Alberti et al., 2005). The actin cytoskeleton defects were accompanied by accumulation of neuronal progenitors in the SVZ and arrested neuroblast migration (Alberti et al., 2005). RMS chain migration was also defective in mice lacking the **actin-binding protein Girdin** (Wang et al., 2011b). Girdin is normally membrane-associated via an interaction with phosphoinositides, and is translocated to the lamelipodium upon phosphorylation where it crosslinks actin filaments (Enomoto et al., 2006). Girdin$^{-/-}$ mice appear developmentally normal but have distinct alteration in the neurogenic niches of the brain, displaying disoriented and dispersed RMS neuroblasts compared to control (Wang et al., 2011b). The numbers of neuroblasts in the SVZ and RMS were increased in the mutant mice; furthermore, a significant number of neuroblasts migrated into the adjacent striatum, suggesting that Girdin regulates several aspects of RMS migration (Wang et al., 2011b).

The tubulin cytoskeleton system also has essential roles in cell migration and the **microtubule-associated protein DCX**, is highly expressed by RMS neuroblasts (Gleeson et al., 1999, Ocbina et al., 2006). DCX inhibition reduced neuroblast migration *in vitro* (Ocbina et al., 2006). Deletion of DCX *in vivo* resulted in a thicker RMS, with a proportional increase in GFAP and PSA-NCAM immunoreactivity, although accompanied by increased cell death and neuronal differentiation within the RMS (Koizumi et al., 2006). A decreased number of interneurons reach the glomerular layer in the olfactory bulb. Live imaging of RMS in cultured slices revealed that the control cells had a bipolar shape and saltatory migration, while mutant cells had a multipolar morphology and their migration was interrupted by long pauses. Further studies revealed that DCX mutant mice displayed less frequent nuclear translocation (Koizumi et al., 2006).

### Adhesion molecules

Cell-cell and cell-matrix interactions, regulated by cell adhesion molecules, are important in maintaining the structure of the RMS and controlling migration. Both embryonic and adult SVZ neuroblasts express **PSA-NCAM**, which have a central function in RMS chain migration (Ono et al., 1994, Hu et al., 1996). The post-transcriptional addition of PSA to NCAM results in increased space between neuroblasts, which in turn reduces homophilic NCAM interaction, and thereby cell-cell adhesion (for review see Bonfanti, 2006). Reducing cell-cell adhesion makes the cells slippery and allows the cells to slide along each other as neuroblasts do during chain migration. In NCAM deficient mice the organization of the RMS was disrupted, displaying increased expression of GFAP processes, disoriented axons and less neuroblast chains. The RMS was thicker and the migrating neuroblasts clustered close to the SVZ (Ono et al., 1994, Hu et al., 1996, Chazal et al., 2000). Enzymatic removal of PSA resulted in a substantial decrease in migration distance *in vitro* (Chazal et al., 2000, Hu, 2000). Addition of PSA increases extracellular space between cells and, some studies suggest there is an extra-large space between RMS neuroblasts and glial tube astrocytes (Lois et al., 1996, Peretto et al., 1999, Bonfanti, 2006).

**Galectins** are another family of proteins modulating cell-cell and cell-matrix adhesion and can both enhance and reduce adhesion through different interactions with for example laminins and integrins (Hughes, 2001). **Galectin-3** is highly expressed in the glial tubes of the RMS and live imaging of migration in Galectin-3 knockout mice revealed an aberrant, slower migration pattern (Comte et al., 2011).

A similar role for **A disintegrin and metalloprotease 2** (ADAM2) in RMS migration has been described (Murase et al., 2008). The multifunctional ADAM transmembrane proteins often have protease- and/or integrin binding activity and can regulate the activity of molecules such as Notch and TGFα (Tomczuk et al., 2003, Yang et al., 2006). The ADAM2 knockout mouse has a smaller OB and decreased migratory speed that is detected both *in vivo* and *in vitro* (Murase et al., 2008).

### Chemoattractive/repulsive factors

Factors repelling neuroblasts are present surrounding the SVZ whereas neuroblast attractants are secreted in the OB. Slit proteins are present in the septum and choroid plexus, and **Slit1** is able to repel SVZ neuroblasts (Nguyen-Ba-Charvet et al., 2004, Kaneko et al., 2010). Slit signalling through their **Robo receptors** is also involved in the organization of the glial tubes (Kaneko et al., 2010).

Several growth factors are chemoattractants for RMS neuroblasts, including **Brain derived neurothropic factor** (BDNF) acting through its receptor **TrkB, glial cell line-derived neurotrophic factor** (GDNF) and **vascular endothelial growth factor** (VEGF), displaying higher expression in the OB than in the SVZ (Paratcha et al., 2006, Chiaramello et al., 2007, Bozoyan et al., 2012). GDNF mRNA is abundantly expressed in the OB and its attractive force is depending on functional NCAM (Paratcha et al., 2006), suggesting various aspects of NCAM activity in the RMS.

**Sonic hedgehog** (Shh) have chemoattractive effects on RMS neuroblasts and grafting Shh expressing cells above the RMS *in vivo* induces their deviation towards grafted cells (Angot et al., 2008). In addition, blocking Shh function decreases SVZ proliferation and increases proliferation of NSPCs in the OB (Angot et al., 2008), suggesting a diverse modulatory function of Shh signalling.

### Ephrins and Eph receptors

EphB are receptor tyrosine kinases which bind transmembrane anchored ligands, ephrins B1-3, and interfering with **EphB2/ephrinB2** interactions disrupts RMS migration (Conover et al., 2000). Intracerebroventricular infusion of a truncated form of EphB2 can activate ephrin ligands and induced SVZ hyperproliferation (Conover et al., 2000), suggesting a strong proliferative effect of the ephrinB2 ligand.

### EGF receptors and neuregulin ligands

Several ligands bind to EGF receptors, the ErbB receptors (ErbB1-4), including EGF, TGFα, epiregulin and neuregulins 1-3, and the receptors are active after homo- or heterodimerization initiated by ligand binding (Yarden and Sliwkowski, 2001). All ErbB receptors are present in the postnatal SVZ, whereas in the RMS, high expression of **ErbB4** and limited expression of **EGFR** (ErbB1) and **ErbB2** have been detected (Anton et al., 2004, Ghashghaei et al., 2006). TGFα treatment reduced neuroblast migration, suggesting a negative effect of ErbB1 stimulation on neuroblast migration (Kim et al., 2009). Contrary to ErbB1, ErbB4 have a supportive role in neuroblast migration. Mice lacking ErbB4 have disorganised neuroblast chains in the RMS and reduced numbers of interneurons in the OB (Anton et al., 2004). Moreover, ErbB4-deficient cells appeared less polarized, directed fewer processes towards the OB and migrated slower (Anton et al., 2004). Furthermore, live imaging revealed that the leading cell processes of migrating neuroblasts in ErbB4 deficient mice changed direction more often than their wild-type counterparts. The features described for the ErbB4 deficient RMS indicate a lack of certain directional cues important for neuroblast migration. In addition, the ErbB ligand **neuregulin NRG1 type III,** which is abundantly expressed in the RMS, had a stimulatory and chemoattractive effect on migration in SVZ explant cultures (Anton et al., 2004, Ghashghaei et al., 2006). Furthermore, intracerebroventricular infusion of **neuregulin 2 (NRG2)** increased the amount of neuroblasts migrating to the OB, whereas NRG1 infusion haltered continued neuroblast migration (Ghashghaei et al., 2006). The halted migration after NRG1 infusion may be due to the chemoattractive force of this ligand. In summary, EGF receptors and their ligands exert a strong influence over the organisation of RMS migration and chemoattraction.

### The GABA receptor

**γ-aminobutyric acid** (GABA) signalling through the **GABA receptor A** modulates RMS migration (Bolteus and Bordey, 2004). Application of GABA decreased migration, whereas inhibition of the $GABA_A$ receptor alone increased migration, suggesting that the presence of GABA in the RMS

tonically activates the GABA receptor. GABA is released by the glial tube astrocytes which also express the GABA transporter GAT4 in their processes and inhibition of GABA uptake decrease migration (Bolteus and Bordey, 2004). **Intracellular $Ca2^+$** signalling is commonly involved in regulating cell migration and blockade of $Ca2^+$ release reduces RMS migration greatly (Komuro and Rakic, 1998, Bolteus and Bordey, 2004). During inhibition of $Ca2^+$ release, no further inhibitory effects could be detected by GABA or $GABA_A$ receptor blockade, suggesting that the effects of GABA is exerted through modulation of Ca2+ release (Bolteus and Bordey, 2004).

### Netrin receptors; neogenin and DCC

Migrating neuroblasts express the netrin receptors **neogenin** and **Deleted in Colorectal Carcinoma** (DCC). Treatment with anti-DCC antibodies in slice cultures altered the direction of the protrusions and reduced the migratory speed. Although not directly proven in this system, it was suggested that DCC contributes to the formation of directed protrusions through an interaction with the ligand netrin-1 (Murase and Horwitz, 2002).

### Detachment signals upon arrival to the OB

Upon arrival to the OB, neuroblasts detach from their migratory chains and start migrating radially to the granular and peri-glomerular layers of the bulb. The detachment is regulated by **reelin**, a large secreted glycoprotein, which is present in the superficial layers of the OB. (Hack et al., 2002). In reelin mutant mice, the RMS neuroblasts accumulate in the anterior RMS and in the center of the OB, unable to proceed to superficial layers (Hack et al., 2002). In addition, **tenascin-R** induces neuroblast detachment from migratory chains thereby triggering radial migration (Saghatelyan et al., 2004). Furthermore, **prokineticin2** may also be involved in the detachment process (Ng et al., 2005).

## NSPCs are present in the RMS and OB

It has long been assumed that type B-and C-cells are only present in the SVZ, however; several research groups have recently shown the presence of **multipotent NSPCs in the RMS** (Gritti et al., 2002, Alonso et al., 2008, Giachino and Taylor, 2009). The presence of NSPCs in the RMS would substantially increase the neurogenic area in the adult brain, since the RMS transverses the entire forebrain (see Figure 2B). This could be important for the recruitment of progenitor cells due to the considerably closer location to, for instance, the frontal cortex. Gritti and colleagues show that the NSPCs of the posterior RMS favour differentiation into the oligodendrocyte lineage, while the anterior RMS-hosted progenitors favour a neurogenic fate (Gritti et al., 2002). However, Alonso and colleagues demonstrated that GFAP expressing NSPCs, along the RMS, give rise to newly formed OB neurons, much like the NSPCs in the SVZ. In addition, a GFAP expressing slowly dividing population was detected in the RMS, resembling NSPCs in the SVZ (Alonso et al., 2008). Furthermore, OB derived neurospheres are multipotent *in vitro* but display limited self-renewal after

passaging (Gritti et al., 2002). However, lineage tracing of NSPCs from the RMS in the OB core showed local generation of neurons in the OB (Giachino and Taylor, 2009). In summary, characteristics of stem cells or progenitor cells in the RMS are still **unclear**.

## EGF signalling in the neurogenic niches of the SVZ and RMS

In addition to the above described effects on RMS migration, EGF receptors and ligands are regulators of proliferation in the adult neurogenic niches. In the SVZ, ErbB1 or EGFR, is expressed mainly by B- and C-cells, but also a small number of neuroblasts in the RMS express low levels of EGFR (Anton et al., 2004, Kim et al., 2009, Gonzalez-Perez and Quinones-Hinojosa, 2010).

### EGF treatment expands a highly proliferative cell population in the SVZ

In the initial *in vitro* studies, EGF was used to propagate cultures of SVZ cells (Reynolds and Weiss, 1992). Furthermore, intracerebroventricular infusion of growth factors such as EGF, FGF-2, transforming growth factor alpha (TGFα), or nerve growth factor (NGF), revealed a specific role for EGF in expanding the proliferative pool in the SVZ in both mice and rats (Craig et al., 1996, Kuhn et al., 1997). Six days of **EGF** or TGFα infusion in mice resulted in 18- and 14-fold **increases in the dividing SVZ population**, respectively (Craig et al., 1996). Similar to mice, infusion of EGF in rats was far more efficient than FGF-2 in increasing the number of dividing cells in the SVZ (Kuhn et al., 1997). Additionally, EGF infusion in rats induced hyperplastic cell compartments lining the ventricles (Kuhn et al., 1997). The hyperplasias induced by EGF infusion contained cells co-expressing GFAP, nestin, Sox2 and Olig2 (Lindberg et al., 2011). Withdrawal of EGF results in normalization of the neurogenic niche, suggesting a transient effect on SVZ progenitors in the presence of EGF (Kuhn et al., 1997).

### EGF decreases RMS neuroblast migration *in vivo*

Although a discrete increase in the formation of new $NeuN^+$ cells in the striatum was reported in the first EGF infusion study (Craig et al., 1996), this has not been reported in subsequent studies. Remarkably, after EGF treatment in mice, close to none of the dividing cells arrived in the OB, suggesting an arrest of neuroblast migration along the RMS (Craig et al., 1996). In rats, there was a 10 fold increase of BrdU incorporating cells in the SVZ directly after EGF infusion, which was markedly decreased 4 weeks after the infusion, without a substantial increase in the number of neurons in the OB or the striatum (Kuhn et al., 1997), suggesting that newly divided cells died and were cleared from the SVZ, or migrated elsewhere to differentiate into non-neuronal cells. Concomitantly, there was **a marked reduction of neuroblasts in the SVZ and RMS** (Kuhn et al., 1997, Doetsch et al., 2002). Furthermore, neurosphere formation *in vitro* was markedly boosted when preceded by EGF intracerebroventricular infusion *in vivo* (Craig et al., 1996). Doetsch and colleagues showed that **EGF** treatment results in downregulation of Dlx2 expression **in C-cells**,

which **reverts them into multipotent stem cells** and ceases neuronal production (Doetsch et al., 2002).

### TGFα decreases RMS neuroblast migration *in vitro*

A more recent study investigated the effects of TGFα on neuroblast migration in the RMS (Kim 2009). A small number of neuroblasts expressed low levels of EGFR, and these had a slower and more complex migration pattern than EGFR negative neuroblasts. Treatment with **TGFα** decreased the percentage of neuroblasts that exhibit a migratory profile, suggesting a **negative impact on neuronal migration**. B-cells and C-cells were not studied after TGFα treatment but were stationary under control conditions (Kim et al., 2009).

### EGF treatment induces aberrant migration of OPC (-like) cells

Parts of **the expanded SVZ population migrate** into the adjacent tissue under EGF or TGFα infusion (Craig et al., 1996, de Chevigny et al., 2008, Gonzalez-Perez and Quinones-Hinojosa, 2010). Cultured SVZ astrocytes treated with EGF induced a population of migratory cells expressing Olig2 and NG2 (Gonzalez-Perez et al., 2009). Similarly, lineage tracing of GFAP expressing NSPCs *in vivo*, revealed generation of Olig2/NG2$^+$ cells **in the septum, striatum and cortex**, several weeks after EGF withdrawal in mice (Gonzalez-Perez et al., 2009, Gonzalez-Perez and Quinones-Hinojosa, 2010). Olig2/NG2$^+$ cells are often considered **OPCs**; however, when spotted in the SVZ, this expression profile indicates resemblance to the **C-cells**. Interestingly, only NG2$^+$ cells in the naive SVZ and RMS, and not cortex, express the EGFR and overexpression of the EGFR in any NG2$^+$ cell induced cell migration (Aguirre et al., 2005). Furthermore, *in vitro* studies showed that the EGFR and ECM components (laminin, fibronectin and vitronectin) synergistically increased NG2$^+$ cell migration (Aguirre et al., 2005).

Similar to EGF infusion, two weeks of intrastriatal TGFα infusion into the dopamine depleted brain induced a proliferative wave of nestin/EGFR/Olig2$^+$ cells into the striatum (de Chevigny et al., 2008). Although, the majority of the TGFα induced progeny was nestin/EGFR/Olig2$^+$, close to 50% of these expressed Mash1$^+$ (de Chevigny et al., 2008). Again, these markers are usually expressed by and employed in the analysis of OPCs.

The **EGF expanded population continues to differentiate into both neuronal and glial lineages** after withdrawal of growth factor stimulation (Doetsch et al., 2002, de Chevigny et al., 2008). Two weeks after withdrawal of TGFα infusion, increased numbers of neuroblasts were found in the striatum, in addition to newly formed astrocytes (de Chevigny et al., 2008).

## Regenerative and inflammatory responses after stroke

CNS injury such as a cortical **stroke** produces an initial wave of **high inflammatory activity** as well as neural injury through energy failure resulting from reduced oxygen and nutrients. This results in

**repair reactions** such as activation of glial cell types which proliferate and accumulate around the infarct core to form a barrier between the damaged and healthy tissue (Schroder et al., 1995, Stoll et al., 1998, Mabuchi et al., 2000, Gregersen et al., 2001). Reactive astrocytes appear during the first days, displaying thicker GFAP and vimentin positive processes, and remain for several months past injury (Schroder et al., 1995, Stoll et al., 1998). The inflammatory response was first considered entirely detrimental but evidence for a beneficial role is currently emerging (Matsuo et al., 1994, Lalancette-Hebert et al., 2007).

### Microglia; the resident immune cell of the brain

During late embryonic development and the early postnatal period, the **resident immune cells** of the brain, the **microglia**, migrate from the blood into the brain parenchyma and continue to reside there in adulthood (Barron, 1995). Although often referred to as **'resting'** or 'quiescent', the microglia population in the healthy brain is highly responsive, scanning the microenvironment with thin, highly ramified processes (Nimmerjahn et al., 2005). Apart from surveying the environment, microglia clear the healthy brain from apoptotic cells and debris (Nimmerjahn et al., 2005). Moreover, microglia rapidly become activated in response to infections or injuries and have a role in restoring the normal tissue homeostasis (Mabuchi et al., 2000, Monje et al., 2003).

### Microglia are activated after stroke

The inflammatory response after stroke includes a rapid expansion of the microglia population in the vicinity of the lesion and these effects are evident prior to neuronal apoptosis (Rupalla et al., 1998, Mabuchi et al., 2000, Schilling et al., 2003). Already 24h **after stroke, resident microglia are activated and proliferate**, whereas infiltrating immune cells appear around day 3 (Schroeter et al., 1997, Schilling et al., 2003). Microglia are responsible for phagocytosis of dead and dying neurons during these first 3 days (Schilling et al., 2005). Quiescent microglia can be detected *in vivo* with antibodies against **ionized calcium-binding protein-1 (Iba-1)**. Activated microglia express **CD68**, **galectin-3** and **CD11b** in addition to Iba1 (Sedgwick et al., 1991, Lalancette-Hebert et al., 2007, Yan et al., 2009). Under normal conditions macrophages express higher levels of CD45, however; macrophages are difficult to distinguish from resident activated microglia, since the latter then upregulate CD45 (Sedgwick et al., 1991). Furthermore, previous studies show that, two weeks after stroke, approximately 30-50% of the $Iba1^{+}$ population represent infiltrating cells (Schilling et al., 2003, Thored et al., 2009).

### Both detrimental and beneficial effects are described for the immune system after stroke

The production of complement proteins, chemokines, pro-inflammatory cytokines and neurotrophic factors by activated microglia results in **neuronal damage** but can also **stimulate repair processes**. Initially, inflammation was thought to be entirely detrimental to the outcome after an ischemic lesion as preventing the inflammatory response resulted in reduced infarct size after stroke (Matsuo et al., 1994, Yang et al., 1998, Becker et al., 2001). But surprisingly, selective ablation of proliferating

microglia using CD11b transgenic mice increased the infarct size, reduced insulin-like growth factor 1 (IGF-1) levels and increased numbers of apoptotic cells (Lalancette-Hebert et al., 2007). Furthermore, IGF-1 is known to stimulate neurogenesis (Aberg et al., 2000). Together, these studies suggest a complex immune response after neuronal damage and indicate some beneficial effects of microglia on recovery after stroke. Recently, a dual mode of microglial activation has been proposed, where classically activated, M1, microglia exert detrimental effects, whereas an alternative class of activated microglia, M2, have beneficial effects on neuronal recovery (Butovsky et al., 2006, Hu et al., 2012).

### Proliferation and migration of NSPCs after stroke

After stroke, the proliferation of NSPCs in the SVZ increases and a number of SVZ derived **neuroblasts migrate to the peri-infarct area**, subsequently expressing markers of developing and mature striatal neurons (Arvidsson et al., 2002). NSPCs from the SVZ after stroke give rise to mostly glial progeny; however a small number of mature neurons was detected (Li et al., 2010). Induction of stroke by photo-thrombosis results in a more focal infarct than the commonly used middle cerebral artery occlusion (MCAO) stroke model and does not injure the SVZ or striatum (Watson et al., 1985). However, cortical stroke is sufficient to trigger SVZ proliferation and migration towards the infarct (Ohab et al., 2006, Osman et al., 2011). The migration of NSPCs to the cortex after photo-thrombotic induction of cortical stroke was demonstrated to continue for at least one year after the insult (Osman et al., 2011).

### Oligodendrocyte progenitors and oligodendrocytes after stoke

In response to stroke lesion, OPCs and oligodendrocytes die within the first days of injury (Mabuchi (Mabuchi et al., 2000, Tanaka et al., 2003). However, new OPCs are abundant after 7 days throughout the adult brain (Levine and Reynolds, 1999, Tanaka et al., 2003). Additionally, myelination increases during the first week after the infarct and is accompanied by axonal regeneration (Gregersen et al., 2001). The SVZ NSPCs contribute to the pool of Olig2/NG2$^{+}$ OPCs in the peri-infarct cortex (Li 2010). Focal MCAO as well as cortical stab wound injury **increases the number of cortical Olig2$^{+}$ cells** threefold although only a fraction of these were also expressing NG2 (Buffo et al., 2005). Furthermore, the increased numbers of Olig2$^{+}$ cells after a cortical stab injury were mainly generated through proliferation (Buffo et al., 2005).

## General potential for repair in the human brain

Studying the basic functions of NSPCs may reveal ways to use these highly plastic cells in brain repair after traumatic brain injury, stroke or neurodegenerative diseases. Although rodent models differ in many aspects from the situation in humans, numerous similarities merit research on aspects of future **brain repair in humans**. Multipotent neural stem cells with the ability to form neurospheres *in vitro* and with characteristics similar to rodent SVZ stem cells *in vivo*, were described in the human

subependymal layer (Sanai et al., 2004). A traceable stream of proliferative cells expressing DCX$^+$ and PSA-NCAM$^+$, although no chain migration, was demonstrated in the adult human brain from the SVZ to the OB (Curtis et al., 2007, Wang et al., 2011a). Similar to the rodent brain, stroke increases both proliferation and the number of neuroblasts in **the human SVZ** (Macas et al., 2006). Optimally, deciphering the code to trigger regeneration of neurons from the endogenous human neurogenic niches is a very desirable goal, although this is currently science fiction.

## The cytoskeletal proteins of the Ezrin/Radixin/Moesin family

The amount of proliferation and migration of adult NSPCs requires dynamic rearrangement of the actin cytoskeleton. As described above, migrating neuroblasts in the RMS are highly dependent on actin and tubulin-related proteins such as Girdin and DCX (Koizumi et al., 2006, Ocbina et al., 2006, Wang et al., 2011b). The cytoskeleton linker proteins of **the ERM (Ezrin/Radixin/Moesin) family** have previously been implicated in both proliferation and migration in various tissues (Paglini et al., 1998, Kahsai et al., 2006, Jeon et al., 2009, Valderrama et al., 2012), however; limited studies of ERM proteins have been made in the adult brain.

### ERM proteins provide a link between the actin cytoskeleton and transmembrane proteins

The cytoskeleton linker proteins of the ERM (Ezrin/Radixin/Moesin) family are highly homologous and all contain three main domains (Figure 4); 1. The N-terminal domain, also called the FERM domain, which allows for interaction of ERM proteins with intracellular domains of **transmembrane proteins** or scaffolding proteins located directly beneath the plasma membrane. 2. An extended central helical domain. 3. The C-terminal domain which **binds f-actin** (Sato et al., 1992a, Tsukita et al., 1994, Serrador et al., 1997, Tsukita et al., 1997, Bretscher et al., 2002).

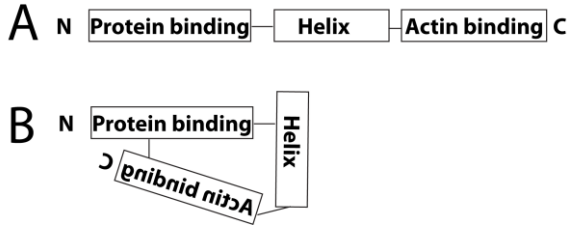


**Figure 4.** The structure of ERM proteins. (A) The N-terminal domain binds to components of the cell membrane or transmembrane proteins. The C-terminal domain binds f-actin. (B) In its closed conformation, the protein binding site of ERM proteins is hidden.

ERM proteins are required for **actin polymerization** during the formation of dynamic actin filament-containing membrane structures such as filopodia and lamellipodia. Formation of filopodia

and lamellipodia are required for cell migration and the extension of cell processes (for details see figure 5).

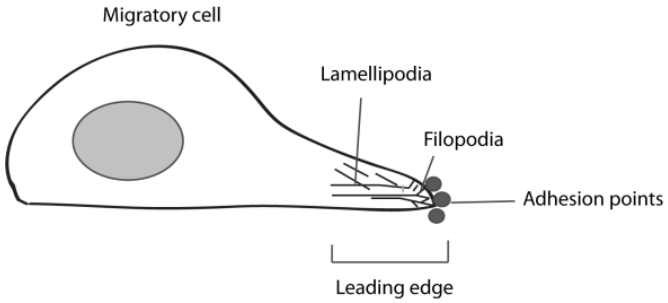


**Figure 5.** Migratory cells regularly display an elongated morphology with actin rich structures such as filopodia or lamellipodia in the leading edge. The filopodia formations are enriched for adhesion proteins, which mediate attachment of the leading edge while the myosin machinery pull the rest of the cell forward.

Furthermore, ERM proteins are concentrated in the cleavage furrow of dividing cells and regulate cell-cell interactions through adhesion molecules (Serrador et al., 1997, Yonemura et al., 1998, Mangeat et al., 1999, Bretscher et al., 2002, Hamada et al., 2003, Niggli and Rossy, 2008).

## ERM function is regulated through phosphorylation and interaction with plasma membrane molecules

The ERM proteins change their structure through **phosphorylation of a threonine residue** (ezrin; T567, radixin; T564 and moesin; T558) in the C-terminal domain. Upon phosphorylation, the **actin-binding and membrane/protein-binding domains are exposed** (Figure 4) (Berryman et al., 1995, Bretscher et al., 1995, Matsui et al., 1998, Hayashi et al., 1999). Phosphorylated ERMs are typically localized to the plasma membrane in cellular protrusions, adherens junctions, cleavage furrows and focal adhesion points (Sato et al., 1992a, Hirao et al., 1996, Yonemura et al., 1999, Yonemura et al., 2002). Furthermore, binding of ERMs to membrane molecules like **inositol (1,4,5)-trisphosphate ($IP_3$)** results in the opening of the closed form of ERM proteins prior to phosphorylation and, enhance membrane protein and f-actin interactions (Niggli et al., 1995, Hamada et al., 2000). Several kinases phosphorylate ERM proteins including **Rho kinase** (Matsui et al., 1998, Haas et al., 2007)Antonie-Bertrand et al 2010), **Protein kinase C** (Simons et al., 1998) and **Leucine-rich repeat kinase 2** (Parisiadou et al., 2009). Furthermore, several soluble factors have been shown to induce ERM phosphorylation such as **stem cell factor** (Yonemura et al., 2002, Jeon et al., 2009) (Koss et al., 2006) and **netrin-1** (Antoine-Bertrand et al., 2011). In addition, the FERM domain of ERM proteins has conserved tyrosine phosphorylation sites and phosphorylation by Src kinase weakens cadherin-based cell adhesion (Takeda et al., 1995, Antoine-Bertrand et al., 2011). Ezrin displays tyrosine phosphorylation upon **EGF stimulation**; however, how this affects conformation and function of ezrin is still unclear (Krieg and Hunter, 1992). In addition, radixin was detected as a substrate for EGF stimulated EGFR tyrosine kinase activity although this has never been studied in vivo (Fazioli et al., 1993).

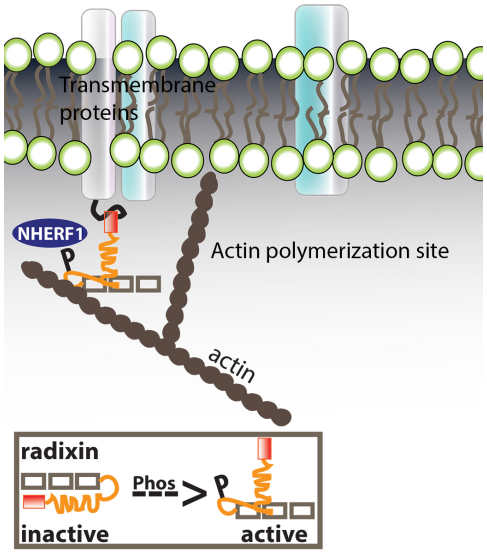


**Figure 6.** Phosphorylated ERM protein binds the actin cytoskeleton and transmembrane proteins. This induces actin polymerization and is sometimes regulated by scaffolding proteins such as NHERF1.

## Differential functions of ERM proteins

Although often studied as a group, the ERM proteins differ in their distribution and activities in different cell types. This may obscure the actual involvement of the individual proteins in specific functions. A high degree of redundancy has been proposed although this may be an artefact occurring in *in vitro* studies. Radixin is generally the lesser-studied ERM protein, possibly due to its low expression in many of the commonly studied cell lines (Yonemura et al., 2002).

**Radixin** was originally isolated from rat liver as a component of cell–cell adherens junctions (Tsukita et al., 1989), and deleting the radixin gene in mouse accumulates high serum levels of bilirubin and results in **liver damage** (Kikuchi et al., 2002). Furthermore, these mice suffer from **deafness** due to degeneration of cochlear stereocilia, which are actin-filament-based apical projections on hair cells. Apart from these features radixin knockout mice appear to grow normally and are fertile (Kitajiri et al., 2004).

**Moesin** knockout mice display no compensatory upregulation of ezrin or radixin in any tissue and remarkably, **no changes** were discerned in ERM related functions such as cell adhesion of platelets, microvilli formation in mast cells and fibroblast migration (Doi et al., 1999).

In contrast, the **ezrin** knockout mouse **does not survive** past weaning, displaying malfunctioning intestinal epithelial membrane formation and **abnormal microvilli** morphology already at embryonic stages (Saotome et al., 2004). The results from the above mentioned knockout experiments suggest that redundancy is not a common feature of ERM proteins *in vivo* although this has often been presumed due to the high homology. Nevertheless, redundancy has been claimed in several *in vitro* studies (Derouiche and Frotscher, 2001, Yonemura et al., 2002, Haas et al., 2007).

## ERM proteins interact with scaffolding proteins

The N-terminal domain binds the cytoplasmic adaptor protein $Na^+/H^+$-exchanger regulatory factor (NHERF), also called ezrin/radixin/moesin binding phosphoprotein 50 (EBP50) (Reczek et al.,

1997). NHERF is an adaptor protein regulating the activity of many G-protein coupled receptors such as the $\beta_2$-adrenergic receptor and the κ-opioid receptor, (Hall et al., 1998, Li et al., 2002). Furthermore, activation of the **EGFR** has been demonstrated to induce EBP50 expression *in vitro* (Lazar et al., 2004). Conversely, increased expression of EBP50 has been shown to downregulate **EGFR** activity suggesting a negative feedback between **EGFR** and EBP50 (Claperon et al., 2012).

### Regulatory function of radixin

The ERM proteins have initially been considered inert structural proteins, linking the actin cytoskeleton to transmembrane proteins. More recently, new binding partners have been identified and emerging evidence suggests that ERM proteins participate in signal transduction. For example, radixin can regulate the **activity of the integrin receptor** (Tang et al., 2007). In the brain, radixin is involved in the **recruitment of the $GABA_A$ receptor** to active membrane domains (Loebrich et al., 2006). In addition, inhibiting all three ERMs in cultures of mouse epithelial cells demonstrated their involvement in **cell-cell adhesion** and **cell-substrate adhesion**, where ERM inhibition resulted in detachment of cell colonies (Takeuchi et al., 1994). Furthermore, inhibition of radixin alone resulted in a complete loss of the ability of these cells to attach and spread, indicating a prominent role for radixin in regulating adhesion (Takeuchi et al., 1994). Additional studies suggest regulatory roles for the ERM protein radixin, such as the spatial regulation of Epac1 which regulate the small G protein Rap-mediated adhesion to the extracellular matrix through integrins (Gloerich et al., 2010).

Recently, **a quinocarmycin analog (DX52-1)** was demonstrated to bind specifically to the C-terminal end of radixin at concentrations below 200nM (Kahsai et al., 2006). Treatment with this chemical in MDCK cells interrupted the ability for radixin to bind actin and the transmembrane protein CD44 (Kahsai et al., 2006). Furthermore, radixin inhibition resulted in decreased wound closure of MDCK cells (Kahsai et al., 2006).

### ERM proteins interact with Rho family members

In many different cell types, ERM proteins also regulate the activity of the Rho small G protein family and vice versa. The Rho family consists of the Rho, Rac and cdc42 subfamilies, which regulate cell morphology, migration and cytokinesis (Hall, 1994). In addition, ERMs can activate Rho family members through direct interaction with the GDP dissociation inhibitor (Rho GDI) (Takahashi et al., 1997). Furthermore, radixin has been implicated in regulation of additional Rho members through recruitment of both positive and negative regulators such as the stimulatory GDP/GTP exchange protein (Rho GEP) Dbl and the Rho GDI (Takaishi et al., 1995, Takahashi et al., 1997, Takahashi et al., 1998). A recent study of prostate cancer cells suggests that radixin levels regulate the transition between a non-migratory morphology with adhesive properties to a migratory morphology through the alteration of the Rho GTPase Rac1 activity (Valderrama et al., 2012).

### ERM proteins in the embryonic brain

In the brain, ERM proteins have been implicated in development of **neural growth cones** and migration of cortical neurons in the embryonic rodent brain; however, limited studies exist of ERM proteins in the adult brain (Gonzalez-Agosti and Solomon, 1996, Paglini et al., 1998, Hamada et al., 2003, Haas et al., 2007, Mintz et al., 2008, Parisiadou et al., 2009). The very first study of ERM proteins in embryonic neural growth cones suggests their association with both microtubules and microfilaments (Goslin et al., 1989).

### Radixin is required for neural growth cone development

Paglini et al demonstrated that **radixin** and moesin, but not ezrin, are important for generating and maintaining growth cones of embryonic pyramidal hippocampal neurons. However, radixin expression could not be detected by western blotting in whole extracts of brains from mice older than P30 (Paglini et al., 1998). A specific role for radixin in **stabilization of growth cone lamellipodia** and **growth cone motility** was also shown in chick dorsal root ganglion neurons (Castelo and Jay, 1999). Similarly, growth cones in embryonic cortical neurons and neuroblastoma cells expressed ERM proteins and Rho kinase inhibition resulted in reduced neurite regeneration, neuronal motility and decreased ERM phosphorylation *in vitro* (Haas et al., 2007). However, the use of the broadly acting Rho kinase inhibitors makes is difficult to know which effects are due to ERM protein inhibition. In embryonic neocortical neurons ERMs were required for **axon guidance** and Semaphorin3a-mediated growth cone collapse (Mintz et al., 2008). Furthermore, ERM phosphorylation was shown to promote actin cytoskeleton rearrangement during neurite outgrowth (Parisiadou et al., 2009).

### Ezrin expression in astrocytes in the RMS

Tangential cell migration in the brain often responds to the same molecules that control axon guidance, and semaphorins have also been implicated in RMS migration (Mendelez-Hererra 2008). In the adult rodent RMS, **ezrin** expression was demonstrated in the astrocytes of the **glial tubes** (Cleary et al., 2006). Additionally, ezrin immunoreactivity was demonstrated in rodent embryonic neurospheres, in neurosphere-derived astrocytes, and in astrocytes of the adult human brain (Gronholm et al., 2005). Radixin and moesin protein expression, however; were not described in these studies of the adult brain (Gronholm et al., 2005, Cleary et al., 2006). Antibodies used in earlier experiments were later shown to have crossreactivity against multiple ERM-proteins, somewhat obscuring the true functions of the individual protein. **ERM proteins in the CNS are so far mostly studied *in vitro*, although it is known that the expression and function of ERM proteins *in vitro* often differ from the *in vivo* situation.**

# General aim

The general aim of this thesis was to discover mechanisms of neuronal migration and cell replacement in the adult brain. For this we studied long-distance migration in the healthy brain and in disease models that cause altered migration in progenitor cell populations. To study this three specific questions were raised and answered in this thesis.

## Research questions

I. Does neuronal progenitor migration in the adult rostral migratory stream involve the cytoskeleton proteins of the Ezrin/Radixin/Moesin family?

II. Can radixin inhibition influence neuronal and oligodendrocyte progenitor migration and proliferation?

III. How do different modulations of the neuronal and oligodendrocyte progenitor populations, in the adult subventricular zone and rostral migratory stream, affect the expression of the cytoskeleton linker protein radixin?

# Materials and Methods

## Chemicals

The quinocarmycin analog DX52-1 (generous gift from Prof. Gabriel Fenteany) was used to block radixin function as described previously (Kahsai et al., 2006). A stock solution was prepared by dissolving the compound in sterile 50% DMSO in PBS which was further diluted with PBS for working solutions (<0.5% DMSO).

**Comments:** DX52-1 specifically binds to the C-terminal of radixin at concentrations lower than 200nM, however at higher concentration this compound also binds with a lower affinity to three additional proteins, one of them are thought to be Galectin-3 (Kahsai et al., 2008). The interaction of DX52-1 with radixin inhibits binding to the actin cytoskeleton as well as the transmembrane protein CD44 (Kahsai et al., 2006).

## Animals

In paper I and IV, postnatal and 2 months old C57BL/6 mice were used for the analysis of radixin expression in the adult brain, neurosphere cultures and after induction of cortical stroke. Eight weeks old Wistar rats were used for studies of DX52-1 and EGF intracerebroventricular infusion in paper II and III, as well as for the explant and neurosphere cultures. All animals were housed in a barrier facility with a 12 hour light cycle with *ad libitum* access to food and water. Experiments were conducted according to protocols approved by the Gothenburg committee of the Swedish Animal Welfare Agency. For immunohistochemical analysis, animals were sedated at the end of experiments using an overdose of pentobarbital and transcardially perfused with 0.9% sodium chloride, then fixed with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (pH 7.4). For generation of primary cell cultures and mRNA analysis, animals were anesthetized using isoflurane and decapitated.

**Comments:** Preliminary data showed no difference between radixin immunoreactivity in mouse and rat adult SVZ or RMS, the main areas in focus of this thesis, and mice were chosen for the general characterization of radixin expression in the adult brain in paper I and for stroke studies in paper III. For intracerebroventricular pump infusion rats were chosen to be able to analyse EGF induced hyperplasias in the SVZ, which are well described in rats (Kuhn et al., 1997, Lindberg et al., 2011).

## Animal models

### Photo-thrombotic stroke model (Paper IV)

Animals were initially anesthetized with 5% isoflurane in a mixture of air and oxygen (1:1), and then mounted on the stereotaxic apparatus and anaesthesia was maintained with 2-2.5% isoflurane. The

body temperature was maintained at 37°C with a heating pad. Ischemia was induced in the cortex (left hemisphere) using the photo-thrombotic stroke model previously described (Watson et al., 1985). An incision was made over the skull, the skin flaps were retracted, and then periosteum was removed to allow exposure of the skull. Rose Bengal dye (0.1 mL of 10 mg/mL in 0.9%NaCl) was injected intraperitoneally (IP) 5 minutes prior to laser illumination. The laser source was placed at 3 cm distance to the skull (Cobolt Jive™). The skull and underlying tissue were illuminated for 10 min with the laser beam (Power: 50 mW; Wave length: 561 nm) at the following coordinates relative to bregma: +2.7 mm lateral and +1mm anterior. Control animals were sedated and left unlesioned. Stroke animals with a lesion not including the cortical layer 6 or involving the corpus callosum were excluded from the study. The experiment was ended at 1, 2, 6 or 12 weeks after induction of stroke.

**Comments:** Compared to other animal models of stroke, the photo-thrombotic model is highly reproducible, enable generation of small cortical infarcts and allow for induction of stroke without intrusive operations. This model generates an ischemic core and initiates reactive gliosis and activates the immune system, providing a suitable model for studying microglial activation and reactions of NSPCs in the cortex.

### Intracerebroventriucular infusion (Paper II and III)

Male wistar rats, 7-8 weeks old, were used for intracerebroventricular infusion experiments. Surgeries were performed under ketamine (33 mg/mL Ketalar, Pfizer) and xylazine (6.67 mg/mL Rompun, Bayer Healthcare AG) anaesthesia, and all efforts were made to minimize suffering. For paper II, animals were divided into two groups receiving DX52-1 (1.3 μg/day) or vehicle (0.5% DMSO in PBS) for 4 days. For paper III, the animals were divided into six groups receiving either vehicle (artificial cerebrospinal fluid, aCSF) or EGF (360 ng/day), for 1, 7, or 14 days. Osmotic minipumps (Model 1002; Alzet-Durect) and infusion cannulas (Brain Infusion Kit 2; Alzet-Durect) were filled with EGF, aCSF, DX52-1 or vehicle. Cannulas were inserted intracerebroventricularly using a stereotaxic instrument (anteroposterior [AP] +8.5 mm, lateral +1.2 mm from the center of the interaural line at flat skull position; cannula length, 5 mm below skull) and the minipumps were placed subcutaneously. For paper III, all animals received three intraperitoneal injections of 50 mg/kg of bromodeoxyuridine (BrdU) during the last 24 hours before perfusion.

**Comments:** Delivery of EGF through pump infusion into the ventricles targets the cells of the SVZ first due to their location close to the ventricle wall. However, after continued infusion EGF will diffuse into most parts of the brain. Cells in the SVZ will most likely be exposed to the highest EGF concentration. The cannula causes a wound in the needle tract which should be considered when studying areas close by. The procedure gives rather reproducible results, although some factors may give rise to variations between animals. For instance, even small misplacements of the pump may result in damage of the SVZ in the ventricle wall; furthermore, clogging of the pump will impair infusion.

## Immunohistochemistry (Paper I-IV)

After perfusion, brains were removed, postfixed for 24 hours in 4% PFA and thereafter kept in 30% sucrose at 4°C until they were cut in serial sections on a sliding microtome (Leica SM 2000R) for subsequent free floating immunostainings. For coronal sections, mouse brains were cut at 20-µm whereas rat brains were cut at 40-µm thickness. Sagittal sections were cut thinner to get more material for analysis of the neuroblast chains in the RMS. Mouse sagittal sections were cut at 20-µm whereas rat sagittal sections were cut at 25-µm thickness. Sections were stored at 4°C in a cryoprotective solution (glycerol, ethylene glycol, and 0.1 M phosphate buffer, pH 7.4, 3:3:4 by volume).

For diaminobenzidine (DAB) staining, sections were washed in tris-buffered saline (TBS), incubated for 30 minutes in 0.6 % $H_2O_2$, the non-specific binding blocked by incubation for 30 minutes with 3 % normal donkey serum in 0.1 % Triton X-100. Sections were incubated in primary antibodies (see table) for 24-48 hours at 4°C. After subsequent TBS washing, sections were incubated with appropriate biotinylated secondary antibody for 1 h (JacksonImmunoResearch Lab), followed by incubation in avidin-biotin solution (1:100; Vectastain ABC Elite kit, Vector Laboratories) for 1 hour. The stain was developed with 3-3´ diaminobenzidine tetrahydrochloride (DAB, 1:100; Saveen Werner AB) and mounted using NeoClear® and NeoMount® (Merck).

For BrdU stainings a DNA denaturation step was added. The sections were incubated for 30 minutes in 2M HCl at 37°C, followed by 10 minutes rinsing in 0.1M Borate Buffer, before continuing with the blocking procedure.

All radixin staining was preceded by pretreatment for 30 minutes with sodium citrate (pH 6.0) at 97°C. Immunofluorescence staining was continued with TBST washes, followed by blocking in 3% donkey serum (JacksonImmunoResearch Lab) and 0.2% triton X-100 in TBS for 30 minutes at room temperature. Sections were then incubated at 4°C for 48-72 hours with the primary antibodies (see Table 1), followed by thorough washing and 2 hours incubation with secondary antibodies (see Table 1). ToPro-3 (1:1000; Molecular Probes) was used as a nuclear counterstain when necessary. Sections were mounted onto glass slides and coverslipped with the antifade reagent ProLong Gold DAPI (Molecular Probes).

| Antibody | Source | Affinity | Application | Dilution | Company |
|---|---|---|---|---|---|
| βIII-tubulin | mouse | Neuroblasts, mature neurons | ICC | 1:1000 | Sigma aldrich |
| BrdU | rat | Detect dividing cells in S-phase after BrdU adminitration | IHC | 1:500 | Serotech |
| CD11b | rat | Activated microglia/macrophages | ICC | 1:250 | AbD Serotech |
| CD68 | rat | Activated microglia/macrophages | ICC | 1:200 | AbD Serotech |
| CNPase | mouse | Mature oligodendrocytes | IHC | 1:200 | Abcam |
| Doublecortin | goat | Neuroblasts, immature neurons | IHC | 1:500 | Santa cruz Biotech |
| Ezrin | mouse | | IHC | 1:50 | Sigma aldrich |
| Galectin-3 | mouse | Activated microglia/macrophages | IHC, ICC | 1:250 | eBioscience |
| GFAP | mouse | Astrocytes, stem cells | IHC, ICC | 1:1000 | Chemicon |
| GFAP | rabbit | Astrocytes, stem cells | IHC, ICC | 1:1000 | DACO |
| Iba-1 | goat | Microglia/macrophages | IHC, ICC | 1:500 | Abcam |
| Moesin | mouse | | IHC, ICC | 1:50 | Abcam |
| NG2 | mouse | OPCs | IHC | 1:500 | Millipore |
| Olig2 | goat | OPCs | IHC, ICC | 1:500 | R&D Systems |
| PSA-NCAM | mouse | Neuroblasts | IHC, ICC | 1:500; 1:1000 | Chemicon |
| phosphorylated Ezrin/Radixin/Moesin | rabbit | Active ERM proteins | IHC, ICC | 1:500 | Cell signalling |
| Phosohorylated Histone3 | rabbit | Dividing cells in M-phase | IHC, ICC | 1:1000 | Millipore |
| Radixin | mouse | | IHC, ICC | 1:250 | Abnova |
| Radixin | rabbit | | IHC, ICC, WB | 1:200; 1:250 | Abcam |
| Sox-2 | rabbit | NSPCs | IHC, ICC | 1:250 | Millipore |
| Sox-2 | goat | NSPCs | IHC | 1:250 | Santa cruz Biotech |
| Vimentin | chicken | Reactive astrocytes, ependyma | IHC | 1:250 | Nordic Biosite |
| Biotinylated tomatolectin | | Blood vessels | IHC | 30ug/ul | Sigma aldrich |
| Alexa Fluor secondary antibodies | donkey | primary antibody species | IHC, ICC | 1:1000 | Molecular probes |
| CF555 donkey anti rabbit | donkey | Rabbit IgG | IHC, ICC | 1:1000 | Biotium |
| CF555 donkey anti goat | donkey | Goat IgG | IHC, ICC | 1:1000 | Biotium |

*Table 1. Primary antibodies used for immunohistochemistry, immunocytochemistry and western blot.*

**Comments:** Immunohistochemistry enables analysis of localization of protein expression in a preserved cytoarchitecture which is important in evaluation of, for example, cell-cell interactions and specialized niches *in vivo*. The thick sections used in free floating immunostainings makes it possible

to study several intact cell layers, however; some antibodies do not penetrate the entire tissue thickness and comparison between sections and groups should be made with caution. In this thesis, it was found necessary with an antigen retrieval step for proper analysis of radixin immunoreactivity which is a disadvantage since a number of antibodies do not work in combination with antigen retrieval. Furthermore, even after blocking procedures primary and secondary antibodies can bind unspecifically to cells. To address this possibility, well characterized antibodies were used whenever possible and the morphology of cells was simultaneously evaluated. On occasion, several antibodies against the same target were used to assure specificity. In addition, negative controls with only secondary antibodies were performed.

## Apoptosis detection (Paper II)

To study apoptotic cell death in the SVZ and RMS after DX52-1 infusion, the ApopTag Fluorescein Direct in situ Apoptosis Detection kit (Millipore) was used. Briefly, fixed free floating sections were mounted onto glass slides and pretreated with ethanol:acetic acid (2:1) for 5 minutes. This was followed by 1 hour of incubation in terminal deoxynucleotidyl transferase at 37°C, and labelling of fragmented DNA with a digoxigenin-nucleotide. The reaction was stopped by washing and the sections were incubated for 30 minutes with a Fluorescein-conjugated Anti-Digoxigenin antibody at room temperature. The slides were coverslipped with ProLong Gold DAPI (Molecular Probes).

**Comment:** The ApopTag kit is based on terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), which detects cells with single- and double-strand breaks of the DNA. Mainly DNA fragmentation is associated with apoptosis and detection of necrotic cells is very small.

## Cell culture

### Neurosphere cultures (Paper I and II)

The SVZ was microdissected from each hemisphere and transferred into ice cold Leibowitz's L15 medium (Gibco). The tissue was pooled and finely chopped with a scalpel blade followed by enzymatic dissociation using a solution containing 0.01% Papain (Worthington/Cell systems), 0.1% Dispase II (Roche), and 0.01% DNase I (Worthington/Cell systems). The cells were washed twice in unsupplemented Neurobasal A (Invitrogen) and then cultured in serum-free Neurobasal-A medium containing B27 (1:50), Penicillin-Streptomycin (PEST; 100 U penicillin, 100 µg streptomycin), and Glutamax (2 mM; all from Invitrogen) at a cell density of 80 000 cells/ml. The medium was supplemented with 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech), 20 ng/ml epidermal growth factor (EGF; Invitrogen), and 2 µg/ml heparin (SigmaAldrich) every second day. For passaging, cells were collected by centrifugation (500 x g, 5 min), and the pellet was resuspended in TrypLE express (Invitrogen), followed by incubation at 37°C for 2 x 3 minutes with intermittent trituration to obtain a single cell suspension. Cells were then re-suspended in fresh media and

centrifuged as above. Viable cells were counted by trypan blue exclusion using a hemocytometer and then reseeded at 80 000 cells/ml.

**Comments:** Neurosphere cultures have been used as a method to study self-renewal and multipotency of adult NSPCs since early 1990s. The neurosphere culture assay is very useful for certain applications, however the method has some drawbacks. Notably, the spheres contain a mixture of stem and progenitor cells and long term treatment with EGF turn most SVZ derived cells into the C-cell stage (Doetsch et al., 2002) which should be taken into account when analysing neurosphere assays.

### Neurosphere and explant migration assay (Paper I-III)

Neurospheres from passage 4 or tissue pieces, explants, dissected from the SVZ, approximately 100 µm diameter pieces, were mixed with Matrigel (3:1, BD Bioscience). The Matrigel mixture was dispensed on the bottom of 8 well chamber slides (BD Bioscience), polymerized for 10 minutes at 37°C, followed by 48-72 hours culturing in Neurobasal A medium, supplemented with B27 and Glutamax, PenStrep (all Invitrogen) at 37°C in 5% $O_2$ and 1% $CO_2$. The experiments were ended by fixating the cultures with 4% PFA and immunocytochemistry was used to distinguish between different populations migrating from neurospheres and explants.

The migration distance of migratory chains was measured for PSA-NCAM$^+$ and Sox2$^{high}$ cells after treatment with a concentration series of the radixin inhibitor DX52-1 (0, 10, 50, 100 and 250nM for 72 hrs). The three longest migratory chains per explant were used to estimate the maximum migration distance under DX52-1 treatment. Furthermore, the percentage of PSA-NCAM$^+$ and Sox2$^{high}$ cells leaving the explants were quantified by determining the number of PSA-NCAM$^+$ and Sox2$^{high}$ cells for at least 100 cells in migratory chains emerging from the explants. Cells were visualized with the nuclear stain ToPro-3 and n=4 were used for all explant quantifications.

**Comments:** In the neurosphere and explant migration assay, cells start to migrate out from the spheres or explants into the Matrigel. The SVZ explant migration assay is commonly used to study aspects of neuronal progenitor migration and resembles the migration in the RMS with neuroblasts migrating along each other in chains. In addition, a large proportion of glial cells migrate from explants and spheres and immunocytochemistry is needed to properly distinguish between the different cell populations. A few cells migrate one by one or detach from chains and continue to mature in the gel.

### Primary microglia cultures (Paper IV)

P1-P2 mouse brains were used for generation of primary microglia cultures. After mechanical dissociation of the brain tissue, cells were cultured in 20% fetal bovine serum (FBS; HI FBS, Gibco) in DMEM (Invitrogen) and PenStrep (Invitrogen). After 7 days in culture, the concentration of FBS was decreased to 10% and the cultures grown for an additional 7 days before shaking the flasks for 3 hours to select for microglial cells. Detached microglial cells were plated in 2% FBS, and treated with

0 or 100 nM lipopolysaccaride (LPS; List biological lab) after 24 hours. The cells were either collected 24 hours later and processed for RNA extraction or processed for immunocytochemistry.

**Comments:** Primary microglia cultures have been widely used as a model in studying microglial properties for over 20 years. Due to the complex environment in the brain, it is sometimes preferred to study microglia activation *in vitro*. Microglial cells are for instance difficult discern from other immune cells *in vivo*. To study the effects of microglia activation, many researchers use the bacterial endotoxin lipopolysaccaride (LPS) which mimics the infection of Gram-negative bacteria. In primary microglial culture, LPS stimulation induces the secretion of cytokines and increased numbers of amoeboid round cells; resembling characteristics of microglial activation *in vivo*. One disadvantage in studying primary microglial cultures is that the cells are ranging from low to moderately activate already under control conditions and contain a mixture of differently activated microglia. Furthermore, results from the artificial milieu created in cell culture can be difficult to compare to the *in vivo* situation. Therefore, cell cultures should be considered a model system, serving as a complement to *in vivo* studies.

## SVZ wholemount preparations (Paper II)

To study the overall cytoarchitecture of the SVZ in control and radixin inhibited rats, the brains were removed and placed in warm HBSS (Invitrogen). The whole ventricular wall of the contralateral hemisphere, including the underlying parenchyma, was carefully dissected out and fixed in cold 4% PFA/0.1%TritonX-100 for 24 hours before washing and blocking unspecific binding in 10% Donkey serum/2%TritonX-100 in PBS for 1 hour (Mirzadeh et al., 2010). The wholemount was incubated for 48 hours with primary antibodies (see table), washed thoroughly in 0.1% TritonX-100 in PBS, and subsequently incubated for 24 hours in secondary antibodies (see table). After completing the staining, a sliver of the SVZ was cut out from the underlying parenchyma and coverslipped with Prolong Gold DAPI (Molecular Probes).

## Immunofluorescence (Paper I-IV)

Cell and tissue cultures were fixed in 4% PFA followed by rinsing in 0.2% triton X-100 in Tris buffered saline (TBST) and blocking the non-specific binding with a solution of 3% donkey serum (JacksonImmunoResearch Lab) for 30 minutes. After blocking, cells were incubated with primary antibodies for 24-72 hours, depending on the antibody and culture type. Subsequently, cells and explants cultures were carefully washed in TBST followed by 2-4 hours incubation with Alexa Fluor secondary antibodies (Molecular Probes), CF555 donkey anti goat (Biotium) and ToPro-3 as nuclear stain, in room temperature. After additional TBST washing steps, the cultures were coverslipped with Prolong Gold with DAPI (Molecular Probes) and analysed with a confocal microscope.

**Comments:** Immunocytochemistry has the advantage of displaying the presence and cellular distribution of different proteins. As for immunohistochemistry, unspecific binding were carefully considered and well characterized antibodies used and negative controls included. Special care should be taken in the analysis of immunostained tissue cultures since unperfused tissue autofluorescence and the Matrigel can give rise to high background fluorescence.

## Microscopic analysis and quantifications

DAB immunoreactivity was analysed using light microscopy and cell number counts were assessed using stereology software (Stereo Investigator; MicroBrightField Inc.). Using systematic random sampling principles, a representative fraction of the sample is analysed, in our case cortical cells in tissue sections in paper IV. Generally the fraction is acquired by overlaying the analysed structure with a grid of evenly spaced counting frames, in which cells are detected, omitting cells touching two of the frame borders. Furthermore, cells in the entire thickness of the section are analysed. The counting volume is calculated by multiplying the area of all counting boxes with the section thickness. The cell density is the total number of cells counted divided by the counting volume. In paper IV, total numbers of cells in a defined area was counted per section, in every $6^{th}$ section, and the cell density was multiplied with 6 to estimate the total number of cells.

Fluorescent immunolabelling was imaged using a confocal laser scanning microscope (Leica TCS SP2, Leica Microsystems). When using several fluorescently labelled secondary antibodies, the labels were imaged serially to eliminate detection of bleed-through. Double-labelling was assumed when either direct co-localization of signals were observed or when separate processes originating from the same cell body were labelled with individual markers.

## Western blot (Paper I)

Olfactory bulb, olfactory tract, RMS and SVZ tissue were microdissected from one P14 C57/Bl6 mouse and one adult C57/Bl6 mouse and placed in sodium dodecyl phosphate (SDS) extraction buffer (1 mM $Na^+$ orthovandate, 1 mM phenylmethanesulfonyl, 2 % SDS, and protease inhibitors; Roche). The tissue was sonicated and protein concentration was determined using the Bicinchioinic Acid Protein Assay kit (Sigma Aldrich). For blotting, 50 µg of protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) using NuPAGE 4-12 % Bis Tris gels, SDS running buffer and NuPAGE antioxidant (Invitrogen). Proteins were transferred to polyvinylidene fluoride membrane (Invitrogen) using NuPAGE transfer buffer supplemented with NuPAGE antioxidant in 30 % methanol. MagicMark™ XP Western protein standard (Invitrogen) was used for molecular weight estimations. The membrane was blocked for 1h in room temperature in blocking solution (5 % bovine serum albumin in TBS containing 0.05 % Tween) and subsequently incubated with primary antibodies, monoclonal rabbit anti-radixin (Abcam) and mouse anti-beta actin (Abcam) in blocking solution overnight at 4°C. The membrane was rinsed thoroughly in TBS containing 0,05%

Tween, then incubated for 1h in room temperature with horseradish peroxidase conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse, Jackson ImmunoResearch Laboratories) in blocking solution. After subsequent rinsing, the immunoreactive bands were detected using SuperSignal West PICO Chemilumi Substrate (Fisher) and a LAS3000 CCD camera (Fujifilm).

## Quantitative PCR (Paper III and IV)

Quantitative polymerase chain reaction (qPCR) was performed in paper II, III and IV. Total mRNA was extracted using the RNAeasy Mini or Micro kit (Qiagen) and DNAse I (Qiagen) was used to remove genomic DNA. cDNA was synthesized from 0.3 μg total RNA in a PCR cycler (Biometra T3Thermocycler, Biosite) by reverse transcription using the Revert Aid Premium first strand cDNA synthesis kit (Fermentas) which includes a mixture of dNTPs, random primers, Oligo dT primers, RNase inhibitor and reverse transcriptase. In addition, negative controls without reverse transcriptase were processed for each RNA sample. Reverse transcription was initiated by a 10 minute incubation at 25°C, followed by 15 minutes at 60°C and terminated by heating at 85°C for 5 minutes. cDNA samples were stored at -80°C until processed for qPCR. Primer sequences were generated using Primer express software and synthesized by Eurofins MWG Operon. Primer sequences were designed with a melting temperature of 60°C, spanning an intron site and the efficiency of all primers was tested using a 5-point dilution curve (Tabel 2). A BLAST search (NCBI primer BLAST) was performed to ensure the specificity of the primer pairs. For all primers we used an initial cycle of 15 min at 95°C, followed by repeated cycles of 94, 55, and 72°C (45 in total) in a Light Cycler instrument (480 Roche Diagnostics). No template controls were included in each qPCR run and absence of amplification was acquired from no reverse transcription-controls. Melting curves, starting at 95°C, was acquired to ensure proper primer functionality and detect any primer-dimer formation. $C_q$ values were normalized using two reference genes. Fold changes were calculated using $C_q$ and the $\Delta\Delta C_q$ method (Vandesompele et al., 2002).

| Mouse | Gene | Forward sequence | Reverse sequence |
|---|---|---|---|
| | *β-actin* | AGGCCAACCGTGAAAAGA | CACAGCCTGGATGGCTAC |
| | *s18* | GGCGTCCCCCAACTTCTT | GGGCATCACAGACCTGTT |
| | *radixin* | CTCTTACCACAGCGTGTTTTGG | TTCCCTTAGCATCCCTCTGTGT |
| | *moesin* | CATGCCGAAGACGATCAGTG | AACAGCTGCTTGCCAGTGG |
| | *ezrin* | ATGGACCAACACAAGCTCAGC | AGCATAGCACTGTCCTTGAGCA |
| **Rat** | | | |
| | *β-actin* | TGTGATGGACTCCGGAGACGGG | TGTAGCCACGCTCGGTCAGGAT |
| | *GAPDH* | AACCCATCACCATCTTCCAGGAGCG | ACATACTCAGCACCAGCATCACCCC |
| | *radixin* | CCTACCACAGCGTGTTTTGGA | TCCCCCTGTGTTCTTCATGC |
| | *DCX* | AAAGCCCAGGCCCAATGCGC | ACAAGTCCTTGTGCTTCCGCAGAC |

*Tabel 2. Primer sequences used for qPCR.*

**Comments:** In quantitative PCR the exponential amplification of DNA during the polymerase chain reaction is measured by simultaneous amplification of fluorescent dyes. After reaching a certain threshold, $C_q$, the exponential phase of PCR amplification starts, continuing until it reaches a plateau. For an accurate quantification, the mRNA amount is determined during the exponential phase and is often normalized to at least two stably expressed, reference genes. qPCR can be used to detect small differences of relative mRNA expression levels, however, increased mRNA levels does not always coincide with increased protein translation and additional method are needed to evaluate the regulation of the protein. Radixin has multiple splice variants and for all qPCR runs in this thesis, primers against the radixin splice variant 2 were used.

## Statistics

In most comparisons between two study groups, the Student´s *t*-test was used. When the data were not considered normally distributed Mann-Whitney U-test was used. For comparison of more than three groups, one-way ANOVAs with Bonferroni post hoc test were employed. Statistical calculations and graphical visualizations were performed in GraphPad Prism 5 (GraphPad Software). Data were presented as mean± standard error of the mean (SEM). Differences of $p<0.05$ were considered statistically significant (*).

# Results and discussion

## Radixin expression in the adult brain (Paper I and II)

### Specific expression of radixin in neuroblasts in the SVZ, RMS and dentate gyrus, and in Olig2$^+$ cells throughout the adult brain (Paper I)

In paper I, intense radixin immunoreactivity was demonstrated in neuroblasts in the mouse SVZ and RMS. Radixin$^+$ cells displayed close to 100% overlap with the neuroblast markers PSA-NCAM and DCX in the RMS (Paper I, Figure 1D). Similarly, neuroblasts in the center of the OB and hippocampal neuroblasts situated in the innermost layer of the dentate gyrus expressed radixin (Paper I, Figure 4D). The specific expression of radixin in neuroblasts at these distinct locations indicates a role for this protein in neuroblast migration. Radixin expression in neuroblasts was localized to the cell membrane covering all processes but was also present in the cytoplasm. The localization along the cell membrane suggests active interaction of radixin with membrane proteins or other membrane components. Interestingly, more mature DCX$^+$ cells, positioned inside the hippocampal granule cell layer, did not generally co-label with radixin. On the other hand, no GFAP$^+$ cells in the neurogenic niches displayed co-localization with radixin (Paper I, Figure 1J). Furthermore, radixin expression was not detected in ependymal cells, microglia or endothelial cells in the intact adult mouse brain. In addition, in all brain regions studied, radixin immunoreactivity was present in Olig2$^+$ cells. These regions included the cortex, thalamus, cerebellum, hippocampus, striatum and in the glomerular and periglomerular layers of the olfactory bulb (Paper I, Figure 5). This suggests that radixin is generally present in OPCs and mature oligodendrocytes throughout the adult rodent brain. In Olig2$^+$ cells, radixin was localized only in the cytoplasm indicative of an inactive conformation of the protein in these cells. Except for OPCs and mature oligodendrocytes, Olig2 expression has been reported in C-cells in the SVZ, suggesting that radixin may be present already at this stage in the NSPC lineage.

### Expression of moesin and ezrin in the SVZ and RMS (Paper I)

Moesin displayed a similar expression profile as radixin apart from a lower degree of co-labelling with neuroblasts. Contrary to radixin and moesin, ezrin was detected in GFAP$^+$ astrocytes, both in the SVZ and in the RMS. The specific expression of ezrin in astrocytes has been reported previously (Gronholm et al., 2005, Cleary et al., 2006). This indicates that neuroblasts and astrocytic cells may use different ERM-proteins for actin-polymerizing functions such as cytoskeleton rearrangement during process formation.

### Radixin in migrating neuroblasts *in vitro* (Paper I, II and III).

The numerous neuroblast chains present in the adult RMS makes it difficult to study single chains in detail and *in vitro* models of neuroblast chain migration, like SVZ neurospheres or explants cultured in Matrigel, mimic some of the features of neuroblast migration. In these *in vitro* systems, neuroblasts emerge out from the sphere/explant and migrate in all directions into the Matrigel, making it a useful tool to study individual chains of migrating neuroblasts and isolated migratory features. Radixin labelling was found both in the core of the spheres and explants as well as in the protruding cell chains. In explants, a more heterogeneous population migrates into the Matrigel than from neurospheres, including cells which do not migrate through the RMS such as GFAP$^+$ and Olig2$^+$ cells. The majority of cells migrating in SVZ explant cultures under control conditions express β-III tubulin and Sox2 and a smaller fraction express GFAP (Paper II, Figure 4). Almost all GFAP$^+$ cells were also Sox2$^{high}$ (Paper II, Figure 4). The majority of radixin immunoreactivity is detected in cell chains co-labelling with β-III tubulin, PSA-NCAM and Sox2$^{low}$, confirming the expression profile demonstrated in neuroblasts *in vivo* (Paper I and III). Olig2$^+$ cells migrating in explant experiments exhibited radixin immunoreactivity, whereas no co-labelling could be discerned in GFAP$^+$ cells outside the explant core (Paper III, Figure 11). On the contrary, GFAP$^+$ cells were found migrating a short distance from the edge in the neurosphere migration assay and some astrocytic processes co-labeled with radixin immunoreactivity (Paper I, Figure 6I-L). Differences between the explant and neurosphere migration assays may be due to the presence of additional cell populations in explants compared to spheres or the fact that sphere cells have been treated extensively with EGF during neurosphere propagation. EGF treatment of cells is known to transform C-cells into multipotent stem cells and reduce neuronal production (Doetsch et al., 2002). Common Matrigel contains growth factors including EGF and although the amount is not defined and is batch-dependent it is likely present at a lower concentration than in conditions of neurosphere suspension cultures. Hence, placing neurospheres in Matrigel may expose sphere cells to a decreased concentration of EGF, whereas the explant cells may be exposed to an increased amount of EGF compared to the *in vivo* situation.

For an environment more comparable to the one *in vivo*, the explant migration assay was used in paper II and III. Still, the artificial content in the Matrigel and other components make conditions different from *in vivo*. To evaluate the influence of growth factors in the Matrigel on fate determination, the explant migration assay was performed in traditional Matrigel and in growth factor-reduced Matrigel and the proportion of GFAP$^+$/Sox2$^{high}$ as well as Sox2$^{low}$ migrating cells were quantified. No difference was found in the proportion of glial compared to neuronal progenitor populations in the two conditions (data not shown).

### Radixin inhibition reduces migration of neuroblasts (Paper II)

To determine the influence of radixin inhibition on NSPC migration, we incubated SVZ explants with increasing concentrations of the radixin inhibitor DX52-1 (Paper II, Figure 1).

This quinocarmycin analog inhibits the actin- and transmembrane-binding functions of radixin (Kahsai et al., 2006). For PSA-NCAM$^+$ cells, the average distance migrated was significantly reduced by concentrations over 50 nM of DX52-1 (Paper II, Figure 2A). Under radixin inhibition, the neuroblasts had an abnormal, round morphology instead of the regular elongated shape, and leading processes were short and thick, if present at all. Since a reduced migratory response could be due to toxicity of the radixin inhibitor, we compared LDH activity in control explants with those treated with 100nM DX52-1. Cultures treated with radixin inhibitor displayed similar LDH activity compared to control, thus revealing no additional cell toxicity during treatment with the radixin inhibitor (Paper II, Figure 3).

To test if radixin inhibition specifically affects migration of neuroblasts or other cell types, we also analysed the migration pattern of Sox2$^{high}$ cells, which represent the glial population migrating from SVZ explants under standard conditions. No statistical difference in the migration distance of Sox2$^{high}$ cells in the migratory chains could be discerned under treatment with any concentration of the inhibitor (Paper II, Figure 2B). As a consequence of the reduced migration of PSA-NCAM$^+$ cells, an increased percentage of Sox2$^{high}$ cells surrounding the explants were observed (Paper II, Figure 2D). This further suggests that DX52-1 is not generally toxic and that the effect on migration is restricted to radixin expressing cells.

### Radixin inhibition *in vivo* results in aberrant neuroblast chain formation in the SVZ and RMS (Paper II)

Effects of intracerebroventricular infusion of the radixin inhibitor were analysed in the SVZ and RMS. Radixin inhibition appears to hinder migration since neuroblasts accumulate in the anterior SVZ and in proximal parts of the RMS after DX52-1 infusion (Paper II, Figure 6B). Whole mount preparations of the lateral ventricle wall give a useful overview of the organisation of neuroblast chains in the SVZ. In the dorsal SVZ of naive animals, massive amounts of neuroblast chains are lined up, organised horizontally to the corpus callosum and pointing towards the anterior SVZ (Paper II, Figure 5A). In addition, long cell chains cover the entire SVZ. Under radixin inhibition, the SVZ displayed aberrant neuroblast chain formation with short, intermittently interrupted cell chains pointing in various directions (Paper II, Figure 5C). Furthermore, no characteristic neuroblast chains were present in the dorsal SVZ. DCX immunoreactivity accumulated in the cell body and the leading processes were short and thick (Paper II, 5D).

### Radixin inhibition decreases proliferation in the RMS (Paper II)

The high amounts of radixin present in the cleavage furrow of dividing cells suggest involvement of this protein in cell proliferation (Sato et al., 1991). A decreased number of cells expressing the cell cycle marker phosphorylated histone H3 (PhosH3) were detected in the RMS after radixin inhibition. Furthermore, fewer dividing neuroblasts were present in the RMS after radixin inhibition (Paper II, Table I). However, no difference in proliferation was detected in

the SVZ and no statistical difference in apoptosis could be discerned in the RMS or the SVZ (Paper II, Table I). Above data suggest that radixin has an activating role in migration and proliferation of neuroblasts.

## EGF induces radixin expression in the RMS (Paper III)

### Structural changes in the RMS after EGF treatment (Paper III)

EGF infusion into the lateral ventricles exposes the SVZ cell populations to abnormally high doses of EGF. Previous studies noted an EGF-induced decrease in the number of neuroblasts in the anterior SVZ (Craig et al., 1996, Kuhn et al., 1997, Doetsch et al., 2002). In paper III, we describe major differences in the RMS induced by EGF treatment. We detected that the orderly structure of the RMS was distorted, the boundaries diffuse and RMS cells spread over a larger diameter than in the RMS in controls. However, the cell density was decreased, most likely due to fewer neuroblasts (Paper III, Figure 1A and B). The changes in the RMS were most pronounced in the parts proximal to the SVZ, whereas the RMS distal from the SVZ only exhibited reduced cell density (Paper III, Figure 1C). Differences along the RMS may be the result of a higher EGF concentration proximal to the SVZ and the fact that during the infusion a vast number of neuroblasts already present in the RMS continue to the distal parts. Given that neuroblasts take about 4-7 days to migrate from the SVZ to the OB, a longer infusion period likely bring the same structural changes also to the distal RMS.

### EGF infusion increases radixin expression (Paper III)

Using immunohistochemistry and qPCR, a reduction of DCX expression was confirmed after EGF infusion in paper III. Residual $DCX^+$ cells in the EGF-treated RMS appeared more dispersed, indicating less homophilic interaction between neuroblasts. In the naïve RMS, radixin expression is present specifically in neuroblasts as described above. However, EGF infusion induced a two-fold increase in radixin mRNA expression in the SVZ whereas mRNA expression in the OB remained unchanged (Paper III, Figure 2C and D). In spite of the greatly reduced number of neuroblasts after EGF treatment, nearly all cells in the EGF-treated RMS still expressed radixin. Accordingly, an additional cell population resides in the RMS which upregulates radixin under EGF stimulation.

### EGF infusion induced a new population of cells in the RMS (Paper III)

Radixin expression in the healthy adult brain is present in neuroblasts of the neurogenic niches and in $Olig2^+$ cells scattered throughout the brain, including in the RMS. Interestingly, EGF infusion induced expansion of a very large amount of radixin/$Olig2^+$ cells in the RMS (Paper III, Figure 4). After 7 days of EGF infusion, the proportion of $DCX^+$ cells in the radixin population was decreased by 50%. In contrast, a proportional increase in the ratio of cells

expressing Olig2 was observed (Paper III, Figure 5). In the naïve RMS, few Olig2 expressing cells are situated within the stream. Radixin/Olig2$^+$ cells increased 8-10 fold already after 7 days of EGF treatment, when about 50% of all radixin$^+$ cells in the RMS co-expressed Olig2 (Paper III, Figure 4 and 5). Radixin/DCX$^+$ cells decreased further when the infusion period was increased to 14 days. The new population of radixin/Olig2$^+$ cells was however not further increased after the additional 7 days, suggesting a rapid induction that reached a plateau early. This suggests that the generation of Olig2 expressing cells is a separate event from the decline in DCX expressing cells and not a direct fate shift. However, since the total number of RMS cells was not assessed it is not clear whether the total number of radixin$^+$ cell was altered. As there was no difference in the radixin/Olig2$^+$ population between 7- and 14-day EGF infusions, we continued our analysis in the RMS of animals infused for 7 days. The lack of further expansion of the Olig2 population during the second week indicates that the proliferative capacity of the progenitor may be limited, or that a negative feedback due to the extensive EGF signalling may counteract the early rapid effects. Furthermore, rapidly increasing the proliferation of SVZ progenitors can result in increased cell death (Belvindrah et al., 2002, de Chevigny et al., 2008).

### EGF induced proliferation of radixin/Olig2$^+$ cells in the RMS (Paper III)

To study the proliferative populations in the RMS, BrdU was administered systemically by three i.p. injections during the last 24 hours before perfusion. The origin of EGF-induced Olig2$^+$ cells has not been fully elucidated; however, the presence of BrdU/Olig2$^+$ cells in the distal RMS already after 1 day of EGF infusion suggest that EGF-responsive cells reside in the RMS and are increased, at least in part, by proliferation (Paper III, Figure 10). These data suggest that sufficient amounts of EGF reached the distal RMS in just one day and rules out the possibility that these cells migrated from the SVZ. The ratio of BrdU/Olig2+ cells increased further after 7 days of EGF infusion. Numerous cells in the same location expressed high levels of Olig2 already at 1d, but were not BrdU labelled, suggesting that the Olig2 expression was upregulated. Possibly, cells in the RMS increase Olig2 expression prior to cell division through EGF signalling. This hypothesis is in line with the increased percentage of Olig2/BrdU$^+$ cells seen at 7 days compared to 1 day. Notably, OPCs are generally thought to express high levels of Olig2 protein, whereas mature oligodendrocytes display low levels of Olig2 (for review see Bradl and Lassmann, 2010). Transient induction of Olig2 expression has been demonstrated during intensive proliferative activity in different cell types (Hack et al., 2004, Buffo et al., 2005, Hack et al., 2005, Chen et al., 2008).

At both time points, in both control and EGF-treated RMS, nearly all BrdU cells were also expressing radixin (Paper III, Figure 10E and F). Radixin was detected early on in the cleavage furrow of several cell types, implying a role in structural rearrangement during the mitosis phase of cell division (Sato et al., 1991).

## The radixin/Olig2+ population is generated within the RMS (Paper III)

To examine whether the EGF-responding cells are activated locally or originate from the SVZ, the ratio of Olig2 expressing cells in the BrdU-labelled cells was investigated at two positions along the anterio-posterior axis of the RMS after 1 day of EGF infusion. An increase in the Olig2/BrdU ratio was observed in both the proximal and distal part of the RMS suggesting that the EGF-induced Olig2$^+$ cells are indeed derived from a local progenitor in the RMS (Paper III, Figure 10D). Notably, the RMS sub-region referred to as the RMS elbow, harboured numerous Olig2$^+$ cells suggesting that the RMS may not be completely homogeneous in its composition and that progenitor cells may reside in higher numbers at certain locations along the stream.

A recent study following the generation of Olig2/NG2$^+$ cells from SVZ B1-type cells after EGF infusion using retroviral labelling, report of cells invading the striatum, corpus callosum, septum, cortex and fimbria fornix in mice (Gonzalez-Perez et al., 2009). However, presence of Olig2$^+$ SVZ-derived cells was not reported in the RMS, further indicating that radixin/Olig2$^+$ cells in paper III are locally recruited.

## What type of cell are the EGF-induced radixin/Olig2$^+$ cell?

Olig2 expression is present in C-cells of the adult SVZ (Hack et al., 2005, Menn et al., 2006); however, additional C-cell markers are needed to distinguish these cells from OPCs and mature oligodendrocytes. Previous reports indicate that EGFR activation can shift cell commitment of SVZ C-cells towards the generation of oligodendrocyte-like progenitors (de Chevigny et al., 2008). Olig2$^+$ cells, both in the control and EGF-treated RMS, occasionally expressed CNPase (Paper III, Figure 8A) or NG2 (Paper III, Figure 8B). However, no difference in the distribution of NG2$^+$ or CNPase$^+$ cells was observed (Paper III, Figure 8A and B). Since the radixin/Olig2$^+$ cells were not specifically positive for OPC or oligodendrocyte markers, it seems more likely that they are related to NSPCs in the SVZ.

EGF stimulation has also been demonstrated to transform C-cells into multipotent stem cells (Doetsch et al., 2002) and the production of a resting cell in the RMS, which resembles a type C-cell, may be triggered by EGF infusion. Furthermore, it is possible that a number of neuroblasts in the RMS could be de-differentiated into a rapidly proliferating C-cell stage upon EGF stimulation. Infusion of the EGFR ligand TGFα into the striatum generated a wave of Olig2/Nestin/EGFR/BrdU$^+$ cells that infiltrated the striatum from the SVZ (de Chevigny et al., 2008). Radixin expressing cells extends into the corpus callosum after 2 weeks of EGF infusion, whereas in the control, only a few scattered cells are radixin positive in the corpus callosum (Figure 7A and B). Additionally, the hyperplasias express high levels of radixin in Olig2$^+$ cells (Figure 7D). This population is barely detectable in the control SVZ (Figure 7 C). Furthermore, the majority of cells in the EGF induced hyperplasias in the rat SVZ expressed Sox2 and a large portion expressed Olig2 (Lindberg et al., 2011).

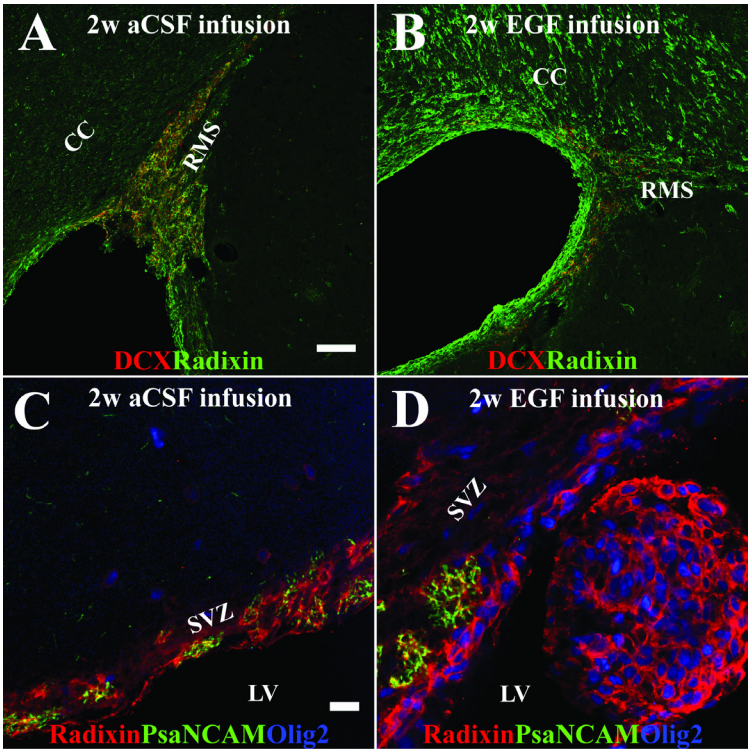


**Figure 7.** Radixin expression in the SVZ after EGF infusion. (A) Radixin expression in DCX+ cells in the anterior SVZ in control. (B) Intensive radixin immunoreactivity in the SVZ, RMS, and corpus callosum after 2 weeks of EGF infusion. (C) In the control, few radixin/Olig2$^+$ cells are located in the SVZ. (D) EGF induced hyperplasias are enriched in radixin/Olig2$^+$ cells. Scale bar in a=50 µm and d=20 µm.

Similarly, EGF-induced radixin/Olig2$^+$ cell in the RMS expressed Sox2 (Paper III, Figure 9B, D and F). Expression of both Olig2 and Sox2 has been detected in C-cells of the SVZ (Ferri et al., 2004, Menn et al., 2006). In conclusion, the presence of dividing Olig2/Sox2$^+$ cells along the RMS in paper III indicates that similar cells may reside in the RMS after EGF treatment.

A number of recent studies report the presence of NSPCs in the RMS (Gritti et al., 2002, Alonso et al., 2008, Giachino and Taylor, 2009). Although the full potential of RMS NSPCs is not clear, it is possible that these cells are of a more restricted fate than SVZ NSPCs. Giachino and colleagues used tamoxifen-inducible NestinCreER transgenic mice to follow a population of NSPCs in the RMS (Giachino and Taylor, 2009). Nestin, Sox2 and GFAP expressing cells appeared early after tamoxifen-induced recombination in the center of the OB; however, such NSPC-like cells were not identified in other parts of the RMS (Giachino and Taylor, 2009). Alonso and colleagues instead used lentiviral green fluorescent protein (GFP) labelling of glial cell types at the elbow of the RMS, after depletion of actively proliferating cells, such as C-cells and neuroblasts, by AraC (Alonso et al., 2008). The GFP expressing cells were positive for GFAP, NG2 and S100β and gave rise to PSA-NCAM and NeuN expressing cells in the RMS and OB (Alonso et al., 2008). However, the lack of precise labelling of a single cell type makes it impossible to draw conclusions regarding possible GFAP expressing NSPCs in the RMS. Furthermore, NG2$^+$ cells in the SVZ have been proven sufficient to produce neurospheres and generated functional interneurons when grafted into the hippocampus in a previous study (Aguirre and Gallo, 2004), suggesting the presence of different progenitor populations in the

SVZ. The similarity of SVZ NG2$^+$ cells, C-cells and the EGF-induced radixin/Olig2$^+$ cells indicate the presence of highly plastic progenitor populations in the adult SVZ and RMS.

### *In vivo* and *in vitro* chain formation suggest migratory properties of EGF-expanded RMS cells (Paper III)

Frequently, and along the entire stretch of the EGF-treated RMS, we observed non-neuroblast cells lined up in chains. These cells were radixin/Olig2$^+$, tightly associated and had an elongated shape similar to chain-migrating neuroblasts. In addition, chain-forming radixin/Olig2$^+$ cells and neuroblasts expressed pERM, unlike non-chain-forming cells in both EGF and control RMS (Paper III, Figure 11A and B). To study the migratory potential of the radixin/Olig2$^+$ cells, we used SVZ explant cultures. After 72 hours in culture, cells had migrated far from the explant core in tight chains. The majority of migrating cells expressed radixin and a small portion was radixin/Olig2$^+$ (Paper III, Figure 11C). These cells frequently formed chains similar to neuroblasts and expressed pERM (Paper III, Figure 11D).

Interestingly, NG2$^+$ cells in the SVZ express the EGFR and these cells are migratory, whereas cortical NG2$^+$ cells are non-migratory and do not express the EGFR (Aguirre et al., 2005). Overexpression of EGFR in NG2$^+$ cells converted non-migratory cortical NG2 cells into migratory cells (Aguirre et al., 2005). Hence, EGF, although primarily associated with induction of proliferation, can additionally induce migratory properties in otherwise stationary cells. However, a small number of neuroblasts in the naïve RMS express low levels of the EGFR whereas the majority of neuroblasts are negative for EGFR. These EGFR expressing neuroblasts migrate at a slower speed in a less directed fashion, compared to EGFR negative neuroblasts (Kim et al., 2009). Furthermore, TGFα treatment was sufficient to decrease the number of motile cells by approximately 40% during time lapse imaging of RMS slice cultures (Kim et al., 2009). Considering that the majority of cells in the RMS are neuroblasts, this provides evidence for a direct regulatory effect of TGFα on neuroblast migration. The four EGF receptors, ErbB1–4, can dimerize in various combinations and can be activated by at least eight known ligands. This complex signal transduction pattern explains some of the diversity in ErbB-mediated cellular responses.

As noted above, intrastriatal infusion of TGFα was shown to induce a wave of C-cell like cells migrating into the striatum originating from the SVZ, and these cells expressed Olig2 (de Chevigny et al., 2008). In addition, increased expression of tenascin C was reported after EGF stimulation, covering the areas occupied by SVZ derived cells (Sakai et al., 1995, Doetsch et al., 2002, Gonzalez-Perez et al., 2009). Tenascin C expression creates a migration-permissive environment in the RMS through reduced cell adhesion (Jankovski and Sotelo, 1996, Thomas et al., 1996) and may facilitate migration of progenitors away from the SVZ. Furthermore, *in vitro* studies showed that the EGFR and ECM components (laminin, fibronectin and vitronectin) synergistically increased the migration of OPCs (Aguirre et al., 2005). The same

ECM components are enriched in the RMS as described above. Similarly, the EGF-treated RMS may provide a migration-permissive environment for radixin/Olig2$^+$ cells which generally do not migrate and the property of the RMS may also be altered considering the structural changes described above. This could explain why chains of radixin/Olig2$^+$ cells were formed along the entire RMS.

### Radixin and the EGF receptor

To our knowledge there is not much evidence for a direct interaction between radixin and EGF receptors. However, radixin was identified as a substrate for the kinase activity of intracellular EGFR-domains (Fazioli et al., 1993), indeed suggesting a direct interaction. Association between the EGFR and f-actin has been proposed in studies of A431 cells (den Hartigh et al., 1992) and this interaction increases in the presence of unknown cytosolic factors (Tang and Gross, 2003) indicating that a larger protein complex forms. EGF stimulation rapidly induces actin rearrangement in these cells (Rijken et al., 1991). The ERM binding protein, NHERF, regulates the function of many transmembrane receptors, including the EGFR (Lazar et al., 2004), and may provide a link between EGF and radixin. Similar to radixin, NHERF expression is induced by EGF stimulation of the EGFR. However, increased NHERF expression was shown to decrease EGFR function, suggesting a negative feedback loop by NHERF (Lazar et al., 2004, Claperon et al., 2012). Furthermore, downstream targets of EGF signalling may induce upregulation of radixin expression.

### Relevance of EGF expanded Olig2$^+$ cells (Paper III)

Previous reports indicate that EGFR activation can shift cell commitment towards the generation of oligodendrocyte-like progenitors (de Chevigny et al., 2008, Ivkovic et al., 2008). Constitutive EGFR signalling in OPCs results in expansion of a proliferative, highly migratory Olig2$^+$ population that does not mature (Ivkovic et al., 2008). Although we can only speculate where the radixin/Olig2$^+$ cells end up after EGF withdrawal, it has been demonstrated that EGF-induced Mash-1/EGFR/Olig2$^+$ cells are partially converted to a neuronal fate after EGFR ligand withdrawal (de Chevigny et al., 2008), indicating that the fate shift of NSPCs towards a glial lineage is reversible. A recent study reports that EGF stimulation after induction of experimental stroke in the striatum increased the number of Mash-1/EGFR$^+$ cells in the peri-infarct region, while the number of neuroblasts decreased (Ninomiya et al., 2006). However, 6 days after EGF withdrawal the number of neuroblasts was markedly increased in the peri-infarct region compared to only stroked animals (Ninomiya et al., 2006). The use of EGF for stimulation of neuronal progenitor cells may induce subsequent tumour formation and is not appropriate for clinical use (Sanai et al., 2005). However, the presence of EGF responsive cells along the entire RMS, with the potential for neuronal differentiation, significantly increases the area of responsive cells in the adult rodent brain.

### Glial tubes and microglia in the RMS are unaffected by EGF treatment (Paper III)

Astrocytes, which form the glial sheaths between chains of migrating cells in the RMS, were analysed by immunofluorescence against GFAP after EGF infusion. Under EGF stimulation, GFAP expressing cells appeared less regular in shape with thicker processes, indicating a more reactive phenotype (Paper III, Figure 8C) but appeared otherwise unaffected.

In addition, resident microglia are found throughout the brain, including in the RMS. Intrastriatal infusion of EGFR ligand TGFα in 6-OHDA lesioned animals was shown to increase Iba1$^+$ microglia in the SVZ and striatum (de Chevigny et al., 2008). However, no changes in the pattern of Iba1$^+$ microglia were observed in the RMS, and no Iba1$^+$ cells co-expressing radixin were found in control or EGF-treated animals (Paper III, Figure 8D).

## Stroke induces radixin expression in the peri-infarct cortex (Paper IV)

### Induction of stroke transiently increases radixin expression (Paper IV)…

Cortical expression of the ERM protein radixin in the intact brain is sparse and scattered, mostly localized to the cytoplasm of Olig2$^+$ cells. Two weeks after induction of photo-thrombotic stroke, we discovered intense protein expression of radixin along the entire peri-infarct cortex and the number of radixin$^+$ cells increased more than two-fold compared to control and contralateral cortex (Paper IV, Figure 1A, E and F). Increased radixin immunoreactivity transiently appeared in the peri-infarct cortex during the first two weeks and declined thereafter (Paper IV, Figure 1I, J, arrows in 1K and L).

### …but do not increased Olig2$^+$/radixin$^-$ cells in the cortex (Paper IV)

Accumulation of Olig2$^+$ OPCs after stroke has been documented previously (Mabuchi et al., 2000, Buffo et al., 2005) and was confirmed in our study. In the peri-infarct cortex, the number of Olig2$^+$ cells was doubled compared to the corresponding area on the contralateral side and to untreated control cortex (Paper IV, Figure 1B, G and H). Considering the general expression of radixin in cortical Olig2$^+$ cell we expected a high number of radixin/Olig2$^+$ cells. Yet, no difference was found in the number of radixin/Olig2$^+$ cells between ipsilateral and contralateral side of the lesioned animals, or between control and lesioned animals (Paper IV, Figure 1C). Accordingly, the proportion of Olig2$^+$ cells within the radixin population was altered. In the control cortex, 51 % of radixin$^+$ cells were Olig2$^+$, while in the ipsilateral cortex of stroke animals the Olig2$^+$ cells accounted only for 25 % of the radixin expressing cells (Paper IV, Figure 2A). It has been noted that proliferating GFAP$^+$ reactive astrocytes express Olig2 after MCAO and cortical injury (Buffo et al., 2005, Chen et al., 2008). Co-localization of radixin and GFAP could not be discerned around the lesion and the increased radixin$^-$/Olig2$^+$ population may represent reactive astrocytes.

### Neuroblasts migrating to the infarct express radixin (Paper IV)

Neuroblasts are known to deviate from the SVZ or RMS towards the ischemic area after stroke (Arvidsson et al., 2002, Ohab et al., 2006, Osman et al., 2011), and we hypothesized that the accumulated radixin$^+$ cells in the peri-infarct cortex were neuroblasts. Indeed, after inducing the cortical stroke lesion we observed ectopic neuroblasts migrating to the ischemic area and the vast majority of DCX$^+$ neuroblasts, 95±8%, expressed radixin in the peri-infarct cortex. Nevertheless, neuroblasts accounted only for 8.5% of the radixin$^+$ cells in the ipsilateral cortex (Paper IV, Figure 2D, E and F).

### Regional radixin expression in resident microglia after stroke (Paper IV)

Radixin expression in the peri-infarct cortex was primarily located to macrophages/microglia, as detected by Iba1. In the control cortex the majority of microglia display a resting, surveying state and none expressed radixin; however, in the peri-infact region, radixin co-localized with 70% of Iba1$^+$ cells after cortical stroke. Of the radixin$^+$ cells in the peri-infarct cortex, 39 % were Iba1$^+$ and close to none were Iba1$^+$ in the contralateral cortex (Paper IV, Figure 2G, H and I). Although we had no means to distinguish between macrophages and microglia, previous studies showed that approximately 30-50% of Iba1$^+$ cells represent invading macrophages (Schilling et al. 2003; Thored et al. 2009). Considering the high proportion of radixin$^+$ cells within the Iba1 population (70%), it is likely that the radixin$^+$/Iba1$^+$ cells correspond, partially or completely, to the resident microglial population. Furthermore, co-expression of radixin and Iba1 was also found in cultures of primary microglia, which do not contain significant amounts of blood-borne cells, further supporting our notion that radixin expression is induced in resident microglia.

### Radixin expression in a subpopulation of activated microglia (Paper IV)

The absence of radixin expressing microglia in control animals suggests that the activation of microglia after stroke is required to induce radixin expression in this cell population. Microglia activation is characterized by altered morphology and expression of markers such as CD68, galectin-3 or CD11b in the Iba1$^+$ population (Sedgwick et al., 1991, Yan et al., 2009). In paper IV, activated microglia expressing CD68 or galectin-3 were observed in the peri-infarct cortex (Paper IV, Figure 3A and 3B). Radixin$^+$/CD68$^+$ microglia were numerous in the peri-infarct cortex, the ipsilateral corpus callosum and scattered in the striatum. However, radixin expression did not correspond exactly to the CD68/Iba1 expressing population and was detected in both non-activated (CD68$^-$) and activated (CD68$^+$) microglia. This suggests that radixin may serve to detect a subpopulation of activated microglial cells and that this population is partially not detectable using CD68 as activation marker. Similarly, radixin only partially corresponded to galectin-3 expression. In the healthy SVZ, microglia are constitutively semi-activated (Goings 2006); however, this level of activation seems not sufficient to stimulate radixin expression in microglia (data not shown). Upon activation, microglia become migratory and proliferate

(Carbonell et al., 2005). Radixin modulates migration and adhesion in other cell types (Takeuchi et al., 1994, Paglini et al., 1998, Valderrama et al., 2012), and may have a similar role in microglia. Furthermore, radixin can regulate the activity of the integrin receptor CD11b, which is expressed in resident microglia (Sedgwick et al., 1991, Tang et al., 2007).

Recent evidence suggests the presence of different subpopulations of activated microglia where the classically activated M1 phenotype induces neuronal death while an alternative activated M2 phenotype produces protective effects (Butovsky et al., 2006, Hu et al., 2012). Investigation of type M1 and M2 activated microglia after ischemic injury showed that the majority adopted the M1 phenotype during the first 7 days after the insult which was sustained for at least 14 days, whereas M2 microglia were only transiently present during the first week (Hu et al., 2012). The radixin/Iba$^+$ population present after cortical stroke in our study was sustained for 14 days and may thus reflect the M1 phenotype. Studying the emerging concept of beneficial and detrimental immune responses may lead to therapies that can increase tissue preservation in the brains of stroke patients. Further studies of subpopulations of activated microglia are needed to test the usefulness of radixin as a new microglia activation marker.

## Differential expression of phosphorylated radixin (Paper II-IV)

The activity of the protein radixin is regulated by conformational changes due to threonine phosphorylation, which is thought to relocate the protein from the cytosol to the membrane (Sato et al., 1992b, Hirao et al., 1996, Yonemura et al., 2002). It has also been suggested that binding to membrane molecules initiates the activation of ERM proteins (Niggli et al 1995; Hamada et al 2000). Only in the phosphorylated state can radixin bind to the actin cytoskeleton and transmembrane proteins as the conformational changes reveal the N-terminal protein-binding site (Matsui et al 1998; Hayashi et al 1999; Berryman et al 1995; Bretscher et al 1995). Visualization of activated radixinin was acquired through co-labelling of radixin with an antibody against phosphorylated ezrin/radixin/moesin (pERM) of a conserved threonine residue in the C-terminal end.

### Neuroblasts, but not cortical Olig2$^+$ cells express phosphorylated radixin in the healthy brain (Paper I and III)

In the naïve adult brain, radixin is present in neuroblasts in the neurogenic niches and in Olig2$^+$ cells throughout the brain (Paper I, Figure 1D and 3D-H). In neuroblasts, radixin and pERM immunoreactivity covered most of the cell membrane, including the fine processes (Paper III, Figure 7A). Similarly, phosphorylated radixin is present in neuroblasts migrating from SVZ explants *in vitro* (Paper III, Figure 11D). In contrast, in cortical Olig2$^+$ cells of the rat brain, radixin was located to the cytoplasm and phosphorylation was not detected. This suggests that in neuroblasts, radixin is actively interacting with components of the cell membrane or

transmembrane proteins. On the contrary, in Olig2$^+$ cells only the inactive conformation appears to be present under control conditions.

### EGF-induced Olig2$^+$ cells display phosphorylated radixin exclusively in the RMS (Paper III)

In the EGF-treated RMS, the majority of radixin/Olig2$^+$ cells display the activated form of the protein (Paper III, Figure 7D). Correspondingly, the morphology of radixin/pERM$^+$ cells in EGF-treated RMS was elongated and displayed the proteins along the cell membrane suggesting active interaction with membrane proteins (Paper III, Figure 11B). Similar to cortical radixin/Olig2$^+$ in the control brain, cortical Olig2$^+$ cells in EGF treated animals were negative for phosphorylated radixin (Paper III, Figure 7B and E). Hence, Olig2$^+$ cells are exclusively radixin/pERM$^+$ in the RMS. Although, occasional radixin/pERM/Olig2$^+$ cells with an elongated morphology were found in the striatum of EGF-treated animals, bordering the SVZ and RMS (Paper III, Figure 7F). EGFR dimerization stimulates its intracellular protein-tyrosine kinase activity and through immune-affinity screening radixin was found to be a substrate for EGF receptor tyrosine kinase activity (Fazioli et al., 1993), however this has to our knowledge never been studied *in vivo*. EGF stimulated phosphorylation of two tyrosine residues has been demonstrated for the ERM protein ezrin; however, these residues are not conserved in radixin. The antibody used in this study detects threonine phosphorylation of ERM proteins.

Furthermore, responsive Olig2$^+$ cells reside throughout the brain (Levine and Reynolds, 1999). However, assuming that EGF reaches most parts of the brain during 7 days of intracerebroventricular infusion, cortical Olig2$^+$ cells seem not to respond to EGF-like cells residing in the RMS.

### Differential expression of phosphorylated radixin after stroke (Paper IV)

Phosphorylated radixin was detected within the Iba1$^+$ population (Paper IV, Figure 5A). In vitro, pERM and radixin were highly expressed in primary microglia in both control and in LPS stimulated cultures (Paper IV, Figure 4G and H). Phosphorylated radixin was present in processes and at branching points, while radixin immunoreactivity was present in both the cytoplasm and covering most of the plasma membrane in Iba1$^+$ cells. After LPS stimulation, intense co-localization of radixin with pERM could be discerned at the plasma membrane (Paper IV, Figure 4H).

In the peri-infarct area, Olig2$^+$ cells located in close proximity to the infarct core were positive for pERM; while in areas further away from the infarct as well as in the surrounding cortex; they were negative for pERM (Paper IV, Figure 5B). Differences in radixin phosphorylation may depend on the local environment around the lesion. Another possibility is that Olig2/pERM$^+$ cells in the peri-infarct region may be under phagocytosis by Iba-1$^+$ cells since a large number of oligodendrocyte progenitors die after stroke (Mabuchi et al., 2000).

In addition, DCX$^+$ cells showed differential phosphorylation of radixin after stroke. Neuroblasts migrating to the lesion lacked pERM expression (Paper IV, Figure 5C); although the RMS regularly harbours DCX$^+$ neuroblasts expressing phosphorylated radixin (Paper IV; Figure 5D; Paper III; Figure 10). This suggests that neuroblasts can migrate without extensive radixin phosphorylation. However, inhibiting radixin function in neuroblasts both *in vitro* and *in vivo* suggests an important role for radixin in their migration and unphosphorylated radixin may have yet unknown functions.

# General conclusion

Based on the data from this thesis we conclude that radixin has an important role in neuroblast migration and proliferation in the adult RMS. The specific expression of phosphorylated radixin in elongated chain-migrating neuroblasts suggests active interaction with membrane proteins in these cells. However, the astroglial population does not rely on radixin function. Accordingly, inhibition of radixin selectively blocked the migration of PSA-NCAM$^+$ cells, while migration of glial cells remained unaltered, suggesting that these populations use different ERM proteins for actin polymerization and that the highly homologous ERM proteins are not redundant *in vitro* nor *in vivo* in the neurogenic niche. Furthermore, EGF treatment induces radixin in Olig2$^+$ cells originating in the RMS. The results from paper III suggest the presence of a progenitor cell in the RMS with many similarities to C-cells. If true, this would significantly increase the zone from which regenerative cells could be recruited considering that the RMS stretches across the entire forebrain.

Contrary to EGF activation, the increased number of cortical Olig2$^+$ cells after stroke did not result in an increased population of radixin/Olig2$^+$ cells around the lesion. The stroke-induced expression of radixin in the peri-infarct cortex was instead located to a subpopulation of activated microglia and may prove useful as a new microglia activation marker. This differential induction of radixin suggests a variety of functions for radixin in different cell types in the adult rodent brain.

### Conclusions to the given research questions

I. We have demonstrated the specific expression of the ERM protein radixin in neuroblasts in the neurogenic niches and the aberrant migration of neuroblasts in the SVZ and RMS after radixin inhibition.

II. In addition to affecting migration, radixin inhibition also decreases proliferation in the SVZ and RMS.

III. EGF treatment stimulates radixin expression and induces radixin phosphorylation in progenitor cells in the SVZ and RMS. However, expansion of OPCs in the cortex after stroke does not involve radixin. Although radixin is still present in ectopic SVZ/RMS neuroblasts migrating towards the stroke lesion, it was only expressed in its inactive conformation. The strongest expression of radixin after stroke was unexpectedly found in activated microglia.

# Acknowledgement

This work was performed at the Center for brain Repair and Rehabilitation, Institute of Neuroscience and Physiology at the Sahlgrenska Academy, Göteborg University. I would like to thank all the co-workers at the Institute of Neuroscience and Physiology for their support. In particular I would like to thank the following people:

**Georg Kuhn**, my supervisor for quickly taking me into your research group when times got rough. Thank you for sharing your great knowledge and for valuable discussions over the years, for your generosity with research money, the independent research environment and introducing me to mac☺. Special thanks for your encouragement and instant response to my email during the last few months!

**Maurice Curtis**, my co-supervisor for inspiring me to start my PhD studies and for help writing the first project plan and valuable comments on the thesis. Thank you for your enthusiasm and optimism, for friendship and good times back in the days in Sweden, New Zeeland and all those incredible places in Africa.

**Linda Paulson**, min nästan bihandledare som ju varit lite väl långt borta lite väl länge. Tack för att du introducerade mig för proteomik i allra första början, för roliga stunder i och utanför labbet, och för att du uppmuntrat och trott på mig!

*In memoriam:* **Peter Eriksson**, som fick mig att starta mina doktorandstudier.. Din forskning och din person inspirerade mig från första dag, du är saknad.

The **Kuhn research group**: Ett stort tack till **Olle Lindberg** för produktivt samarbete – varför började vi inte tidigare ☺?! Tack för din otroliga entusiasm och positiva inställning, som tog min vilja att forska till en ny nivå! **Ahmed Osman**, best room-mate ever, for friendship and fruitful collaboration, fingers crossed that the stroke paper goes into GLIA☺. Thank you for good discussions and for listening. **Anke Brederlau**, för din härliga ironiska humor, för att du drog igång samarbete i gruppen..för råd och stöd, och fick mig att inse att man kan vara för kritisk. **Christi Cooper Kuhn**, for organizing the lab and teaching me how to do immunos and providing me with

probably 112 protocols. **Jenny Zhang**, for sharing your knowledge, cooking proper chinese food for me and friendly chats. Tack också till **Jenny Nyberg, Reza Motalleb, Aidin Shabro, Joan Birules Muntane** och **Susanne Neumann**.

**Charlotta Lindwall,** är så glad att du hittade till CBR, du förgyller stunder både på labb och i fikarummet med ditt glada humör! Tack för stöd, uppmuntran och vetenskaplig input under alla åren och hoppas det blir mer samarbete framöver.

Vet inte riktigt hur jag ska kunna tacka **Ann-Marie Alborn** och **Birgit Linder** nog för allt de gjort för mig under åren.. Tack för er omtanke i stort och smått, personligt och jobbrelaterat, för att ni delat med er av er kunskap och alltid haft tid för mig. **Birgit**, TACK!!! för hjälp med de läskiga råttorna, utan dig hade inget blivit gjort.. Tack också **Rita Grandér**, för stöd och trevliga stunder i fikarummet, ditt glada skatt förhöjer stämningen på CBR! **Gunnel Nordström**, för att du fixar med parkerings- och passerkort och för recept på migräntabletter.

Tack **Nina Hellström, Ina Nodin** and **Henrik Landgren**, för ni så generöst delat med er av er kunskap, för uppmuntran och stöd och trevliga stunder i fikarummet.

Tack nuvarande och forna kollegor: **Giulia Zanni, Niklas Karlsson, Marie Kalm, Malin Blomstrand, glada Martina Boström, fina Karolina Roughton, Lars Karlsson, Helene Andersson, Lina Bunketorp Käll, Michelle Porrit, Anna Wolf, Thomas Padel, Martina Olsson, Josefine Siege, Klas Blomgren, Changlian Zhu, Cuicui Xie, Kai Zhou, Mathilda Zetterström Axell, Cecilia Bull, Karin Gustavsson, Andrew Naylor, Karin Hultman, Karin Gustavsson, Pete Smith, Jonas Fajiersson, Axel Jansson, Fredrik Blomstrand, Michelle Andersson, Michael Nilsson, Sheila Engdahl, Mila Komitova, Johan Lind, Anders Persson, Henrik Ryberg, Milos Pekny, Marcela Pekna, Daniel Andersson, Åsa Widerstrand, Lizchen Li, Ulrika Wilhemsson** och alla som jag glömt nämna, för att ni alla bidrar till den utomordentligt trevliga stämningen på CBR, ser alltid fram emot att gå till jobbet eftersom ni är/var där! **Johanna Zanden**, tack för trevliga stunder i och utanför ACI/CBR, sen du försvann är det ingen ordning på nåt på immunolabb ;-).

Ett stort tack till **Mari Klaesson**, du är en pärla! Tack för att du rett ut både det ena och det andra ekonomiska frågetecknet för mig, ditt snabba agerande och framförhållningen med min opponents arvode, bra jobbat! Tack också till och alla andra på kansliet.

Ännu större tack till IT-support **Oskar Bergström** och **Markus Johansson**, tack för att ni är suveräna och snabba och med en ängels tålamod besvarat mina frågor vid datorbyte och problem.

**Carina Mallard, Hayde Bolouri, Mats Sandberg** and **Jaspal Patil,** for collaboration, valuable discussions and support. **Carina Sihlbom** på Proteomics core facility, för din insats att försöka få klart proteomikstudien innan tryck, bara Murphys law's stod i vägen! och **Sara Hansson** bara för att du alltid är så trevlig och hjälpsam ;-). **Maria Smedh** på center for cellular imaging, för planeringen av diverse imaging försök.

Tack till min familj, släkt och vänner, för att ni förgyller mitt liv!

Speciellt tack till mina **Annor och Tove** för ovärderlig vänskap och uppmuntran under åren.

Tack familjerna **Sandelius/Gabrielsson** för er omtanke och stöd och ert intresse för mitt arbete.

Min kära familj, **Ingrid** och **Bengt,** för att ni alltid ställer upp och all barnpassning senaste månaderna.. mina systrar med familjer **Malin, Fredrik, Olivia** och **Anton, Camilla, Jan, Linus, Simon, Filip** och **Vilgot,** för att ni alltid stöttar och uppmuntrar mig och ger mitt liv utanför jobbet större mening.

Tack min underbara **Martin**, för att du tror på mig och alltid får mig på bra humör även i de allra jobbigaste stunder, för att du gett mig perspektiv ♥, och världens bästa, vackraste och smartaste barn, **Arvid Persson**! Tack för att ni stått ut med mig och mina arbetstider senaste året. Älskar er!

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