Academic Dissertation

Neurogenesis from Neural Stem Cells, Ependymal Cells and Fibroblasts

Karthikeyan Devaraju

2014

With the approval of the Faculty of Medicine, Lund University, Sweden, this thesis will be defended on March 21, 2014 at 09.00 in Segerfalksalen, Wallenberg Neurocentrum, Lund, Sweden.

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Stroke is a major cause of death and disability around the world. Stroke leads to loss of neurons and also other cells in the brain due to lack of blood supply. Currently no therapies are available to treat stroke-related disability. It has been shown that stroke leads to increased neurogenesis, birth of new neurons, within the brain. This increased neurogenesis is not sufficient to restore lost function. There is a need to develop therapies for neuronal replacement by improving neurogenesis within the brain and/or transplanting neurons. Cortical strokes lead to more disability after stroke as compared to those affecting the striatum, and whether cortical neurogenesis occurs after stroke is controversial. Cell transplantation may be the key to cortical repair after stroke.

Reports have identified positive but very few negative regulators of neurogenesis after stroke, and suggested that ependymal cells can also contribute to stroke-induced neurogenesis. Transplantation of neurons generated from different sources such as fetal brain, embryonic stem cells and induced pluripotent stem cells are associated with ethical issues and carry the risk of immune rejection and tumorigenicity. Direct conversion of patient’s own skin cells to neurons could overcome these problems and potentially restore function after transplantation in stroke-damaged brain.

In this thesis we have used transgenic models, viral vectors, electroporation-mediated gene delivery and overexpression of transcription factors to demonstrate neurogenesis from neural stem cells, ependymal cells in the lateral ventricular wall and fibroblasts.

We show that Lnk, a known inhibitor of hematopoietic stem cell self-renewal, is also expressed in the brain. Overexpression or removal of Lnk expression leads to decreased or increased neurogenesis in vitro respectively. When brain is damaged by stroke there is increased proliferation of neural stem cells in animals without Lnk expression. This was not observed in status epilepticus, a severe form of epilepsy. We determined that upregulation of STAT1/3 after stroke leads to increased Lnk expression. Subsequently Lnk inhibits cellular response to increased IGF1 stimulation after stroke, by decreasing Akt phosphorylation. We have identified LNK signaling as a novel mechanism of influencing neurogenic response to stroke.

We next determined if ependymal cells in lateral ventricular wall of adult rat brain contribute to neurogenesis after stroke. We identified FoxJ1 as a marker of ependymal cells in rats similar to mice, and used FoxJ1 promoter in piggyBac system to genetically label these cells with fluorescent reporter proteins GFP or RFP by electroporation. Tracing the lineage of the labeled cells in intact and stroke-damaged brain, we identified that FoxJ1 expressing cells contribute to olfactory bulb neurogenesis while the striatal neurogenic response was not significant. Thus, FoxJ1 expressing cells probably have only a minor role in repair after stroke.

We then tested whether human fetal lung fibroblasts could be directly converted to cortical neurons. We overexpressed sets of transcription factors that are known to be involved in cortical neuron development. We found that overexpression of different sets of these factors in fibroblasts converted them to cortical-like neurons. These neurons expressed markers of cortical neurons and were functional by electrophysiology.

In summary, these results raise the possibility that inhibition of Lnk, a negative regulator of neurogenesis from the brain’s own neural stem cells, and intracortical transplantation of cortical neurons directly converted from fibroblasts could be developed into novel therapeutic strategies for stroke in the future.
Neurogenesis from Neural Stem Cells, Ependymal Cells and Fibroblasts

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2014
The image shows the delaminated and migrating ependymal cells from the lateral ventricular wall in adult rat brain, within the stroke-damaged striatum. The cells express FoxJ1 indicated by the presence of green and red fluorescent proteins while they do not immunostain for BrdU (blue).
To my wonderful family

“In a day, when you don’t come across any problems – you can be sure that you are travelling in a wrong path”

- Swami Vivekananda
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This thesis is based on the following papers and manuscripts


SUMMARY

Stroke is a major cause of death and disability around the world. Stroke leads to loss of neurons and also other cells in the brain due to lack of blood supply. Currently no therapies are available to treat stroke-related disability. It has been shown that stroke leads to increased neurogenesis, birth of new neurons, within the brain. This increased neurogenesis is not sufficient to restore lost function. There is a need to develop therapies for neuronal replacement by improving neurogenesis within the brain and / or transplanting neurons. Cortical strokes lead to more disability after stroke as compared to those affecting the striatum, and whether cortical neurogenesis occurs after stroke is controversial. Cell transplantation may be the key to cortical repair after stroke.

Reports have identified positive but very few negative regulators of neurogenesis after stroke, and suggested that ependymal cells can also contribute to stroke-induced neurogenesis. Transplantation of neurons generated from different sources such as fetal brain, embryonic stem cells and induced pluripotent stem cells are associated with ethical issues and carry the risk of immune rejection and tumorigenicity. Direct conversion of patient’s own skin cells to neurons could overcome these problems and potentially restore function after transplantation in stroke-damaged brain.

In this thesis we have used transgenic models, viral vectors, electroporation-mediated gene delivery and overexpression of transcription factors to demonstrate neurogenesis from neural stem cells, ependymal cells in the lateral ventricular wall and fibroblasts.

We show that Lnk, a known inhibitor of hematopoietic stem cell self-renewal, is also expressed in the brain. Overexpression or removal of Lnk expression leads to decreased or increased neurogenesis in vitro respectively. When brain is damaged by stroke there is increased proliferation of neural stem cells in animals without Lnk expression. This was not observed in status epilepticus, a severe form of epilepsy. We determined that upregulation of Stat1/3 after stroke leads to increased Lnk expression. Subsequently Lnk inhibits cellular response to increased IGF1 stimulation after stroke, by decreasing Akt phosphorylation. We have identified Lnk signaling as a novel mechanism of influencing neurogenic response to stroke.

We next determined if ependymal cells in lateral ventricular wall of adult rat brain contribute to neurogenesis after stroke. We identified FoxJ1 as a marker of ependymal cells in rats similar to mice, and used FoxJ1 promoter in piggyBac system to genetically label these cells with fluorescent reporter proteins GFP or RFP by electroporation. Tracing the lineage of the labeled cells in intact and stroke-damaged brain, we identified that FoxJ1 expressing cells contribute to olfactory bulb neurogenesis while the striatal neurogenic response was not significant. Thus, FoxJ1 expressing cells probably have only a minor role in repair after stroke.

We then tested whether human fetal lung fibroblasts could be directly converted to cortical neurons. We overexpressed sets of transcription factors that are known to be involved in cortical neuron development. We found that overexpression of different sets of these factors in fibroblasts converted them to cortical-like neurons. These neurons expressed markers of cortical neurons and were functional by electrophysiology.
In summary, these results raise the possibility that inhibition of Lnk, a negative regulator of neurogenesis from the brain's own neural stem cells, and intracortical transplantation of cortical neurons directly converted from fibroblasts could be developed into novel therapeutic strategies for stroke in the future.
SVENSK SAMMANFATTNING


Tidigare studier har identifierat många positiva men mycket få negativa regulatorer av nybildning av nervceller efter stroke. Det har också föreslagits att ependymala celler kan bidra till stroke-inducerad neurogenes.

Nervceller till transplantation kan genereras från olika källor såsom fostrets hjärna, embryonala stamceller och inducerade pluripotenta stamceller. Dessa är dock förknippade med etiskt svåra frågor och har risker associerade med avstötning och uppkomst av cancer. Direkt omvandling av patientens egna hudceller till nervceller, och använda dessa till transplantation, skulle kunna övervinna dessa problem och potentiellt återställa funktionen i stroke-skadad hjärna.

I denna avhandling har vi använt transgna modeller, virala vektorer, elektroporation-medierad genleverans och överuttryck av transkriptionsfaktorer för att påvisa neurogenes från neurala stamceller, ependymalceller och fibroblaster.


Vi undersökte sedan hurvida ependymalceller i ventrikelsväggen på vuxna råttor bidrar till neurogenes efter stroke. Vi identifierade FoxJ1 som en markör av ependymala celler i råttor. Vi använde sedan FoxJ1 promotorn och piggyBac systemet för att genetiskt märka dessa celler med fluorescerande reporterproteiner, GFP eller RFP, genom elektroporering. Genom att följa de märkta cellerna i intakt och strokeskadad hjärna, identifierade vi att FoxJ1 uttryckande celler bidrar till neurogenes i luktbulberna men väldigt lite i striatum. Således har FoxJ1 uttryckande celler förmodligen bara en mindre roll i reparation av striatum efter stroke.
Vi testade sedan om humana fetala lungfibroblaster kan direkt omvandlas till kortikala nervceller. Vi överuttryckte olika kombinationer av transkriptionsfaktorer som är kända för att vara inblandade i utvecklingen av kortikala nervceller. Vi upptäckte att överuttryck av olika uppsättningar av dessa faktorer i fibroblaster konverterade dem till kortikal-liknande nervceller. Dessa nervceller uttryckte markörer för kortikala nervceller och visades vara funktionella med hjälp av elektrofysiologi.

Sammanfattningsvis, visar dessa resultat, att hämning av Lnk, en negativ regulator av neurogenes från hjärnans egna neurala stamceller, och intrakortikal transplantation av kortikala nervceller direkt omvandlade från fibroblaster kan utvecklas till nya terapeutiska strategier för stroke i framtiden.
கன்னது பணித்திட்டான்

லகணுக்குரிய சிக்கல்கள் இளவுக்குறிக்கும் பக்தாகம் நூற்றாண்டுகள் காண்பதற்காக இயங்குதலையில் குறிப்பிட்டுகிறது. முதலில் கி.நூற்றாண்டு குறிப்பிட்டது, பின்னர் கி.மூன்றாம் நூற்றாண்டு குறிப்பிட்டது. ஓரும் முறையாக தொடர்ந்து கி.நப்பாலும் கி.முப் பக்தாகம் குறிப்பிட்டது. இன்றைய காலத்தில் இந்த குறிப்பிட்டுக்குரிய பக்தாகம் கி.மு. பிறகு கி.மு. குறிப்பிட்டது. இது மட்டுமல்லே இக்கூற்றின் வல்லைசநிற நிலப்பகுதியில் கி.நப்பால் கி.முறையாக தொடர்ந்து காணப்படுகிறது.

பக்தாகம் குறிப்பிட்டு பெருமளவான பல்வேறு செயற்கைகள் இன்றைய குறிப்பிட்டுக்குரிய பக்தாகம் குறிப்பிட்டது. பல்வேறு செயற்கைகள் கருத்துக்குறிப்பிட்டுக்குரிய பக்தாகம் குறிப்பிட்டது. மேலும் செயற்கைகள் கருத்துக்குறிப்பிட்டுக்குரிய பக்தாகம் குறிப்பிட்டது. இன்றைய காலத்தில் பல்வேறு செயற்கைகள் இன்றைய குறிப்பிட்டு குறிப்பிட்டது. பல்வேறு செயற்கைகள் இன்றைய குறிப்பிட்டு குறிப்பிட்டது. இன்றைய காலத்தில் பல்வேறு செயற்கைகள் இன்றைய குறிப்பிட்டு குறிப்பிட்டது. இன்றைய காலத்தில் பல்வேறு செயற்கைகள் இன்றைய குறிப்பிட்டு குறிப்பிட்டது. இன்றைய காலத்தில் பல்வேறு செயற்கைகள் இன்றைய குறிப்பிட்டு குறிப்பிட்டது.
பதிகியில் பட்டப்பதிகியில் அதிகாரத்துடன் நடந்ததுக் குறிப்பிட்டும்.

சோட்டிகள் பட்டப்பதிகியால் பட்டப்பதிகியாக FoxJ1 அறிவல்
க்காண்பதால், பிந்தைவு பற்றுத்தென நிலைக்கும் போது விளக்கக் குறிப்பிட்டு
க்காண்பதற்கு பதிகியில் குறிப்பிட்டு குறிப்பிட்டு பதிகியில் முழுநிலைக்கு பாதிக்கிறது. பாதிக்கிறது பதிகியில் முழுநிலைக்கு பாதிக்கிறது பாதிக்கிறது பாதிக்கிறது பாதிக்கிறது
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பதிகியில் குறிப்பிட்டு பின்னர் பாதிக்கிறது பதிகியில் குறிப்பிட்டு
குறிப்பிட்டு பாதிக்கிறது பாதிக்கிறது பாதிக்கிறது பாதிக்கிறது பாதிக்கிறது
பாதிக்கிறது பாதிக்கிறது.

ஆனால், சோட்டிகளில் பட்டப்பதிகியில் முழுநிலைக்கு பாதிக்கிறது.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABM</td>
<td>Ascl1, Brn2 and Myt1l</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromo deoxy-Uridine</td>
</tr>
<tr>
<td>CA</td>
<td>Constitutively Active</td>
</tr>
<tr>
<td>CCA</td>
<td>Common Carotid Artery</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immuno Precipitation</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant Negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo Nucleic Acid</td>
</tr>
<tr>
<td>dpi</td>
<td>days post induction</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>GABA</td>
<td>γ Amino Butyric Acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HEFL2</td>
<td>Human Fetal Lung Fibroblasts</td>
</tr>
<tr>
<td>ICA</td>
<td>Internal carotid artery</td>
</tr>
<tr>
<td>iCtx</td>
<td>induced Cortical neurons</td>
</tr>
<tr>
<td>iDA</td>
<td>induced Dopaminergic neurons</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like Growth Factor 1</td>
</tr>
<tr>
<td>iMN</td>
<td>induced Motor Neurons</td>
</tr>
<tr>
<td>iN</td>
<td>induced Neurons</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>LVW</td>
<td>Lateral Ventricular Wall</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle Cerebral Artery Occlusion</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle Cerebral Artery</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal Nuclei / Fox3a</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NSPC</td>
<td>Neural stem and progenitor cells</td>
</tr>
<tr>
<td>OB</td>
<td>Olfactory Bulb</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology</td>
</tr>
<tr>
<td>PTX</td>
<td>Picrotoxin</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>rtPA</td>
<td>Recombinant tissue Plasminogen Activator</td>
</tr>
<tr>
<td>SE</td>
<td>Status Epilepticus</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homolog 2</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>TAP</td>
<td>Transit Amplifying Progenitor cells</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
INTRODUCTION

Neurogenesis

Neurogenesis, birth of neurons, starts from the embryonic stage and continues in adult brain declining with age until death. The precursor of all neurons and macroglia in the central nervous system are the neuroepithelial cells that give rise to radial glial cells. The radial glial cells line the ventricle and extend processes to the pial surface. The radial glial cells expand by symmetric division and then give rise to neural progenitors by asymmetric cell division (Huttner and Brand, 1997). Thus they maintain a pool of radial glial cells while also giving rise to neural progenitors (Gotz and Huttner, 2005). The regional specification of neural precursors into forebrain, midbrain and hindbrain is orchestrated by morphogen gradients and specific gene expression patterns (Sansom and Livesey, 2009; Shimamura and Rubenstein, 1997; Tiberi et al., 2012). The neuronal diversity is determined by specific gene expression patterns. The temporal and spatial expression of genes determines the fate of the neurons, different regions and functions of the brain (Greig, Woodworth et al. 2013).

Adult Neurogenesis

Adult neurogenesis is confined to two regions of the brain, the subventricular zone (SVZ) and the subgranular zone (SGZ). Initial reports of neurogenesis in adult brain were made with findings from H³-Thymidine (Altman and Das, 1965) in hippocampus of adult rats. Later, several reports have clearly shown that neurogenesis from these regions has functional significance (Abrous et al., 2005; Schinder and Gage, 2004). The demonstration that cells grow and self renew in the form of spheres when isolated from SVZ and SGZ has been a hallmark of the functional assay for neural stem cells (Reynolds and Weiss, 1992). Sox2, BLBP, GFAP, CD133, Nestin and Vimentin are markers of neural stem cells, but these are also expressed in other cell types in the brain. So far, there is no unique marker to identify these cells. Partly the problem has been the slow division of cells to give rise to progenitors that rapidly proliferate. These proliferating cells have been identified by BrdU pulse chasing experiments, markers of cell cycle such as p-H3 and Ki-67, and retroviral labeling.

Subventricular zone

The SVZ, present around the lateral ventricles of adult brain, gives rise to new olfactory bulb (OB) neurons. The neural stem cells, probably single ciliated ventricle-contacting astrocytes, reside within the niche consisting of the multiciliated ependymal cells that line the ventricles, proliferating progenitors, migrating neuroblasts and endothelial cells. In mice, the neurogenic SVZ consists of the lateral ventricular wall (LVW) lined by the ependymal cells and ventricle-contacting astrocytes that are arranged in pinwheel architecture (Mirzadeh et al., 2008). The ventricle-contacting astrocytes, express markers of neural stem cells GFAP, Nestin and Vimentin, and are also referred to as type B cells. The B cells give rise to proliferating type C cells that express Mash1, Dlx1 and give rise to doublecortin (DCX) expressing neuroblasts,
referred to as Type A cells. The type A cells migrate along the rostral migratory stream as chains of neuroblasts and end up in the OB becoming interneurons (Alvarez-Buylla et al., 2002; Doetsch, 2003a, b; Doetsch et al., 1999). Depending upon the site of origin within the SVZ they become granule cell or the periglomerular interneurons. They express markers such as calretinin and calbindin while a few of them also express tyrosine hydroxylase and Tbr1/2 (Brill et al., 2009; Ihrie and Álvarez-Buylla, 2011; Kohwi et al., 2005; Kohwi et al., 2007). These new neurons are constantly produced to replace the dying neurons in the granule cell layer and the periglomerular region. Species difference between mice, rats and humans have been observed. In rats the type C cells have not been identified (Danilov et al., 2009). The

Figure 1 Subventricular zone niche.
The subventricular zone consists of the multiciliated ependymal cells separating the cerebrospinal fluid containing lateral ventricles from the parenchyma. The ependymal cell layer also have ventricle contacting astrocytes (type B cells), which have a single cilium. These astrocytes give rise to progenitor cells (type C cells) that proliferate and give rise to migrating neuroblasts (type A cells). These cells are closely associated with blood vessels. Modified from Riquelme et al., (2008).
organization of the SVZ niche in humans is different with a hypocellular gap between the LVW lined by ependymal cells and the astrocytes that give rise to progenitors (Quiñones-Hinojosa et al., 2006). The type C cells have not been identified and there is absence of chain migration of neuroblasts.

**Subgranular zone**

The SGZ of the dentate gyrus in the hippocampus is the other neurogenic region in the adult brain. The GFAP+ neural stem cells give rise to proliferating intermediate progenitors. These intermediate progenitors give rise to DCX+ neuroblasts that integrate into the granule cell layer of the dentate gyrus. These immature cells integrate into the network and mature by establishing connections with the adjacent granule cells and interneurons. They send axonal projections to the CA3 area of hippocampus (Abrous et al., 2005; Zhao et al., 2008). The addition of these new neurons plays a major role in learning and memory function (Deng et al., 2010; Gage, 2002). SGZ neurogenesis is increased after brain damage such as stroke and epilepsy (Bengzon et al., 1997; Parent et al., 1997).

**Stroke-induced neurogenesis**

Stroke is a major cerebrovascular disease that leads to disability and mortality. The most common cause of stroke is ischemic stroke, caused by blockage of arteries that supply the brain. Currently there are no therapies for stroke except recombinant tissue plasminogen activator (rtPA) that is used to lyse the clot causing the stroke (Frey, 2005). But, rtPA has a therapeutic window of 3 – 6 hours only in selected patients to be effective (Wardlaw et al., 2012). There is a need to develop therapies towards functional recovery of stroke-affected brain.

Experimentally occluding the blood vessels supplying the brain physically or chemically induces stroke in animal models. The most commonly used model to mimic clinical condition is intraluminal occlusion of the middle cerebral artery (MCAO) (Durukan and Tatlisumak, 2007; Iosif et al., 2008; Kokaia et al., 1998). Depending on the duration of occlusion the damage extends from striatum only to involving both the cortex and striatum. Occlusion of the distal branch of the MCA induces cortical lesion. Stroke has been shown to induce increased neurogenesis in the SVZ and migration of these neuroblasts towards the peri-infarct region in rodents and humans (Arvidsson et al., 2002; Jin et al., 2006; Martí-Fàbregas et al., 2010; Parent et al., 2002; Thored et al., 2006). The increased proliferation and migration of these neuroblasts is influenced by many factors including IGF1 (Thored et al., 2009), glial cell-derived neurotrophic factor (Kobayashi et al., 2006), vascular endothelial growth factor (Wang et al., 2007), erythropoietin (Tsai et al., 2006) and notch signaling (Androutsellis-Theotokis et al., 2006; Carlen et al., 2009). The migrating neuroblasts integrate into the stroke-damaged brain and become mature neurons expressing markers such as NeuN (Arvidsson et al., 2002; Parent et al., 2002). Only a few of these neuroblasts survive and become functional neurons that also project to distant targets (Arvidsson et al., 2002; Sun et al., 2012b). It has been shown that neurogenic response is necessary for functional recovery and reducing the stroke-induced
tissue loss (Sun et al., 2012a). The increased neurogenic response lasts for two weeks and returns to baseline levels (Thored et al., 2006). Only a few of the migrating neuroblasts survive and mature as neurons (Arvidsson et al., 2002; Parent et al., 2002). Due to this functional recovery is never complete due to the less number of neurons replacing the lost neurons. Thus there is a need to enhance the generation, survival, migration, differentiation and functional integration of these newborn neurons to restore function after stroke. Most importantly the restoration of the phenotype of the lost neurons is critical to restore function. Even a minor improvement in function will enhance the recovery in stroke patients.

**Lnk in Neurogenesis**

The increased neurogenic response after stroke persists for two weeks and returns to basal levels (Arvidsson et al., 2002; Parent et al., 2002; Thored et al., 2006). There is still striatal neurogenesis and migration towards the stroke-damaged brain for few months after stroke. Most of the studies have shown factors that enhance neurogenesis, but factors that reduce the neurogenic response also need attention. It has been shown that TNF-α is an inhibitor of neurogenesis after stroke, but there might be other factors inhibiting neurogenesis (Iosif et al., 2008). Interestingly, in hematopoietic system, Lnk has been shown to inhibit proliferation and expansion of hematopoietic stem cells. Lack of Lnk has been shown to increase the number of hematopoietic stem cells and also B-cell genesis (Buza-Vidas et al., 2006; Takaki et al., 2000). It has been implicated in regulating neovascularization by inhibiting endothelial progenitor proliferation (Kamei et al., 2010; Kwon et al., 2009). The role of Lnk in brain has not been elucidated and its expression in brain is unknown.

Lnk, also known as SH2B3, is an adapter protein belonging to the SH2B adaptor family of proteins. These proteins are involved in signaling by growth factor and cytokine receptors. SH2B3 is conserved across humans, chimpanzee, rhesus monkey, dog, cow, mouse, chicken and zebrafish. It contains Src Homology 2 (SH2) and pleckstrin homology (PH) domains along with a phenylalanine zipper. The SH2 domain helps to bind to phosphorylated tyrosine residues on other proteins and gives rise to their involvement in signal transduction of receptor tyrosine kinase pathways. The PH domain allows them to bind to phosphotidyl inositolts, protein kinase C and βγ subunits of G-proteins, which are involved in intracellular signaling. The phenylalanine zipper allows the proteins to dimerize. Lnk is involved in c-Kit and erythropoietin (EPO) signaling pathways (Tong et al., 2005; Velazquez et al., 2002), which have been shown to influence NSPC proliferation, survival, migration and differentiation (Sun et al., 2004; Tsai et al., 2006). Whether Lnk is expressed in the brain and if expressed, what is its role in neurogenesis in intact and stroke-damaged brain is not known. Loss or gain of function studies with Lnk would help us to identify whether Lnk is a modulator of stroke-induced neurogenesis.

**Ependymal cells and FoxJ1**

The ependymal cells are multiciliated and are an important component of the SVZ niche. They help to maintain the cerebrospinal fluid flow in the ventricles and also maintain a gradient of factors such as BMP and Noggin that allows the migration of neuroblasts to the olfactory
bulb (Sawamoto et al., 2006). Disruption of ependymal cells and the layer integrity leads to hydrocephalus and impaired neurogenesis (Jimenez et al., 2009). Ependymal cells have also been shown to be neural stem cells (Chiasson et al., 1999; Johansson et al., 1999) but their contribution to neurogenesis has been controversial. Studies have shown that ependymal cells also give rise to OB and striatal neurogenesis after stroke in a notch dependent manner (Carlen et al., 2009). In mice the ependymal cell derived cells become gliogenic differentiating to astrocytes or oligodendrocytes. In rats displaced ependymal cells have been observed after stroke but not much is known about their fate (Danilov et al., 2012). Interestingly in intact human brain displaced ependymal cells are observed in the astrocytic ribbon layer of the SVZ niche (Quiñones-Hinojosa et al., 2006). Thus it is possible that these cells can improve neurogenic response and survival of the migrating neuroblasts into the peri-infarct region. Ependymal cells express multiple markers such as Vimentin, S100β, β-Catenin clusters, CD24, CD133 and LRP2. But these markers are not unique to ependymal cells as these are also expressed by astrocytes and neural stem cells. To study these ependymal cells genetically a unique marker is essential.

FoxJ1 has been shown as a marker of ependymal cells in mice and is necessary for development of ependymal cells from radial glia (Jacquet et al., 2009). FoxJ1 is a marker of ciliated cells in the body (Ostrowski et al., 2003) and is conserved across many species including chimpanzee, Rhesus monkey, dog, cow, mouse, chicken, and zebrafish. It belongs to the forkhead family of proteins and regulates the transcription of genes involved in motile ciliary assembly. Studies have shown that prenatal and early postnatal OB neurogenesis can happen from FoxJ1+ cells (Jacquet et al., 2011). Using the FoxJ1-cre mice it has been shown that the FoxJ1+ ependymal cells contribute to striatal neurogenesis after stroke (Carlen et al., 2009). The stroke lesion size and neurogenic response are robust in rats compared to mice models. Since lesion size determines neurogenic response, the variability of lesion size in mice leads to varied neurogenic response within the same experimental setup. Thus it is even more important to study the neurogenic response and mechanisms in rats. The lack of transgenic rats is a huge drawback and hence there is a necessity to use both mice and rats to study stroke therapies. Whether FoxJ1 is expressed in rats is not known and if expressed whether it could be used to study ependymal cells after stroke needs to be studied. Genetic labeling experiments using FoxJ1 could help us to study rat ependymal cell response after stroke.

Cortical neuron replacement

Cortical damage causes major disability compared to striatal damage following stroke in humans (Delavaran et al., 2013). Thus restoration of cortical function is important to functional recovery. There have been conflicting reports about cortical neurogenesis in stroke-damaged brain. The presence of neuroblasts in stroke damaged cortex was not observed in some reports (Arvidsson et al., 2002; Parent et al., 2002). On the other hand, there are reports showing that SVZ derived neuroblasts migrate to the stroke-damaged cortex (Jiang et al., 2001; Kreuzberg et al., 2010; Ziv et al., 2007). The subpial cortical layer I progenitor cells were shown to proliferate and give rise to GABAergic neurons after stroke (Ohira et al., 2010). These variations can be
attributed to factors including stroke model, infusion of growth factors and extent of lesion. Given the complexity of the cortex with multiple neuronal phenotypes and connections this endogenous neurogenic response may not be sufficient for neuronal replacement after stroke. Replacement strategies by cell transplantation remain the viable option for replacing the lost cortical neurons. Fetal brain tissue transplantation in humans restores function in Parkinson’s disease (Lindvall et al., 1990). Transplantation of cells derived from embryonic stem cells (ESC) (Espuny-Camacho et al., 2013) show that they follow development patterns and establish proper connections in mouse brain. Transplantation of cortically fated induced pluripotent stem cells (iPSC) have been shown to restore function after stroke (Tornero et al., 2013). Fetal brain tissue derived cells have ethical problems and are a limited source of transplantable cells. ESC and iPSC carry the risk of tumorigenicity from undifferentiated pluripotent cells. Direct conversion of fibroblasts to neurons is a viable alternative to the ESC and iPSC derived neural transplants. Similar to iPSC technology (Takahashi and Yamanaka, 2006), fibroblasts can be converted to functional neurons without going through pluripotent state, by using a defined set of transcription factors (Vierbuchen et al., 2010). The greatest challenge is to derive the different subtypes of neurons present in the six-layered cortex.

Cortical neurons

The cortex is divided into six distinct layers, layers I - VI, dorsoventrally in rodents and superoinferiorly in humans, based on the morphology, function and gene expression pattern. The cortex is further divided into distinct functional areas, each having different combinations of transcription factor expression. The functional or regional specification within the cortex is determined by gene expression patterns and adhesion molecules expressed (Greig et al., 2013; Srinivasan et al., 2012). For example Bhlhb5 and Lmo4 transcription factor expression along with cadherin-8 (Cdh8) determine the boundary between sensory and motor areas (Azim et al., 2009; Cederquist et al., 2013; Greig et al., 2013). The projection neurons in the different layers project to the thalamus (layer VI), hindbrain and spinal cord (layer V) and to inter- or intra-hemispheric projections (layer IV). Functional recovery in damaged cortex requires restoration of the different layers and their connections in a given region of the brain. This requires that the cells transplanted have the necessary molecular and phenotypic signatures as observed in cortical neurons. Understanding the cortical development and determining the key molecules involved in this process could help us to develop transplantable cells. Cortical development could be divided into three phases, 1) the development of specific layers, 2) the development of different functional regions within the cortex and 3) the molecular mechanisms of how these neurons find their targets and maintain these connections.

The first step in developing layer-specific neurons would be the identification of key molecules that determine cortical neuron specificity. Fezf2 (Arlotta et al., 2005; Rouaux and Arlotta, 2010), Ctip2 (Chen et al., 2008), Satb2 (Alcamo et al., 2008), Tbr1 (Hevner et al., 2001) and Cux2 (Franco et al., 2012; Molyneaux et al., 2009) have been identified as critical molecules for cortical layer specification. Whether the expression of these molecules in fibroblasts will convert them to cortical neurons is not yet explored.
Figure 2. Laminar organization and functional areas of the rodent cortex.

The representative figure illustrates the complexity of the cortex in an adult rodent brain. The different functionally distinct areas, namely auditory (A), motor (M), somatosensory (S) and visual (V) areas in the cortex are depicted on the left hemisphere along the rostro-caudal axis of the brain. The coronal section on the right hand side shows the laminar organization of the cortex into six layers along with their known projections. The different neuron phenotypes mostly found in these layers along with their characteristic molecular markers are depicted. The other neuronal subtypes and cells are not shown. Adapted from Greig et al. (2013) and Molyneaux et al. (2007).

Direct conversion to functional neurons

Direct conversion of somatic cells to neurons, called induced neurons (iN), has been shown in mouse and human cells (Marro et al., 2011; Pang et al., 2011; Pfisterer et al., 2011b; Vierbuchen et al., 2010). The overexpression of proneural genes such as Ascl1 or Ngn2 along with other transcription factors (Ladewig et al., 2012; Vierbuchen et al., 2010) in somatic cells gives rise to iN cells. Subtype-specific neurons such as dopaminergic neurons (iDA) (Caiazzo et al., 2011; Liu et al., 2012; Pfisterer et al., 2011a) and spinal motor neurons (iMN) (Son et al., 2011) have been demonstrated. These cells were derived by expression of proneural genes along with subtype-specific transcription factors. Transplantation studies have shown that these neurons survive in the host brain (Torper et al., 2013) and reverse the functional deficits in a mouse model of Parkinson’s disease (Kim et al., 2011). Since this is an emerging technology, their safety and efficacy to restore function in brain damage needs to be analyzed in detail. Even though these studies demonstrated the feasibility of deriving mixed population of neurons (Vierbuchen et al., 2010) or subtype-specific neurons, the number of neurons derived or neuronal conversion
efficiency from fibroblasts is less. To be useful for disease modeling and clinical transplantation studies, more neuronal cells have to be derived. Using synergistic SMAD (Mothers against decapentaplegic homolog) inhibition, the conversion efficiency has been enhanced manifold (Ladewig et al., 2012). Thus, a highly enriched population of neurons can be derived from somatic cells in mice and humans. The possibility of reprogramming fibroblasts to cortical neurons would open up options for transplantation studies in stroke. Whether combining the proneural factors with cortical layer specific transcription factors would give rise to cortical neurons remains to be explored.
AIMS OF THE THESIS

The main objective of the thesis has been to explore different approaches for neuronal replacement in the stroke-damaged brain. The main strategies were to investigate the neurogenic response from endogenous neural stem cells and ependymal cells after stroke, and direct conversion of fibroblasts to neurons. The specific aims of the thesis have been:

I. To investigate the role of Lnk in adult neurogenesis from subventricular zone after stroke (paper I)

II. To investigate the role of ependymal cells in lateral ventricular wall in adult neurogenesis after stroke (paper II)

III. To derive cortical neurons by direct conversion of human fetal lung fibroblasts for transplantation after stroke (paper III)
RESULTS AND DISCUSSION

Adaptor protein Lnk is a negative regulator of brain neural stem cell proliferation after stroke (paper I)

Adult neurogenesis is modulated by pathological conditions such as stroke and epilepsy. SVZ neurogenesis increases for the first two weeks after stroke and plateaus later. The mechanisms behind the decrease in neurogenesis are not fully understood. Cytokines such as C-KIT and EPO are potent modulators of NSPC after stroke. Lnk, an adaptor protein, negatively regulates cytokine pathways including C-KIT and EPO. The role of Lnk in normal and pathological brain has not been elucidated.

Lnk expression in brain and influence on neural stem cells

Lnk belongs to adapter protein family that has SH2 and PH domains with a C-terminal tyrosine phosphorylation site. The expression and role of Lnk in hematopoietic system has been delineated. So far it has been detected in embryonic rat cortex (Wang et al., 2011), but the presence and function in human or mouse SVZ has not been determined. We detected Lnk expression in mouse and human SVZ tissue and also in neurospheres derived from mouse SVZ and human fetal ganglionic eminences. The expression was observed in SOX2+, DCX+ and IBA1+ cells of SVZ but also in other cell types that did not express these markers. We then used the Lnk⁻/⁻ mice and could not detect abnormalities in the gross structure and fine cytoarchitecture of brain. We grew neurospheres from SVZ of Lnk⁻/⁻ mice and observed that over subsequent passages, the numbers of proliferating cells were higher compared to Wt mice. Data from BrdU flow cytometry showed that Lnk⁻/⁻ cells proliferate faster by shortening G0/G1 phase. We then overexpressed Lnk in neurospheres from Wt mice and found the size of the Lnk-overexpressing neurospheres was dramatically reduced along with more TUNEL+ cells. These results clearly showed that Lnk is an inhibitor of NSPC proliferation.

Lnk negatively regulates stroke-induced proliferation of NSPCs

Having found that Lnk inhibits NSPC proliferation in vitro, we then determined if Lnk inhibits NSPC proliferation in vivo. We used BrdU pulse chase analysis coupled with p-H3 immnufluorescence in Lnk⁻/⁻ and Wt mice. We could not detect any differences in the p-H3+ and BrdU+ population in SVZ of these mice, indicating that Lnk has no influence on NSPC proliferation in SVZ of intact mice. Having shown that stroke induces transient increase in NSPC proliferation in SVZ (Arvidsson et al., 2002; Thored et al., 2006), we decided to check the role of LNK in stroke-induced neurogenesis. We compared the NSPC proliferation and survival of newly formed cells 7 days after stroke in Lnk⁻/⁻ and Wt mice by p-H3 and BrdU staining. We found p-H3+ and BrdU+ cells were increased in Lnk⁻/⁻ SVZ compared to Wt mice. We also found significant increase in Sox2+/BrdU+ cells and an increase in DCX+/BrdU+ cells, while no increased proliferation of CD31+ endothelial cells, Iba1+ microglial or FoxJ1+ cells were observed. The stroke lesion size was not different between the Lnk⁻/⁻ and Wt mice, since lesion size also determines neurogenic response. We then determined if the increased NSPC
proliferation is confined to SVZ or also to SGZ. SVZ neurogenesis is also increased after status epilepticus (SE), a severe model of epilepsy that mimics the condition seen in humans. We induced SE in Lnk−/− and Wt mice and analyzed the SVZ for NSPC proliferation. We did not detect any differences in SVZ cell proliferation between Lnk−/− and Wt mice. We then compared the SGZ cell proliferation in the stroke and SE lesioned brains of Lnk−/− and Wt mice. We found increased cell proliferation in SGZ of Lnk−/− mice but no differences were observed between stroke and SE lesions. We did not find any differences in the numbers of Iba1+ microglia in the intact, stroke or SE lesioned brains of Lnk−/− and Wt mice. This ruled out the possibility of Lnk suppressing NSPC proliferation by microglial activation.

**Upstream regulators of Lnk and downstream effectors of Lnk after stroke**

To determine the influence of stroke on Lnk upregulation, we checked the Lnk expression levels in intact, stroke- and SE-lesioned mice brain by qPCR. Lnk expression was upregulated after stroke while downregulated after SE. We analysed the Lnk promoter region for transcription factor binding motifs and found SP1, E2F1, Stat1 and Stat3 binding sites. We detected by qPCR that Stat1 and Stat3 were upregulated after stroke, indicating that Stat1 or Stat3 could influence Lnk upregulation after stroke. We transfected CA-Stat1 and DN-Stat1 plasmids to Wt SVZ derived neurospheres and found increased Lnk expression in CA-Stat1 transfected cells. By ChIP analysis with Stat1 antibody, we found that Stat1 was specifically binding to first exon of Lnk. Stat1 and Stat3 work in synergy, hence we cloned the Lnk genomic sequence including the Stat1 and Stat3 binding region to a luciferase reporter plasmid. We also mutated the Stat1 and Stat3 binding sites of the above-mentioned Lnk sequence. These plasmids were transiently expressed in 3T3 and Stat1-deficient U3A cells and also separately co-transfected CA-Stat1 and CA-Stat3 plasmids. In the presence of CA-Stat1 and CA-Stat3 plasmids, we found increased luciferase activity in Lnk sequence while the mutated Lnk sequence had decreased luciferase activity. These findings showed that Stat1 and Stat3 regulate increased Lnk expression after stroke.

To identify mechanisms through which Lnk might suppress stroke-induced NSPC proliferation, we first investigated using qPCR the expression of known and potential Lnk targets in SVZ tissue from intact mice and at 1 week after stroke or SE. Gene expression of Epo, FgfR1, FgfR2, Egfr, Fgf2 and Igf1 were upregulated only after stroke and not in SE. Based on the specific alterations of gene expression induced by stroke, we hypothesized that Lnk may act on the EPO, EGF, FGF and IGF1 signaling pathways.

Lnk is known to inhibit the phosphorylation cascade in receptor tyrosine kinase signaling by direct interaction with receptors (Takaki et al., 2000; Tong et al., 2005) or with downstream signaling (Bersenev et al., 2008). One common signaling pathway of EGF, FGF, and EPO is the phosphorylation of ERK1/2, while IGF1 signaling leads to Akt phosphorylation by PI3K. We analyzed the degree of phosphorylation of ERK1/2 and Akt in neurosphere cells from SVZ of Lnk−/− and Wt mice in response to EGF/FGF, IGF1, and EPO stimulation. We found marked increase of AKT phosphorylation in Lnk−/− neurospheres stimulated with IGF-1 compared with Wt controls. We then transfected CA-Akt or DN-Akt plasmids into the lateral ventricle of Wt mice by in vivo electroporation. 3 days later, animals were injected with BrdU, and SVZ
proliferation was assessed using BrdU and p-H3 immunohistochemistry. Consistent with a regulatory role of AKT on NSPC proliferation, we found that CA-Akt increased while DN-Akt decreased the number of BrdU+ and p-H3+ cells in the transfected SVZ.

The Lnk-induced changes in growth factor signaling could lead to alterations in gene expression and subsequently in cell proliferation. To explore this further, we analyzed the expression of several genes that have been implicated in NSPC proliferation and maintenance in the Lnk-overexpressing neurosphere cells. We found a significant reduction in Hes5 and Gli1 gene expression.

Together, our findings provide evidence that Lnk inhibits IGF1 signaling by attenuating phosphorylation of AKT, possibly also EGF/FGF signaling via ERK1/2, and decreases gene expression of Hes5 and Gli1, resulting in reduction of stroke-induced NSPC proliferation.

Discussion

We have shown here that Lnk is expressed in adult brain and has a functional role in regulating NSPC proliferation after stroke. Interestingly, NSPC proliferation is regulated in stroke and not in SE. Previous reports in hematopoietic system have shown that Lnk is a negative regulator of HSC self-renewal and proliferation (Buza-Vidas et al., 2006; Seita et al., 2007), and B-cell progenitor proliferation (Takaki et al., 2003). It is also involved in regulation of endothelial cell proliferation (Kamei et al., 2010; Kwon et al., 2009). Mutations in Lnk gene have been observed in myeloproliferative neoplasms (Oh et al., 2010). We did not find increased endothelial cell proliferation or vascularization after stroke in Lnk−/− mice. We found no evidence for altered microglial activation in SVZ of Lnk−/− mice after stroke, while in absence of immune cells or microglia, Lnk overexpression in neurospheres decreased NSPC proliferation. Therefore, observed effect of increased NSPC proliferation in Lnk−/− mice cannot be attributed to effect of other cell types. Since Lnk is expressed in NSPCs, LNK influences NSPC proliferation cell autonomously.

We found that Lnk does not influence NSPC proliferation in intact brain of Wt or Lnk−/− mice in vivo. We observed increased proliferation of neurospheres from Lnk−/− mice compared to Wt mice. This could be due to the abundance of growth factors in the culture conditions similar to what is observed after stroke. We found that Lnk expression increased after stroke and decreased after SE, indicating that it is regulated in brain after pathological insults. We also found increased levels of Stat1 and Stat3 after stroke indicating that they upregulate Lnk after stroke. STAT1 (Takagi et al., 2002) and STAT3 upregulation after stroke is mediated by cytokine release after insult (Liesz et al., 2011; Yamashita et al., 2005). Cytokines such as IL-6 and IFN α and γ are known to activate STAT family members (Takeda and Akira, 2000). Thus STAT1 / 3 might mediate Lnk upregulation after stroke.

The increased levels of FGF, EGF, EPO and IGF1 after stroke but not after SE might be responsible for increased neurogenesis in Lnk−/− mice after stroke. After stroke Igf-1 levels are increased (Thored et al., 2009) and mediate NSPC proliferation (Yan et al., 2006). LNK mediates receptor tyrosine kinase signaling by inhibiting or decreasing phosphorylation of
downstream kinases (Bersenev et al., 2008; Takaki et al., 2000; Tong et al., 2005). We found increased levels of AKT phosphorylation after IGF-1 stimulation and ERK1/2 phosphorylation after EGF and FGF stimulation in Lnk<sup>-</sup>- neurospheres. Similarly, we found increased and decreased NSPC proliferation in SVZ after CA-Akt and DN-Akt electroporation, respectively. Thus, LNK might inhibit ERK1/2 and AKT phosphorylation in response to growth factor signaling. In hematopoietic stem cells and endothelial precursors LNK inhibits AKT and ERK1/2 phosphorylation (Seita et al., 2007; Tong et al., 2005; Wan et al., 2006). The targets of AKT and ERK1/2 are Hes and Gli1 proteins, involved in NSPC proliferation (Cayuso et al., 2006; Gregory et al., 2010; Stecca and Ruiz i Altaba, 2009), which we also observed to be downregulated with Lnk overexpression. Thus Lnk inhibits NSPC proliferation by inhibiting the IGF1-AKT pathway.

**FoxJ1-expressing cells contribute to neurogenesis in forebrain of adult rats: Evidence from in vivo electroporation combined with piggyBac transposon (paper II)**

The SVZ neurogenic niche consists of multiciliated ependymal cells and ventricle contacting astrocytes with single cilium along with the vasculature (Mirzadeh et al., 2008). These single ciliated astrocytes are putative neural stem cells that give rise to the neural progenitors. The ependymal cells considered to be post-mitotic, have been shown to be neurogenic especially after stroke (Carlen et al., 2009). But the fate of these ependymal cell derived neuroblasts has not been determined. Whether the ependymal cells in adult rat brain contribute to stroke induced neurogenesis is not known. In mice FoxJ1 has been shown to be a marker of ependymal cells. Whether FoxJ1 is expressed in rat brain and if they contribute to neurogenesis is not yet known. Genetic labeling studies of FoxJ1-expressing cells in adult rat brain would help us to answer these questions.

**Specific labeling of LVW cells in adult rat brain by electroporation**

The LVW consists of the ependymal cell and ventricle-contacting astrocytes. Labeling of LVW cells has relied upon viral vectors (Johansson et al., 1999) but these vectors label the entire ventricular wall. Electroporation has been shown to effectively label the LVW in adult mouse brain (Barnabe-Heider et al., 2008; Carlen et al., 2009). We attempted to label the LVW in adult rat brain using electroporation. We observed GFP<sup>+</sup> cells in the LVW of adult rat brain and found the method to be safe without causing any damage to the neurogenic niche and neurogenesis. After optimizing the electroporation parameters, we observed efficient labeling of the cells in LVW with 175 V/cm pulse of 50ms duration at 1Hz frequency.

**FoxJ1+ cells in the adult rat brain contribute to olfactory bulb neurogenesis**

FoxJ1 is a key transcription factor in functionally motile cilia development (Ostrowski et al., 2003). It is important for the development of ependymal cells and a subset of astrocytes from radial glia (Jacquet et al., 2009). FoxJ1 has been used as a specific marker of ependymal cells in the adult mouse brain (Carlen et al., 2009; Jacquet et al., 2009). We detected the expression
of FoxJ1 in the LVW of adult rat brain by using FoxJ1 antibody. Next, a Cre-expressing plasmid under FoxJ1 promoter, along with a floxed reporter construct was electroporated. Based on the Cre recombinase system, we identified that FoxJ1 is expressed in ependymal cells and also in a subset of astrocytes lining the LVW.

Figure 3: Electroporation in lateral ventricular wall of adult rodent brain.

Electroporation is a method of delivering DNA to the target cells by applying an electric potential. The plasmid is first injected into the lateral ventricle of brain by stereotaxy. Then the electrodes with conductive gel is applied on the head parallel to the injected site and electroporated. DNA is taken up by cells along the side of cathode due to the transient permeability caused by electroporation. The lateral ventricular wall cells are later analyzed for plasmid expression.
Having established the expression of FoxJ1 in adult rat brain, we needed to establish a stable genetic reporter for lineage tracing of FoxJ1+ cells. We used the piggyBac transposon system (Cadinanos and Bradley, 2007) to genetically label the FoxJ1+ cells and its progeny. The piggyBac transposon, with FoxJ1 promoter based donor (pPB-FoxJ1-eGFP) and helper (pFoxJ1-mPB) plasmids, was electroporated to the LVW of adult rat brain. We also used donor plasmid with RFP reporter (pPB-UbC-DsRed2) along with pFoxJ1-mPB for lineage tracing. We observed expression of both GFP+ and RFP+ cells in the LVW. Most of the GFP+ and RFP+ cells were FoxJ1+, while some of the RFP+ cells were DCX+ and GFAP+. We also found RFP+ and GFP+ cells in the OB of the electroporated brains and most of the cells were NeuN+. We did not observe any disturbances in the LVW and shows that OB neurons would have originated from the LVW cells. These results clearly showed that the piggyBac system is ideal for lineage tracing in adult rat brain. Most importantly we found that FoxJ1+ cells in the LVW of adult rat brain contribute to OB neurogenesis.

Stroke-induced striatal neurogenesis from FoxJ1+ cells

Stroke induced striatal neurogenesis involves recruitment of migrating neuroblasts from the RMS to the stroke-damaged striatum. Since we found FoxJ1+ cells contributed to OB neurogenesis, we electroporated the pFoxJ1-mPB along with pPB-FoxJ1-eGFP and pPB-UbC-DsRed2. One week later, we induced stroke by 30 minutes and 2 hour MCAO models. One week following MCAO, we administered BrdU for seven days to detect proliferating LVW cells. We observed an increased neurogenic response after stroke, but very few GFP+/RFP+ cells were observed in the striatum. We observed both GFP+/RFP+ and GFP-/RFP+ cells with some BrdU+ among the GFP-/RFP+ cells. We could not determine the phenotype of these migrating cells. The OB neurogenesis from labeled cells was intact and was similar when compared to the intact brain.

Taken together our findings show that FoxJ1 expressing cells in the LVW of adult rat brain contribute to olfactory bulb neurogenesis in intact and stroke-damaged brain, while some ependymal cells delaminate and migrate into the stroke-damaged striatum.

Discussion

We have used genetic lineage tracing with electroporation to show that FoxJ1 expressing cells in LVW contribute to neurogenesis in adult rat forebrain.

We show that electroporation is a safe and valuable method to label LVW of adult rat brain similar to what is described mice (Barnabe-Heider et al., 2008). No evidence for damage to the SVZ, rat brain or the rats in general from electroporation was found. Markers used to identify ependymal cells S100β, Vimentin, CD24 and CD133 are also expressed by astrocytes and NSPCs (Järlestedt et al., 2010; Pfenninger et al., 2007; Pruszak et al., 2009). FoxJ1 has been used to identify ependymal cells in mice (Carlen et al., 2009; Jacquet et al., 2011; Jacquet et al., 2009). We also determined by immunostainings and using FoxJ1-Cre (Carlen et al., 2009) and Cre-reporter plasmid electroporation that the ependymal cells express FoxJ1. A small population of astrocytes expressing FoxJ1 is consistent with similar findings in FoxJ1EGFP
mice (Jacquet et al., 2009).

We found long-term expression, 12 weeks in our study, of reporter proteins and also in the progeny of FoxJ1+ cells. We show the first evidence for in vivo labeling in adult brain using piggyBac transposon as an effective genome-targeting tool. piggyBac is known to stably express transgenes in human and rodent genomes (Cadinanos and Bradley, 2007; Ding et al., 2005; Jang and Behringer, 2007; Saridey et al., 2009; VandenDriessche et al., 2009; Wilson et al., 2007). With piggyBac transposon labeling, we were able to detect OB neurons arising from FoxJ1 expressing cells. This has not been detected so far in the rat brain. Previously, FoxJ1+ ependymal cells and their progeny in perinatal period were shown to be necessary for adult OB neurogenesis (Jacquet et al., 2011). FoxJ1+ cells have been shown to give rise to neurospheres (Jacquet et al., 2011) and these could have arisen from FoxJ1+ astrocytes. Since ependymal cells are post-mitotic, it is probable that the OB neurons observed here are from the FoxJ1+/GFAP+ cells. We observed that GFP expression under FoxJ1 promoter was weak in labeled neuroblasts and OB neurons, while RFP expression under FoxJ1 promoter was strong in all labeled populations. These results clearly showed that for effective labeling transposase expression should be under cell specific promoter.

Ependymal cells have previously been shown to give rise to neuroblasts (Carlen et al., 2009) and transform into radial glia after stroke (Zhang et al., 2007). They have also been shown to generate progeny by asymmetric division after 6-hydroxydopamine lesion (Gleason et al., 2008). We performed MCAO one week after electroporation with the GFP and RFP donor plasmids. GFP+/RFP+/BrdU- cells were found in the stroke-damaged striatum 6 weeks after stroke. We also found GFP-/RFP+/BrdU+ neuroblasts-like cells in the striatum but their fate could not be determined. This clearly shows that these cells originate from FoxJ1 expressing cells, while loss of GFP expression shows that FoxJ1 is downregulated after giving rise to progeny. We also observed GFP+/NeuN+ and RFP+/BrdU+ OB neurons after stroke. The GFP+/RFP+/BrdU- cells could be similar to the displaced ependymal cells found in striatum after stroke in rats (Danilov et al., 2012). Thus we show that FoxJ1 expressing cells contribute to OB neurogenesis in intact and stroke-damaged brain, but contribution to striatal repair after stroke in these experimental conditions is limited.

Direct conversion of fibroblasts to functional cortical neurons (paper III)

Reprogramming of mature somatic cells to pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006) and neurons (iN) (Vierbuchen et al., 2010), have opened up possibilities for patient specific transplantation to diseased brain. Most importantly these are also powerful tools to create human disease models in vitro. Many diseases such as neonatal hypoxia, head trauma, stroke and Alzheimer’s disease affect the cortex and cause cortical dysfunction. Cortical neuronal transplants would be useful to restore function in diseased brain. Cells produced by direct conversion probably are not tumorigenic and do not require long-term immunosuppression after transplantation. Thus, cortical neurons from fibroblasts (iCtx) would be an invaluable source for intracerebral transplantation.
Conversion of human fibroblasts to functional pyramidal-shaped neuron-like cells

Cortical projection neurons are glutamatergic and exhibit pyramidal morphology. We decided to use the transcription factors that are critical for development of projection neurons with glutamatergic phenotype. Brn2 (B), Zic1 (Z), Ctip2 (C), Fezf2 (F) and Tbr1 are critical for the formation of cortical projection neurons from layers II - VI. The combination of the transcription factors Ascl1 (A), Brn2 and Myt11 (ABM) has been shown to reprogram mouse fibroblasts to Tbr1+ neurons (Vierbuchen et al., 2010), a marker of layer VI cortical neurons. Brn2 and Zic1, part of transcription factor combinations (BMZ) for iN reprogramming (Vierbuchen et al., 2010) are expressed in layers II – V (Molyneaux et al., 2007). We initially tested whether the combinations ABM or BMZ would give rise to layer V and layer VI neurons alone or in combination with Fezf2 and/or Ctip2. However, we observed increased cell death and hardly any cells survived at 25 days after doxycycline addition.

We continued to search for combinations of transcription factors that could give rise to surviving neurons with pyramidal morphology. Factors critical for glutamatergic phenotype and NeuroD1 (Nd) have been shown in reprogramming studies to be more effective in converting fibroblasts to neurons (Pang et al., 2011). Brn2 and Myt11 have been shown to produce iN cells without Ascl1 in combination with other transcription factors (Vierbuchen et al., 2010). Hence, we included Brn2 and Myt11 along with cortical transcription factors to test if they would give rise to cortical layer-specific neurons. We observed surviving neurons and the combinations were analyzed for neuronal conversion efficiency and cortical projection neuron morphology. qPCR analysis for expression of cortical layer markers revealed that compared to other combinations, maximal expression was observed for Satb2, Cux2, Fezf2 and Tbr1. Combinations with NeuroD1 had more GAD1 expression compared to vGlut1 and vGlut2 expression. Whole-cell patch-clamp recordings revealed that cells from these combinations were able to generate action potentials. Based on the electrophysiological and morphological analysis along with the expression of layer specific markers, we identified BMNgn2, MNgn2 and BMF as the most promising combinations for inducing cortical layer-specific iCtx cells.

Layer- and region-specific functional cortical neurons

We evaluated three combinations in more detail for their ability to generate iCtx cells expressing cortical layer- and region-specific markers. Cultured cells were transduced with SynI-GFP to distinguish neurons i.e. iCtx cells, from fibroblasts. Cells were FACS sorted for GFP expression and qPCR was performed in BioMark HD with 48.48 chip. First combination of transcription factors expressed Bhlhb5, Emx2 and Satb2 maximally while Tbr2, Pax6, Cux2, Ctip2 and Tbr1 were expressed at lower levels. Second combination expressed weakly Emx2, Tbr2, Bhlhb5, Satb2 and Ctip2. Interestingly, third combination expressed Tbr2, Satb2 and Ctip2 at very low levels. We could not detect expression of Fezf2 in any of the conditions. Electrophysiological recordings revealed that most of the iCtx cells from the three combinations exhibited the characteristics of mature neurons. We also found that BMF or BMNgn2 combinations were more responsive to Glutamate and GABA application compared to MNgn2. Taken together, these findings show that the neurons derived from human fetal lung fibroblasts by direct con-
version exhibit the characteristics of mature neurons and express functional glutamate and GABA receptors. By immunostaining we detected that cells reprogrammed with BMNgn2 and BMF were immunopositive for Satb2 and Ctip2 respectively.

Taken together, our findings show that cortical transcription factor combinations can induce cortical projection neuron phenotype from human fetal lung fibroblasts that is layer- and region-specific.

Discussion

We have shown that expression of specific combinations of transcription factor can lead to specific cortical projection neuron subtypes. The cerebral cortex has six functionally distinct layers (layers I to VI) with combination of transcription factor expression. The initial iN study (Vierbuchen et al., 2010) showed that majority of mouse iN cells were Tbr1+ and glutamatergic. Tbr1 is a marker of layer VI neurons (Hevner et al., 2001) and is also expressed by olfactory bulb interneurons (Brill et al., 2009) and cerebellar neurons (Fink et al., 2006). Evaluation of other layer specific markers such as Sox5 (layer VI), Fezf2 (layer V), Ctip2 (layer V), Satb2 (layer IV) and Cux2 (layer II – III) (Greig et al., 2013; Molyneaux et al., 2007) is necessary to confirm if these iN cells are cortical in nature. We did not have surviving neurons with ABM combination to evaluate their cortical phenotype, which is in agreement with other reports (Pang et al., 2011; Qiang et al., 2011).

The iCtx cells expressed layer specific genes Satb2, Cux2, Tbr1 and Fezf2. Interestingly, the iCtx cells also expressed markers of cortical neuron development, Emx2, Pax6 and Tbr2. These molecules are necessary for rostro-caudal patterning in the mouse brain (Bishop et al., 2000; Gulisano et al., 1996; Hamasaki et al., 2004; Muzio and Mallamaci, 2003). They also expressed Bhlhb5 critical for sensory area development in the brain (Joshi et al., 2008). During cortical development, layer VI neurons are formed first followed by layers V, IV, III and II (Greig et al., 2013). It is probable that these fibroblast-derived cells also exhibit patterns observed in cortical development. We have previously shown that iPSC-derived cortically fated neurons express Tbr1 in vitro and also in vivo after transplantation (Tornero et al., 2013). Studies on human ESC-derived cortical neurons (Espuny-Camacho et al., 2013) have also reported that differentiation follows the cortical development pattern. Thus, a complex phenotype such as cortical neurons could be derived by forced expression of sets of transcription factors.
CONCLUDING REMARKS AND PROSPECTS

Stroke is a major disabling disease and till date there are no therapies to enhance the functional recovery. Rehabilitation helps in improving the plastic response within the brain after stroke, but is not sufficient to improve the recovery in many stroke patients. Enhancing endogenous neurogenesis and neuronal replacement by cell transplantation could potentially promote functional recovery after stroke. In this thesis, we have analyzed three different approaches for neuronal replacement after stroke. We have identified a negative regulator of neurogenesis, a new subset of cells that contribute to adult OB neurogenesis and transcription factor combinations that can convert fibroblasts to cortical neurons.

Lnk is an inhibitory regulator of NSPC proliferation after stroke in rodents. Since we observed Lnk expression in human SVZ, it is probable that Lnk also suppresses increased neurogenesis after stroke in humans. Targeting Lnk could potentially be developed into a therapeutic strategy for stroke by increasing neurogenesis. Given its expression in other cells such as microglia, neuroblasts and endothelial cells in the brain, it is probable that Lnk acts upon these cells too. Even though we show that Lnk inhibits post-stroke SVZ neurogenesis by inhibiting the IGF1 – AKT pathway, it is probable that other pathways may also be involved. Recovery in the post-stroke brain involves functional and structural reorganization such as plasticity of surviving neurons, reorganization of vasculature in the peri-infarct region and SVZ, and activation of the immune system. Lnk might also influence these mechanisms. It remains to be explored in more detail how manipulation of Lnk can be used to stimulate post-stroke recovery.

We show that FoxJ1 expressing cells contribute to olfactory bulb neurogenesis in the adult rat brain. FoxJ1 is predominantly a marker of ependymal cells and we also found a subset of astrocytes that express FoxJ1. Rats are useful animal models for many neurodegenerative diseases and electroporation enabled us to trace the lineage of the LVW cells in intact and stroke-damaged brain, thereby overcoming the lack of genetic models. This opens up the possibility to do genetic labeling or ablation studies in rat LVW that was not possible before. We show that the OB neurons arise from the FoxJ1+ cells, but whether these FoxJ1+ cells are a finite source of these OB neurons is not known. It remains to be established whether these OB neurons arise from the FoxJ1+/GFAP+ subset. The functional significance of neurogenesis from FoxJ1+ cells in intact brain is not known. We found that ependymal cells delaminated from the LVW and migrated into the striatum after stroke. How these cells delaminate and what factors stimulate their migration is not known. The contribution to striatal neurogenesis from the FoxJ1+ cells is minor, whether it could be enhanced for functional recovery remains to be studied.

Cortical strokes cause more disability than other types of stroke and we tried to formulate a replacement strategy using patient’s own somatic cells. Cortical neurons expressing Satb2 and Ctip2 were derived from human fetal lung fibroblasts by using defined sets of transcription factors. These cells also express markers for cortical layer and area development. It remains to be explored if these cells would integrate into the intact cortex and also reconstruct the stroke-damaged cortex. Improvement of conversion efficiency of these neurons to achieve
the numbers necessary for transplantation is needed. The peri-infarct region is characterized by glial scar and increased gliogenesis. Whether these astrocytes can be converted into cortical neurons \textit{in situ} by gene delivery, leading to improved functional recovery, also needs to be explored.

To conclude, the results in this thesis raise the possibility that inhibition of Lnk, identified here as a negative regulator of neurogenesis from the brain’s own neural stem cells, and intracortical transplantation of fibroblast-derived cortical neurons could be developed into novel therapeutic strategies for stroke in the future.
EXPERIMENTAL PROCEDURES

Animals

All experimental procedures were approved by the Malmö-Lund Ethical Committee for the use of laboratory animals and were conducted in accordance with European Union directive on the subject of animal rights. Animals were housed under 12 h light/12 h dark cycle with unlimited access to food and water. Wild type and transgenic mice (paper I) as well as adult male Wistar rats (paper II) were used for the thesis.

Experimental models

Stroke

Mice and rats were subjected to experimental stroke by intraluminal filament MCAO model. In mice (paper I), under isoflurane anesthesia, the left external carotid was ligated and temporary sutures were placed around the common and internal carotid arteries. An 8–0 monofilament (Alcon) coated with silicone was advanced through the internal carotid artery until it blocked the blood flow in the MCA. For reperfusion, mice were re-anesthetized after 40 min of occlusion and the filament was removed (Iosif et al., 2008). Rats were subjected to experimental stroke one week after electroporation (paper II), on the electroporated side (ipsilateral side). Briefly, under isoflurane anesthesia, the right common carotid artery (CCA) and its proximal branches were isolated. The CCA and external carotid artery (ECA) were ligated, while the internal carotid artery (ICA) was temporarily occluded using a metal microvessel clip. A nylon monofilament was inserted and advanced through the CCA and ICA until resistance was felt, past the origin of middle cerebral artery (MCA). The nylon filament was carefully removed 30 min or 2 h after the start of occlusion, and ECA was ligated permanently (Kokaia et al., 1998). Both the mice and rats were allowed to awake in between occlusion and reperfusion.

Status Epilepticus

Mice were subjected to experimental epilepsy by induction of SE (paper I), a severe form of epilepsy observed in humans. Mice were anesthetized and implanted with a stimulating/recording electrode (Plastics One) unilaterally into ventral hippocampal CA1–CA3 region. Ten days later, mice were subjected to electrically induced SE. Animals received 1 h of suprathreshold stimulation consisting of 10 s trains of 1ms biphasic square wave pulses at a frequency of 50 Hz. Stimulation was interrupted for 1 min every 10 min to allow for electroencephalographic (EEG) recording and measurement of afterdischarges (MacLab). After ending the stimulation, all mice exhibited self-sustained, continuous ictal activity in EEG, and associated motor behavioral convulsions. Epileptic activity was arrested with pentobarbital at 2 h after stimulation offset (Iosif et al., 2006).
**In vivo electroporation**

Electroporation is a safe method of transfecting plasmids to cells. It is used extensively *in vitro* for transfecting cells in culture and *in utero* for transfecting developing embryo. Recently it has been shown to be effective in transfecting the cells lining the lateral ventricular wall (LVW) of adult brain (Barnabe-Heider et al., 2008). The mechanism behind electroporation is widely accepted to involve transient pore formation in the cell membrane when electric potential is applied across the cell (Neumann et al., 1999). Since the electric current travels from negative electrode to positive electrode, the plasmid DNA molecule, being negatively charged, travels along with the electrical current through the pores in the cell membrane towards the positive electrode. Since the electric potential is applied for a fraction of a second and pores are transient, the plasmid DNA is retained inside the cells. For *in vivo* transfection of LVW and subventricular zone (SVZ) cells, CUY21 EDIT square wave electroporator (NEPA Gene, Japan) with platinum electrodes was used. Plasmids were injected stereotactically into the lateral ventricle of adult mice or rats under isoflurane anesthesia. Immediately after injection, electrodes were applied with electro-conductive gel, the cathode (positive electrode) on the same side as the intraventricular injection (ipsilateral side) and anode (negative electrode) on the opposite side (contralateral side) to complete the circuit. Three to five electrical pulses (100-200 V/cm) of 50 ms duration with 1Hz frequency were applied. Electrodes were then taken off followed by removal of the injection needle. The wound was closed with Histoacryl (Braun, Germany) and animals were allowed to recover. Grooming behavior, food and water intake and signs of burns or scars at the site of electrode application were monitored (*paper I and II*).

**Human tissue**

Human tissue was obtained from Skåne University Hospitals at Lund and Malmö according to the guidelines approved by the Lund-Malmö Ethical Committee. Dead aborted human fetuses 6- to 9-weeks post-conception were obtained from Skåne University hospital, Lund, with informed consent. Ganglionic eminences (GE) (*paper I*) and cortex (*paper III*) were subdissected from brain while the lung tissue (*paper III*) was microdissected, under a stereomicroscope (Leica, Germany) in ice-cold hibernation medium. Anonymized adult human brain samples of SVZ from routine clinical diagnostic autopsy for neuropathological investigations were obtained in accordance with national regulations.

**Dissection and cell culture**

**Neurospheres**

Neurospheres were generated from SVZ of intact 8- to 12-week-old mice (*paper I*). The SVZ was microdissected and single cell suspension was made by enzymatic digestion of the tissue. Human fetal GE was subdissected from the fetal brain, mechanically dissociated and
cultured. For neurosphere formation, cells were grown at clonal density (10 cells/μl) in DMEM/F12 with N2, 2 μg/ml heparin, 10 ng/ml leukemia inhibitory factor (Sigma-Aldrich), and 20 ng/ml epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Differentiation of neurospheres was performed by plating on poly-D-lysine (PDL)/laminin coated coverslips and culturing in medium without growth factors but with N2 and 1% fetal bovine serum (FBS).

**Human fetal lung fibroblasts**

Human fetal lung fibroblasts (henceforth referred to as HEFL2) were isolated from aborted human fetuses 7-9 week post-conception (paper III). After removal of the central nervous system and spinal ganglia, the trachea was exposed and resected at the bifurcation of the mainstem bronchi. The sub-dissected pulmonary tissue was digested with 0.25% trypsin-EDTA (Sigma-Aldrich) at 37°C for 10 minutes and manually triturated to reach a single cell suspension. Cells were plated onto 0.1% gelatin (Sigma-Aldrich) in DMEM 4.5g/L glucose supplemented with 2mM glutamax and 10% FBS (all from Life technologies) and were passaged after reaching confluence with 0.25% trypsin-EDTA. The HEFL2 cell line was used for neuronal differentiation protocols after passage 3 to avoid contamination from neural tissue and other cell types (Pfisterer et al., 2011a; Vierbuchen et al., 2010).

**Astrocytes**

Fetal cortical-derived astrocytes obtained from dead aborted fetuses were plated onto PDL/Laminin (both 10μg/mL; Sigma-Aldrich) coated glass coverslips at a density of 20,000 cells / cm² and maintained in DMEM:F12 (1:1 ratio) supplemented with 10% FBS until co-culturing (paper III).

**Plasmid constructs**

Plasmids were constructed by standard molecular methods, extracted with phenol-chloroform and resuspended in 10mM Tris chloride for all experiments. The promoter sequences for cloning were amplified by PCR from genomic DNA (paper I) or from transgenic targeting plasmid construct (paper II). The Lnk promoter sequence was mutated with Quickchange Lightning Kit (Agilent) (paper I).

**piggyBac transposon**

*piggyBac* transposon was used for genetic labeling of the LVW cells (paper II)(Cadinanos and Bradley, 2007). Transposons are mobile DNA elements, remnants of viral genome. They can integrate or excise genetic sequences within inverted terminal repeats into / from the host genome. The mobility of transposons is conferred by the transposase enzyme that transposes the genetic sequences, within inverted terminal repeats of a helper plasmid or genetic sequence, into the genome. The transposase enzyme can also cleave out the inserted sequence thus having the ability to safely insert and excise a given sequence from the genome (Elick et al., 1996; Elick et al., 1997). This enables genetic labeling of target cells that could be
removed later. Transposons are found in all organisms and have the ability to insert even 100 kb sequence into the host genome. piggyBac system was identified in grasshoppers and later shown to be effective in targeting mammalian genomes including human genome (Jang and Behringer, 2007; Li et al., 2011; Lu et al., 2009; Wilson et al., 2007). The advantages of this system over other transposon system are its highly efficient genomic targeting and that there is no footprint upon excision in the genome (Wu et al., 2006). We used the helper plasmid mPB with donor plasmid pPB-UbC-eGFP (UbC: Ubiquitin-C promoter; kind gift of Dr. A. Bradley, The Wellcome Trust Sanger Institute, UK). The FoxJ1 promoter from pTg-FoxJ1-CreGFP was amplified by PCR and cloned to generate the pPB-FoxJ1-eGFP and pFoxJ1-mPB donor and helper plasmids, respectively. pPB-UbC-DsRed2 was generated from pPB-UbC-eGFP by replacing the eGFP with DsRed2 from pIRES2-DsRed2 (Clontech). The plasmids pFoxJ1-mPB with pPB-UbC-DsRed2 or pPB-FoxJ1-eGFP were used in 1:1 ratio for fate mapping experiments in intact animals. The plasmids pFoxJ1-mPB with pPB-UbC-DsRed2 and pPB-FoxJ1-eGFP were electroporated in 1:1:1 ratio, (total DNA concentration 36 μg) for fate mapping experiments following MCAO.

**Viral vectors**

All viral vectors were handled in a class III biosafety laboratory and a multiplicity of infection (MOI) of 2 for each vector was used for all viral transductions. All the vectors were packaged with the VSVG capsid and produced at the Viral Vector Core, Lund Stem Cell Center, Lund University.

**Retroviral vectors**

Murine Stem Cell Virus (MSCV) encoding IRES-GFP (MIG) or IRES-GFP-Lnk (MIG-Lnk), kindly provided by Drs. J. Lodish and W. Tong, were used to transduce neurospheres (paper I). Transduced cells were allowed to grow for an additional 5 days in vitro before cell sorting.

**Lentiviral vectors**

The lentiviral vectors encoding the murine transcription factors Ascl1, Brn2 and Myt1l immediately downstream of the tetracycline response element (TRE) in FUW-TRE--WPRE and pLD-puro-2A-M2rtTA-TcVA encoding the tetracycline activator expression were obtained from Addgene (paper III). The human consensus coding sequences (CCDS) of cortical transcriptino factors were codon optimized for human / mouse codon usage and synthesized commercially (Genscript, CA, USA). The synthesized sequences were cloned into pBOB-TRE--WPRE, immediately downstream of the TRE. pHG-hSynl-GFP, a kind gift of Dr. Cecilia Lundberg, was used to transduce reprogrammed neurons for further analysis (paper III).

**Neuronal reprogramming**

The human fetal lung fibroblasts cells were transduced with pLD-puro-2A-M2rtTA-TcVA
(Mak et al., 2010) (paper III). The transduced cells were selected with puromycin (1 μg/ml; Life Technologies) for rtTA expression and grown to 90% confluency before passage. Upon passage, cells were transduced with the respective pool of transcription factor expressing lentiviral vectors in microcentrifuge tubes and plated 1 h later along with the viral particles. The cells were plated to 6 well plates with coverslips, T75 flasks or T175 flasks and 48 h after transduction the medium was changed to DMEM / 10% FBS with Doxycycline (2.5 μg/ml; Sigma-Aldrich) to induce expression of transcription factors. The expression of the pool of transcription factors would induce cortical neurons from fibroblasts (iCtx). Three days after doxycycline was added, the medium was changed to neuronal induction medium (iCtx medium: Neurobasal medium, 2% B27 without vitamin A, 0.5mM Glutamine and 10U/ml Pen/Strep; all from Life Technologies). The medium was changed once every 3 days. Laminin (1μg/ml; Life Technologies) was added to the culture medium every week to improve neuronal cell survival. Cells were cultured in iCtx medium until 25 - 38 days after doxycycline addition (dpi). Cells were transduced with pHG-hSynI-GFP lentiviral vector one week before electrophysiology analysis and cell sorting. Cells grown on gelatin-coated coverslips were used for electrophysiology and immunocytochemistry.

Cell Sorting and flow cytometry

**Fluorescence-activated cell sorting**

Neurospheres were passaged and SVZ tissue dissociated by enzymatic digestion (paper I) while the iCtx cells were trypsinized (paper III) to make a single cell suspension. Cells were washed and resuspended in PBS containing 30mM glucose and 5% FBS (paper I) or L-15 medium containing 0.1% IgG-free BSA (Jackson ImmunoResearch), 10U/ml DNaseI (Sigma-Aldrich) and 1:10 ratio Citrate-Phosphate-Dextrose (Sigma-Aldrich) (FACS buffer; paper III). iCtx cells were incubated at room temperature with APC conjugated CD44 antibody (BD Biosciences). Suspensions were passed through a 70μm filter and cells were sorted using fluorescence-activated cell sorting (FACS) Aria (BD Biosciences). The sorted cells were directly sorted to medium or lysis buffer for cell culture or RNA extraction, respectively.

**Flow cytometry**

WT and Lnk−/− neurospheres were pulsed with BrdU and cells harvested at 3, 6, and 10 h (paper I). The harvested cells were labeled with FITC conjugated BrdU antibody and propidium iodide. Cells were analyzed for BrdU incorporation and cell cycle with the data acquired on an LSR II flow cytometer, with Diva software version 6.0 (Becton Dickinson). The cytometry data was further analyzed on FlowJo software version 9.4.9 (TreeStar).

**Magnetic activated cell sorting**

One week after transduction with SynI-GFP lentiviral vector, iCtx cells were trypsinized
and made into single cell suspension in FACS buffer (paper III). The cells were incubated with the human anti-fibroblast antibody labeled with magnetic beads (Miltenyi Biotec, Germany) according to manufacturer’s instructions and the cell suspension was passed through a separation column held within a magnet, by gravity. The column was removed from the magnet and remaining cells eluted out by gravity with FACS buffer. The sorted cells were used for co-culture experiments with human astrocytes.

RNA extraction and Quantitative PCR

RNA was isolated from sub-dissected SVZ primary tissue, expanded neurospheres, (paper I), fluorescence-activated sorted cells (paper I and III) or cultured cells (paper III). Total RNA was isolated using RNAeasy kit (Qiagen) with DNase treatment according to manufacturer’s instructions. RNA was reverse transcribed using oligoDT primers and superscript-II (Invitrogen) (paper I) or iScript advanced cDNA synthesis kit (Bio-Rad) (paper III). For some experiments (paper III) the cDNA was pre-amplified with TaqMan PreAmp Master mix (Applied Biosystems) according to manufacturer instructions. PCR was performed with cDNA using the primers listed in Table 1 for some of the experiments (paper I). Quantitative-PCR (Q-PCR) was performed with TaqMan gene expression assays (Applied Biosystems) on iQ5 thermal cycler (Bio-Rad) (paper I and III), or with 48.48 microfluidic chips BioMark HD (Fluidigm, CA) (paper III). cDNA input was normalized to GAPDH (paper I) or GAPDH, βActin, UBC, TBP, HPRT and YWHKAZ (paper III). Relative gene expression was calculated using the ΔΔC(t) method (paper I). ΔC(t) values were log transformed to base of 2 (log2 ΔC(t)) and then z-scores, calculated by subtracting the ΔC(t) of a sample with the mean ΔC(t) value of all samples for a given gene and dividing it by the corresponding standard deviation, were calculated. The z-score values or log2 ΔC(t) were visualized as a heat-map using Gene-E software (http://www.broadinstitute.org/cancer/software/GENE-E/index.html). Hierarchical clustering was performed for initial set of qPCR results in Gene-E by average linkage of one minus Pearson’s correlation values to identify the clustering of transcription factor combinations with respect to cortical markers (paper III). The list of TaqMan assays used in this thesis are listed in Table 2.

Transcription factor binding site prediction

DNA sequences for SH2B3 (LNK) (paper I) corresponding to conserved regions between the human, mouse, rat, horse, and dog species upstream of the 5′ UTR and including a part of the 5′ UTR were retrieved using the VISTA Browser (http://pipeline.lbl.gov) (Mayor et al., 2000) and the UCSC Genome Browser (http://genome.ucsc.edu/) (Kent et al., 2002). These regions corresponded to the following positions in the human genome: Human SH2B3 (March 2006) chr12: 110319473–110319590, chr12: 110320884–110320980, chr12: 110325532–110325770, and chr12: 110327105–110328342. We applied MotifScanner 3.1.1 (http://med.kuleuven.be/lcb/toucan/help/WebServices/motifscanner.htm) (Aerts et al., 2005) for binding site prediction using matrix files from TRANSFAC and murine and human conserved noncoding sequences as background model (paper I).
Chromatin immunoprecipitation PCR

Neurospheres were fixed with 1% formaldehyde for 10 min at RT, and the process stopped by the addition of glycine at a final concentration of 0.0125 M for 5 min. Cells were washed and resuspended in 1 ml lysis buffer with protease inhibitor mixture and 1 mM serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF; Roche). Sonication was performed with a Branson 450 probe, yielding DNA fragments with a bulk size of 50–300 bp. The debris-cleared lysate was frozen at -80°C. Magnetic Dynabeads (Invitrogen) were incubated (1:2) with blocking buffer (Lonza) containing 1 mg/ml herring sperm DNA (Promega) and 1 mg/ml bovine serum albumin (Sigma-Aldrich) overnight at 4°C on a rotator. For each reaction, beads in 100 µl of the slurry-blocking buffer mix were separated with a DynaMag magnet (Invitrogen) and incubated for 20 min at room with rabbit anti-STAT1, normal rabbit IgG (both from Cell Signaling Technology), or H2O, diluted in 200 µl Binding & Washing Buffer (Invitrogen), and washed with Binding & Washing Buffer. For immunoprecipitation, 100 µl cell lysate was diluted with 900 µl of radioimmunoprecipitation assay buffer containing PMSF and protease inhibitor, and incubated with the prepared beads for 2 h. Beads were washed three times with Washing buffer. Cross-linking was reversed by resuspending beads in 250 µl digestion buffer containing 10 mM Tris, 10 mM EDTA and 1% SDS, addition of proteinase K to a concentration of 50 µg/ml, and incubation for 2 h at 68°C. 10 µl of raw lysate were diluted in 240 µl digestion buffer and treated equally to the ChIP samples, served as input control. Finally, the material was phenol-chloroform extracted and ethanol precipitated. DNA was resuspended in 50 µl of nuclease-free water and 2 µl was used as a template for PCR (paper I).

Enzyme Linked Immunosorbent Assay (ELISA)

Neurosphere cells were grown adherent in 96-well plates in expansion medium until semiconfluent, when growth factors were withdrawn overnight. Cells were stimulated with 10 ng/ml EGF and 10 ng/ml bFGF, 50 ng/ml IGF1, or 5 U/ml EPO for 10 min. Levels of total and phosphorylated ERK1/2 and AKT were analyzed using FACE in-cell Western phospho ELISAs (Active Motif) (paper I).

BrdU administration

BrdU (10 µM) was used for cell cycle analysis by flow cytometry (paper I). BrdU (50 mg/kg, i.p.), dissolved in PBS, was given four times with a 2 h interval to intact mice and at 7 d after SE, and animals were killed 2 h thereafter (paper I). Mice subjected to stroke received BrdU once daily for 7 d after the insult and were killed the following day (paper I). For BrdU label retention studies, mice received two daily BrdU injections during day 9 and 10 after stroke and were killed 8 weeks later (paper I). Rats received BrdU (50 mg/kg, i.p.) one week after stroke, twice daily for seven days and were killed 4 weeks after the last injection (paper II).
Immunostaining

Immunocytochemistry in vitro

Neurospheres or cells attached to coverslips or slides were fixed with 4% paraformaldehyde (PFA), pre-incubated in potassium PBS (KPBS) with 0.025% Triton X-100 and 5% serum for 1 h, and incubated with primary antibodies overnight at 4°C (Table 3). After washing, cells were incubated with respective secondary antibodies conjugated to Cy2 / Alexa488, Cy3 and Cy5 / Alexa 657 fluorophores. Biotinylated secondary antibodies were incubated with fluorophore conjugated streptavidin. The cells that had been labeled with biocytin from electrophysiology experiments (paper III) were stained with Streptavidin conjugated Alexa488 or Alexa647 along with the secondary antibodies. After rinsing thrice with PBS, cell nuclei were stained with Hoechst 33342 for 10 min in room temperature. Coverslips were mounted on glass slides or slides were coverslipped with PVA-Dabco after rinsing with distilled water (papers I and III).

Immunohistochemistry in vivo

Animals were perfused transcardially and brains were removed, post-fixed overnight in PFA, and cryopreserved in 20% sucrose solution. Free-floating frozen sections (30 μm thick) were cut on a sliding microtome (Leica, Germany) in the coronal plane. Sections were pre-incubated in 0.25% Triton X-100 in phosphate-buffered saline (PBS) containing 5% serum for 1 h. Sections were then incubated with primary antibody overnight at 4°C (Table 3). Sections were washed with PBS and then incubated for 2 h with secondary antibodies conjugated to Cy2 / Alexa488, Cy3 and Cy5 / Alexa 657 fluorophores at room temperature (RT). Sequential staining was carried out if two of the primary antibodies were made in the same species. For BrdU staining, DNA was denatured in 1N HCl for 30 min at 65°C (paper I) or in 2N HCl at RT for 1 or 2 h (paper I and II respectively), washed three times with PBS, and incubated overnight with rat anti-BrdU (1:100; OBT0300, AbD Serotec, Germany). Biotin-conjugated donkey anti-rat (1:200; Jackson ImmunoResearch) secondary antibody was incubated for 2 h at RT followed by incubation with Streptavidin-conjugated Alexa-647 for 2 h at RT (paper II). The sections were washed with PBS after secondary antibody incubation and stained with Hoechst 33342 for 10 minutes at RT. The sections were washed three times, mounted onto gelatin coated slides, air-dried and coverslipped with PVA-DABCO.

TUNEL staining

TUNEL staining was performed using the in situ cell death detection kit (Roche) according to manufacturer’s instructions. The sections were rinsed three times in ice-cold PBS and then in ice-cold permeabilization solution (0.1% Triton X-100 in 0.1% Sodium Citrate) for 2 min. Sections were rinsed three times in PBS and incubated in TUNEL enzyme and label mix for 1 h at 37 °C. After rinsing once in PBS, nuclei were stained with Hoechst 33342 (10 μg/ml). The sections were then rinsed twice in PBS, air-dried and coverslipped with PVA-DABCO mounting medium (paper I and II).
Microscopical analysis

Immunofluorescence was analyzed in Olympus BX61 (Olympus, Germany) epifluorescence microscope, images were obtained with CellSens Dimension software (Olympus, Germany). Cells were counted in four evenly spaced coronal sections that contained the SVZ (paper I) or LVW (paper II), or eight evenly spaced olfactory bulb sections (paper II). Confocal images were obtained for co-localization of markers on Leica TCS-SP2 (paper I and II) or Zeiss LSM 780 (paper III). Cell counts on MCAO lesioned animals were also performed on confocal images (paper II).

Conversion efficiency

In vitro quantification was done in at least 20 regions of interest from three different coverslips per condition in an epifluorescence microscope (paper III). The total number of MAP2/βIII Tubulin positive cells with neuronal morphology was quantified 25-30 days after infection. Transduction efficiency was calculated as previously described (Pang et al., 2011; Vierbuchen et al., 2010). Briefly, average number of neuronal cells present in 20 randomly selected 20x visual fields was estimated. The area of 20x visual field was then used to determine MAP2+/βIII Tubulin+ cell density in the entire dish. This number was divided by the number of plated cells during transduction to get the percentage of starting population of cells that acquired neuronal-like characteristics (paper III).

Cell soma size

The cell soma size was measured from 60 cells randomly chosen from 3 coverslips for each condition in CellSens imaging software (Olympus, Japan). The images were obtained on using 20X objective and the outline of the cell body was delineated manually within the software. The area covered by the outline corresponding to the cell soma size was calculated by the software and used for analyzing the average cell soma size in the given transcription factor combinations (paper III).

Pyramidal morphology index

The pyramidal morphology index (PMI) was defined as the ratio between the width of the largest process and the total number of processes crossing a sampling circle of fixed diameter (Ø25 μm) (Hand et al., 2005) in CellSens imaging software (Olympus, Japan). To determine the PMI, at least 60 cells per condition were randomly chosen. The number of processes crossing the sampling circle was counted and the width of the widest neurite was measured. The index was calculated for the multipolar cells that had more than two processes. These cells were used for analyzing the percentage of cells that were multipolar in a given condition (paper III).

Immunoreactivity assay

Assessment of MAP2/βIII Tubulin immunoreactivity was done using cellSens Dimension (Olympus, Japan) imaging software, to determine the neurite density. 20 images of randomly chosen regions in three representative coverslips were acquired. Using a 10X objective, 15
fields were chosen randomly in 3 coverslips for each condition. In each coverslip, areas of MAP2/βIII Tubulin immunoreactivity were identified using defined representative ranges of threshold for specific signal. Using these defined parameters, the images of each area were analyzed by software, which calculated the total area covered by the specific immunopositive signal (X in μm²). The number of MAP2/βIII Tubulin cells (y) that had Hoechst 33342 positive nuclei in each field was counted. This number was then used to calculate the immunoreactivity area per cell (X/y, μm² / cell) to get a measure of the neurite density. The cell density in a given coverslip was calculated using y/X (cells / μm²) (paper III).

**Electrophysiological analysis**

Whole-cell patch-clamp recordings were performed with EPC10 double patch clamp amplifier (HEKA Elektronik, Germany) using PatchMaster software (HEKA Elektronik, Germany) for data acquisition. Cells grown on coverslips were transferred to the recording chamber mounted on Olympus BX62 upright microscope. The cells for recording were identified using a 40X water immersion lens with infrared differential interference contrast optics, while cells labeled with SynI-GFP virus were visualized with fluorescence optics. The coverslip was constantly perfused (1ml/min) with carbogenated artificial cerebral spinal fluid (aCSF, in mM : 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 26 NaHCO₃, 1.25 NaH2PO₄, and 25 glucose, pH 7.2-7.4, 295-300 mOsm) at 34ºC. Recording pipettes were filled with intracellular solution containing (in mM): 122.5 KGlu, 2.5 KCl, 1.0 HEPES, 8.0 NaCl, 2.0 MgATP, and 0.3 Na₂GTP for recordings of intrinsic properties, 135 CsGlu, 10 HEPES, 10 NaCl, 1 MgCl₂, 2 MgATP, and 0.4 Na₂GTP for recordings of evoked excitatory post synaptic currents (EPSC), and 135 CsCl, 10, HEPES, 10 NaCl, 2 MgATP, and 0.3 NaGTP for recordings of evoked inhibitory post synaptic currents (IPSC). Intracellular solutions had a pH of 7.2–7.4, an osmolarity of 285–295 mOsm and had a resistance of 2.5-9.5 MΩ. Biocytin (2-4 mg/ml) was added to the internal solution prior to recording for post-hoc identification of the recorded cell. Voltage values were not corrected for the liquid junction potential, which were 13.82 mV, 15.55 mV and 5.10 mV for KGlu-, CsGLu- and CsCl-based internal solutions, respectively. Voltage- and current-clamp recordings were used for the electrophysiological characterization. Sodium and potassium currents were evoked by a series of 200 ms long voltage steps (from -70 mV to +40 mV in 10 mV steps) and their sensitivity to respectively, 1 μM TTX, (Tocris, UK) and 2-10 mM TEA (Tocris) were determined. A series of current steps (0-200 pA in 10 pA steps) lasting 500 ms were performed from a membrane potential of around -70mV (current was injected when needed to keep the membrane potential around -70mV) to determine the cells ability to generate action potentials. EPSCs and IPSCs were evoked by puff application (0.5-0.75 bar) of 100 mM glutamate or 100 mM GABA lasting 0.5-1 s using a pneumatic drug ejection system (PDES-02DE-2, NPI electronic, Germany). AMPA and NMDA receptors were blocked by 5 μM NBQX and 50 μM D-APV, respectively. GABA receptors were blocked by 0.1 mM PTX. Data was analyzed offline with FitMaster and IgorPro (paper III).
Statistical analysis

Statistical analysis and graphs were made in Prism software (GraphPad, CA, USA). Comparisons were performed using paired or unpaired Student’s t-test, ratio paired Student’s t-test, two-way ANOVA followed by Scheffé’s post hoc test, Tukey’s multiple comparisons test or Uncorrected Fisher’s LSD, Kruskal-Wallis test followed by Dunn’s multiple comparison test and Wilcoxon matched-pairs signed rank test where appropriate. Differences are considered significant with $P < 0.05$. 
**Table 1. RT-PCR primer sequences**

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<th>Gene</th>
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Sequences are in 5’ to 3’ orientation
Table 2. List of Taqman Gene Expression Assays

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Table 3. List of primary antibodies

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* Antigen retrieval with 1 or 2 N HCl is required

#Antigen retrieval with Citrate buffer is required
REFERENCES


Caiazzo, M., Dell’Anno, M.T., Dvoretzskova, E., Lazarevic, D., Taverna, S., Leo, D., Sotnikova, T.D., Menegon,


Gulisano, M., Broccoli, V., Pardini, C., and Boncinelli, E. (1996). Emx1 and Emx2 Show Different Patterns of Expression During Proliferation and Differentiation of the Developing Cerebral Cortex in the Mouse. European Journal of Neuroscience 8, 1037-1050.


