Generation of cortical neurons through reprogramming technology

Miskinyte, Giedre

2018

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Miskinyte, G. (Accepted/In press). Generation of cortical neurons through reprogramming technology. Lund: Lund University, Faculty of Medicine.

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Generation of cortical neurons through reprogramming technology
Generation of cortical neurons through reprogramming technology

Giedre Kvist

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden. To be defended on December 19th at 13.00, GK-salen, BMC, Lund, Sweden.

Faculty opponent
Associate Professor Anna Falk
Department of Neuroscience, Karolinska Institutet, Stockholm
Abstract
The human cortex is affected by several debilitating acute and chronic neurodegenerative disorders such as stroke, traumatic brain injury, amyotrophic lateral sclerosis and Alzheimer’s disease, which target specific types of cortical neurons. Emerging evidence indicates that stem cells and reprogrammed cells can be used to generate human cortical neurons both for cell replacement by transplantation, and for disease modeling and drug screening. Several laboratories have established in vitro protocols for the derivation of excitatory pyramidal neurons, the principal type of neuron in the adult cortex, from human pluripotent stem cells (hPSCs). Efficient production of corticofugal projection neurons (CfuPNs) from ES cells has also been reported. Alternatively, human fibroblasts have been directly converted into induced neuronal (iN) cells. However, cortical neurons have not been produced by this method. Also, it remains to be assessed how closely the derived cortical neurons resemble their in vivo counterparts, as well as, whether the generated cells are capable of integrating into human neuronal circuits.

The work presented in this thesis demonstrates that cortical neuronal cells can be produced from different types of starting cells: human fibroblasts, human ES cells and human iPSC-derived NSPCs. Regardless the starting cell context and derivation protocol, we have produced in all studies neuronal cells pyramidal in shape, expressing key cortical markers and functional in vitro. More importantly, we showed that these cells are capable of forming synaptic connections with adult human cortical neurons. It remains to be assessed whether cells derived in this thesis are capable of projecting to correct brain regions in vivo. Nonetheless, by providing the first evidence that cortical neurons derived here integrate in adult host neural networks also in a human-to-human grafting situation, this thesis represents an early but important step in the clinical translation of neuronal replacement to promote recovery in the injured brain.

Key words: cortex; reprogramming; human ES cells; transcription factor programming; cortical projection neurons; human adult cortical slices

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature Date 2018-11-13
Generation of cortical neurons through reprogramming technology

Giedre Kvist
Coverphoto by Giedre Kvist

“Cortical layer symphony No 6”.

This picture is a fusion between neuroscience and music. It shows the transformation of an ideal starting cell to an ideal cortical neuron that would be suitable for transplantation, drug screening, etc. This magical (“musical”) cell transformation occurs within the typical five-line stave that defines musical pitch. In this particular picture, five-lined stave also divides the paper into six spaces that represent six cortical layers (Roman numbers at the end of each stave). In addition, the transformation is instructed by sharp key signatures (Brn2, Ngn2, Satb2 and Myt1l), common time (Ctip2) and dynamics “forte” (Fezf2), which in this picture appear as main transcription factors, known to drive neuronal as well as cortical fate in different types of cells within the cell reprogramming field.
To my family
# Table of Contents

Summary .................................................................................................................. 11  
Populärvetenskaplig Sammanfattning ................................................................. 12  
Santrauka lietuvių kalba .................................................................................... 13  
Original papers and manuscripts ..................................................................... 14  
Abbreviations ....................................................................................................... 15  

## Introduction

- Neuronal diversity in the mammalian cerebral cortex ...................................... 17  
- Cerebral cortex in neurodegenerative disorders ............................................... 20  
- Stem cell-based approaches for neuronal replacement .................................... 21  
- Generation of cortical neurons from stem cells and reprogrammed cells ........ 23  
- Cellular reprogramming .................................................................................. 24  

## Aims of the thesis

- Experimental procedures .................................................................................. 29  
- Animals and surgical procedures (paper III) .................................................. 29  
- Cell Culture ..................................................................................................... 30  
- Derivation of human organotypic slice cultures ............................................. 34  
- Co-culture of *in vitro* derived cortical neurons with adult human cortical organotypic slice ................................................................. 35  
- RNA extraction and qPCR ............................................................................ 36  
- Immunocytochemistry ..................................................................................... 39  
- Microscopy ...................................................................................................... 41  
- Electrophysiology ............................................................................................ 43  
- Data representation and statistics ................................................................... 44
Results ......................................................................................................................... 45
  Derivation of functional excitatory cortical neurons from human
  fibroblasts (paper I) ...................................................................................................... 45
  Derivation of functional excitatory cortical neurons from human
  embryonic stem cells (paper II) .................................................................................. 47
  Human iPSC-derived cortical neurons integrate into adult human cortical
  neural circuitry (paper III) .......................................................................................... 49

Discussion ................................................................................................................... 51
  Instructing cortical fate in somatic and pluripotent cells .................................... 51
  Direct comparison of derived cells with bona fide cortical neurons ..................... 53
  Electrophysiological properties of functional in vitro derived cortical
  neurons ......................................................................................................................... 53
  Integration of in vitro derived cortical neurons into human neuronal
  networks ....................................................................................................................... 54

Concluding remarks .................................................................................................. 57

Acknowledgments ...................................................................................................... 59

References .................................................................................................................. 61
Summary

The human cortex is a structure of the brain that plays a crucial role in processing of sensory and motor information, memory, cognition, language, and consciousness. It can be affected by several neurodegenerative disorders such as stroke, traumatic brain injury, amyotrophic lateral sclerosis and Alzheimer’s disease, which cause movement, and mental function impairment. These symptoms occur primarily due to cortical neuronal cell death, and because the human brain cannot efficiently produce new neurons and replace damaged ones. Emerging evidence indicates that stem cells can be used to generate human cortical neurons in vitro both for cell replacement by transplantation and for disease modelling and drug screening. Several laboratories have established in vitro protocols for the derivation of excitatory pyramidal neurons, the principal type of neuron in the adult cortex, from human pluripotent stem cells. However, these protocols are often time-consuming and inefficient, and the functional properties of the generated cells have been incompletely characterized. Moreover, it remains to be assessed how closely the derived cortical neurons resemble their counterparts in the healthy human brain, as well as, whether the generated cells can integrate into human neuronal circuits.

The work in this thesis demonstrates that cortical neuronal cells can be produced from different types of starting cells such as human fibroblasts or human embryonic stem (ES) cells. Regardless of the starting cell and derivation protocol, we have produced in all studies neuronal cells pyramidal in shape, expressing key cortical markers and functional in vitro. More importantly, we showed that these cells are able to form synaptic connections with adult human cortical neurons. It remains to be assessed whether cells derived in this thesis are capable of connecting with correct brain regions in vivo. Nonetheless, by providing the first evidence that cortical neurons derived here integrate into adult host neural networks also in a human-to-human grafting situation, this thesis represents an early but important step in the clinical translation of neuronal replacement to promote recovery in the injured brain.
Populärvetenskaplig Sammanfattning


Arbetet i denna avhandlingen visar att kortikala neuronceller kan framställas från olika typer av startceller: humana fibroblaster, humana ES-celler. Oberoende av startcells kontext och framställningsprotokoll har vi i alla studier producerat neuronalna celler i pyramidial form som uttrycker nyckelmarkörer och är funktionella in vitro. Vi visar även att dessa celler kan bilda synaptiska kopplingar med vuxna humana kortikala neuroner. Det återstår att granska om cellerna i denna avhandling har förmågan att skapa kopplingar med den korrekta hjärnregionen in vivo. Genom att tillhandahålla de första bevisen att kortikala neuroner som erhållits här kan integrera i den vuxna värdens nervnätverk och även i en människa till människa transplantationssituation, representerar denna avhandling ett tidigt men viktigt steg i den translationella kliniska forskningen av neuronal ersättning för främjande återhämtning hos den skadade hjärnan.
Santrauka lietuvių kalba

Žmogaus galvos smegenų žievė – tai smegenų dalis, gyvybiškai reikalinga perdirbti jutimo ir judesio informacijai bei formuotis atminčiai, kalbai, sąmonei bei pasaulio pažinimui. Smegenų žievė gali būti pažeista insulto, galvos traumas, išėtinės sklerozės ar Alzheimerio ligos metu. Šių būklės sukeltos pažeidos pasireiškia dėl žievė sudarančių ląstelių, vadinamų žievės neuronais, žūties ir dėl to, kad žmogaus smegenys negeba efektyviai atsinaujinti pagamindamos naujų žievės neuronų, kurie galestį ligos metu žuvusias ląstelas. Naujausi moksliniai tyrimai rodo, kad žievės neuronus įmanoma įtraukti į kamieninių ląstelių, ir panaudoti jas netik ląstelių transplantacijai į pažeistas smegenis bet ir žmogaus smegenų ligų tyrimams laboratorioje. Deja, metodai, naudojami žievės neuronams įtraukti laboratorioje kol kas yra nepakankamai efektyvūs, ląstelių gamyba užtrunka ilgai, o pagamintų ląstelių savybės nėra iki galo apibūdintos. Taip pat, kol kas nėra duomenų apie panašumus tarp laboratorioje pagamintų ir natūraliai susiformavusių žmogaus žievės neuronų, ar jų gebėjimą integracijos į likusių gyvų neuronų tinklą žmogaus smegenyse.

Original papers and manuscripts

**Paper I**

Direct conversion of human fibroblasts to functional excitatory cortical neurons integrating into human neural networks


*Stem cell research & therapy (2017) 8:207.*

**Paper II**

Transcription Factor Programming of Human ES Cells Generates Functional Neurons Expressing Both Upper and Deep Layer Cortical markers


**Paper III**

Integration of human iPS cell-derived cortical neurons into adult human cortical neural circuitry


*Manuscript*

* These authors contributed equally

& These authors have shared senior authorship
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BMC</td>
<td>Brn2, Myt1l and Ctip2</td>
</tr>
<tr>
<td>BMF</td>
<td>Brn2, Myt1l and Fezf2</td>
</tr>
<tr>
<td>BMNd</td>
<td>Brn2, Myt1l and NeuroD1</td>
</tr>
<tr>
<td>BMNgn2</td>
<td>Brn2, Myt1l and Neurogenin 2</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post induction</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic Stem</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>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HEFL</td>
<td>Human Fetal Lund Fibroblasts</td>
</tr>
<tr>
<td>iCtx</td>
<td>Induced Cortical Neurons</td>
</tr>
<tr>
<td>iN</td>
<td>Induced Neurons</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced Pluripotent Stem Cells</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule Associated Protein 2</td>
</tr>
<tr>
<td>MNgn2</td>
<td>Myt1l and NeuroG2</td>
</tr>
<tr>
<td>N</td>
<td>Neurogenin 2</td>
</tr>
<tr>
<td>NF</td>
<td>Neurogenin 2 and Fezf2</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NS</td>
<td>Neurogenin 2 and Satb2</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>PTX</td>
<td>Picrotoxin</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of Mean</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
</tbody>
</table>
Introduction

Neuronal diversity in the mammalian cerebral cortex

The cerebral cortex is one of the most complex structures in mammalian brain. It is composed of hundreds of different cell types that connect to perform the most sophisticated tasks. Neurons of the cerebral cortex belong to two broad classes: excitatory pyramidal (or projection) neurons (PNs) and inhibitory interneurons (INs). Excitatory neurons constitute the majority (85%) of them and can be categorized further into many subtypes, each of which is characterized by a specific morphology, functionality and connectivity (1). Cortical neurons are organized spatially into specific cortical areas such as somatosensory cortex (processes sensory modalities), auditory cortex (processes sound), visual cortex (processes the sense of light), etc., which can predict their properties (2). Within the areas, cortex displays unique cytoarchitecture defined by six horizontal layers, that contain different classes of neurons and vary in thickness and tissue architecture depending on their areal identity (3).

Projection neuron diversity in the cerebral cortex

Cortical PNs are excitatory, glutamatergic neurons that connect the cerebral cortex to the entirety of its distal intracortical, subcortical, and subcerebral targets. The nomenclature of these cells is primarily based on the target of their axons (Figure 1) and can be broadly divided into associative PNs (extend axons within cortex of the same hemisphere), commissural PNs (extend axons across the midline to the contralateral hemisphere), corticofugal PNs (extend axons away from the cortex) (4). Most commissural PNs cross the midline through the corpus callosum (CC) and are called callosal projection neurons (CPNs) and reside primarily in layer II/III, with fewer residing in layer V and VI, and extend axons to mirror-image locations in the same functional area of the contralateral hemisphere (1). Associative PNs include short-distance intrahemispheric PNs, which extend axons within a single cortical column or nearby columns and are present in all layers.
Cortical PNs can be broadly divided into commissural PNs and corticofugal PNs. The main subtype of commissural PNs is callosal projection neurons (CPNs) that project to contralateral hemisphere (blue cell), whereas the main subtypes of corticofugal PNs include subcerebral projection neurons (SCPNs) that project to the pons, superior colliculus and spinal cord (red cell), and corticothalamic projection neurons (CThPNs) that project to the thalamus (green cell). Additional level of complexity in PN nomenclature is their expression of molecular determinants, transcription factors (TFs), which is summarized in the table (left). Ctx- cortex, CC- corpus callosum, Hip- hippocampus, Tha- thalamus, Cpu- caudate putamen (striatum), Gpe- globus pallidus, Hyp- hypothalamus, SN- substantia nigra, Sc- spinal cord. Drawing by Bengt Mattsson

Corticofugal PNs include corticothalamic PNs (CThPNs), mainly found in layer VI and project to different nuclei of the thalamus, and subcerebral PNs (SCPNs), mainly populating layer Vb and projecting their axons to distinct targets below the brain, predominantly to the pons (corticopontine PNs), superior colliculus (corticoretical PNs (CTPNs)) and to the spinal cord (corticospinal motor neurons (CSMNs)) (3, 4).
The classes of PNs can be also defined by the expression of molecular determinants such as different transcription factors (TFs). Among the best-characterized PNs are CPNs and CSMNs. Examples of these molecular identifiers are Fezf2, Cntn6, Ctip2, Crim1, Pcp4 and Ldb2 for CSMNs (5, 6) and Cux1, Cux2, Inhba, Lpl, PlexinD1, Dkk3 for CPNs (7).

**Generation of cortical neuronal subtypes during development**

All PNs of the cortex develop from progenitors located in the germinal zone of the dorsal telencephalon, within the anterior neural tube (8). There are two main progenitor populations responsible for the generation of the vast majority of cortical excitatory neurons: ventricular zone (VZ) progenitors or radial glia cells (RGCs) that arise from (NE) neuroepithelial cells, and subventricular zone (SVZ) or intermediate progenitors (IPs) (9). Most PN classes are born between E12.5 and E16.6 in mice, during a series of temporally restricted, but overlapping waves of neurogenesis (10). After the generation of neurons in the VZ and SVZ, they migrate radially toward the pial surface along RGC processes and seed a region called the cortical plate (11). Next, CThPNs and deep layer commissural neurons arrive and form layer VI. Layer V SCPNs and CPNs are born next followed by CPNs of the upper layers (II/III). These neurons migrate radially to the cortical plate, continue through the previously established deep layers, and seed the more superficial layers in an ‘inside-out’ manner.

The molecular control of cortical neurogenesis involves the interplay of extrinsic and intrinsic cues that coordinate the pattern of neural progenitor division and differentiation (12). First, the area patterning is initiated by expression of morphogens from patterning centers at the borders of the neocortical primodium (13). At E9.5 in mice, fibroblast growth factor (FGF) 8 and FGF17 are secreted rostromedially by the commissural plate, while caudomedially, WNT and bone morphogenic protein (BMP) family members are secreted from the cortical hem and, laterally, the WNT antagonist secreted frizzled-related protein 2 (SFRP2) is secreted from the antihem (14). Together these growth factors induce graded expression of TFs in VZ progenitors, which in turn control the relative size and position of cortical areas. Among the key patterning TFs is paired box gene 6 (Pax6), empty spiracle homeobox 2 (Emx2), trans-acting transcription factor 8 (Sp8) and chicken ovalbumin upstream promoter transcription factor 2 (Couptf1; also known as Nr2f1). Although manipulation of these TF gradients is sufficient to change the size and position of cortical areas, the neuronal identity is established in a normal manner. This suggests that the ultimate fate of RGCs and IPs is established by a second network of TFs that direct post-mitotic acquisition of cell identity (3).

Four key genes - Fezf2, Ctip2, Tbr1, and Satb2 are involved in post-mitotic specification of PN identity (15). Satb2 regulates the identity of CPNs, whereas Fezf2
and Ctip2 regulate the identities of layer V SCPNs and Tbr1 expression is crucial for CThPN development.

Fezf2 is a TF expressed in all SCPNs from early stages of development through adulthood (5, 16) and is required for specification of all SCPNs (17, 18). Without Fezf2, SCPNs up-regulate Satb2, switch fate, and extend axons across the CC (19, 20), suggesting that Fezf2 represses Satb2 and the acquisition of a callosal identity. In addition, Fezf2 instructs CFuPN identity (3).

Ctip2 functions downstream of Fezf2 to control the differentiation of SCPNs. The absence of Ctip2 expression leads to defects in axon outgrowth, fasciculation and pathfinding of SCPNs with very few axons reaching the brainstem and none reaching the spinal cord (5). Also, the expression of Ctip2 is repressed by several CPN identity determinants such as Satb2 and Couptf1, indicating that Ctip2 is a critical target for transcriptional regulation during the development of SCPNs.

Tbr1 acts in opposition to Fezf2 and Ctip2 to specify CThPN identity (21, 22). In the absence of Tbr1, early-born neurons that would normally develop into CThPNs express aberrantly high levels of Fezf2 and Ctip2 and extend axons towards subcerebral targets instead of thalamus (22). In addition, Tbr1 directly represses Fezf2 expression at least in part preventing SCPN specification (23).

Satb2 is crucial for specification of CPNs and repression of other fates such as SCPN and CThPN. Satb2 is expressed at high levels by CPNs but also by associative neurons in all layers of cortex (19, 20). Satb2 directly represses Ctip2, therefore, Satb2-null mice have superficial layer neurons expressing high levels of Ctip2 as well as a number of other genes characteristics of SCPNs.

Cerebral cortex in neurodegenerative disorders

The human cortex is affected by several debilitating acute and chronic neurodegenerative disorders such as stroke, traumatic brain injury, amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease, which target specific types of cortical neurons. For example, stroke remains as one of the leading causes of death and the main cause of adult disability worldwide. In more than 90% of cases, stroke occurs due to the restricted blood supply to defined brain areas (ischemic stroke). Stroke could also by caused by cerebral bleeding due to the ruptured blood vessel (hemorrhagic stroke). As a result, there is neuronal cell loss of given brain area leading to sensory, motor and cognitive impairments depending on the location of the lesion (24). Cortical damage causes a major disability compared to striatal damage following stroke in humans (25). Currently, the main treatment for acute stroke therapy is thrombolysis through intravenous administration of tissue plasminogen (tPA), which has been approved
within a narrow time window of few hours. Mechanical removal of blood clot from large vessels (thrombectomy) is also efficient treatment in acute phase of ischemic stroke with also narrow therapeutic window (26). After the acute time period, focused physical rehabilitation is the primary current therapy that is proven to be effective for the improvement of patient’s condition (27). It should be noted that some degree of spontaneous recovery occurs in virtually all patients, which reflects the complex nature of post-stroke recovery. It involves plastic changes in surviving neurons and neurons on the contralateral side, redistribution of brain representation, release of growth factors and anti-inflammatory factors from immune cells, synaptogenesis as well as generation of new neurons, glial and endothelial cells from endogenous stem cells (28, 29). While rehabilitation and spontaneous recovery can be encouraging, the extent of neurological recovery is still limited and novel approaches to augment the functional recovery are required.

Stem cell-based approaches for neuronal replacement

The damage of central nervous system (CNS) leads to neuronal loss and debilitating pathologies associated with a significant health and economic burden. Current treatments rely on supportive care to alleviate symptoms and promote brain functional plasticity (30). However, these treatments do not offer a substantial functional recovery and other strategies are highly warranted. Advancement of knowledge about stem cells has drawn attention towards stem cell-based approaches as a promising therapeutic option for the treatment of CNS disorders (31). The clinical trials of stem cell therapy in Parkinson disease (PD) patients, have provided proof of principle that the neuronal replacement is feasible in the human brain and can induce long-lasting improvement (32). Also, transplantation of various types of stem cells or their progeny has been reported to improve behavioral impairments in animal models of stroke (33). Several possible mechanisms underlying these improvements have been proposed such as neuronal replacement, modulation of inflammatory responses, trophic action and stimulation of plasticity in host brain neuronal circuitries as well as angiogenesis and gliogenesis (34).

The pioneering studies transplanting fetal midbrain dopaminergic neurons into the striatum of PD animal models or later in patients demonstrated survival and maturation into dopaminergic neurons of the correct subtype within the host parenchyma (35, 36). Later on, transplantation and neuronal integration of fetal projection neurons into homotopic cortical areas of the adult brain after injury has been explored (37-39). Other exciting studies revealed for the first time, how transplanted fetal projection neurons can project through host parenchyma primed by an injury (40, 41) and highlighted a role of area identity of the donor neurons on directing their projections (41). In addition, a recent study showed for the first time a correct and
remarkably precise circuit integration of fetal neurons transplanted in the primary visual cortex of adult mouse after an injury. The newly formed circuits were functional and resembling those of visual cortex, as demonstrated by in vivo calcium imaging experiments during visual stimulation (30, 42).

Together these studies revealed a strong capability of fetal neuronal cells to survive transplantation by overcoming growth inhibitors of axonal regeneration in the adult brain and project to the correct target areas. However, fetal neuron-based cell therapies for neurological disorders are hampered by limited tissue availability and ethical concerns. These issues have been addressed by exploring the use of alternative expandable stem cell sources.

**Expandable neural stem/progenitor cells (NSPCs)**

Human fetal NSPCs can be derived from human fetal brain and expanded in vitro as neurospheres or monolayer culture offering a renewable source of cells for transplantation. These cells have shown to be promising candidates for establishing stem cell replacement therapies due to their capacity of differentiating into mature neurons and innervating the regions of interest after transplantation into lesioned striatum or cortex of rats subjected to ischemic stroke (43, 44). More importantly, in addition to integration capacity, human fetal NSPCs have been shown to improve the functional recovery after transplantation into stroke-injured rat cortex (45, 46). Recently, a phase 1, first-in-human clinical trial results using allogeneic transplantation of a human fetal brain-derived immortalized NSPC line (CTX-DP) was reported (47). Some improvement in neurological and functional outcomes were seen in the 24 months after transplantation into putamen of chronic ischaemic stroke patients, and there were no cell-related adverse events.

**Embryonic stem (ES) cell-derived and induced pluripotent stem cell (iPSC)-derived neurons.**

The isolation of human ES cells (48) has fueled a great effort to generate a variety of specific neuronal subtypes relevant for cell replacement therapy and to test their functional integration into the animal models of disease. Given that ES cells still pose considerable ethical issues, an alternative way of producing iPSCs has been proposed (49). These cells were induced by reprogramming human fibroblasts with four TFs raising a possibility that cells for transplantation could be generated from the patients’ own fibroblast. The iPSCs, are capable to differentiate into specific neuron types, similar to ES cells, such as dopaminergic neurons (50, 51), or motor neurons (52) (for review see (53)). Alternatively, a long-term expandable neuroepithelial-like stem (lt-NES) cells have been generated from ES cells (54) as well as iPSCs (55). These cells
possess extensive self-renewal, clonogenicity and stable differentiation features, offering a renewable source of NSPCs for cell transplantation purposes. These iPSC-derived Lt-NES cells (alternatively iPSC-derived NSPCs) were shown to generate mature, functional neurons after transplantation into stroke-damaged mouse striatum (56). When primed to cortical fate and then transplanted to stroke-injured rat cortex, iPSC-derived NSPCs formed functional cortical neurons and promoted post-stroke motor recovery (57). Importantly, our group recently showed that intracortically grafted iPSC-derived cortical neurons in stroke-injured rat brain respond to tactile stimulation of nose and paw and are integrated in host thalamocortical neural circuitry (58).

**Generation of cortical neurons from stem cells and reprogrammed cells**

Over the past few years, pluripotent stem cells (PSCs) such as ES cells and iPSCs have emerged as promising tool for neurobiology, allowing the directed differentiation of specific neuronal subtypes (59), modeling human brain diseases and for the design of novel brain repair strategies. The PSC differentiation protocols towards specific cortical PN fate are designed based on knowledge of cortical development and adapted to *in vitro* settings. Interestingly, previous studies have indicated that the *in vitro* differentiation of human PSCs towards cortical neuronal fate follows a similar temporal order as observed in the intact developing cortex. Neurons expressing deep layer markers occur first, followed by neurons having a phenotype of upper cortical layers (60, 61). The majority of ES cell- and iPSC-derived PNS express markers of layer V and VI neurons, like Ctip2, Tbr1 or FoxP2 and a smaller population express upper layer markers like Cux1 and Satb2. This is also true for human ESC-derived cortical progenitors (62-64). Importantly, the integration capacity and layer diversity as well as ability to project to correct brain targets was further confirmed by *in vivo* transplantation experiments into mouse brain (62, 63, 65, 66). These findings demonstrate that the complex events leading to the generation of neurons displaying different layer-specific patterns of identity can take place outside of the developing brain and rely mainly on a cell population-intrinsic pathway (67).

It is worth noting that while the temporal generation of neurons belonging to the different cortical layers is largely maintained *in vitro*, and the presence of neurons belonging to specific layers has been found, the proportion of cells characteristic of each layer varies considerably depending on the method used (61, 67). Interestingly, it also holds truth when ESC-derived cortical progenitors are cultured *in vitro* (68), which suggests that other extrinsic molecular cues are required for the generation of proper proportions of different layer subtypes of PNs (12). In addition, the cues instructive for
particular neuronal types may be present but require a specific pattern of cytoarchitecture or polarity that may be less developed in a purely in vitro system (67).

Cellular reprogramming

During development, cell fate is established and maintained by complex regulatory networks of TFs that promote expression of cell type-specific gene products and repress regulators of other lineages. In this process, a pluripotent cell can only become more restricted over time, moving towards a more specialized cell type. Once established, cellular identity is remarkably stable despite numerous intrinsic and extrinsic perturbations (69). This, long lasting and widely accepted, dogma of cell biology has been challenged by somatic cell nuclear transfer (SCNT) experiments demonstrating that nuclei from endoderm cells of tail-bud stage frog embryos could successfully control the development of new tadpoles (70, 71). However, it was not clear whether these results were due to the unique molecular properties of oocytes or to the inherent plasticity of epigenetic modifications acquired during development (69). Later, experiments with DNA methylation inhibitor 5-azacytidine, demonstrated spontaneous differentiation of fibroblasts to muscle and fat cells (72). This suggested that DNA methylation is important for preventing expression of genes of alternative fates. About a decade later, Davis and colleagues showed that TF MyoD is sufficient to convert fibroblasts into myoblasts (73). It was a proof of principle that one TF is enough to control the differentiation of specific cell type. Another dramatic proof of this concept was results from ectopic expression of TF Pax6, a master regulator of eye development, which led to generation of functional eyes at various sites on the body of Drosophila (74).

Reprogramming to iPSCs

The initial works on cell fate restriction and alteration led to a remarkable finding of Takahashi and colleagues (49, 75). Performing a systematic screening approach, they identified a minimal combination of four TFs, Oct4, Sox2, Klf4 and cMyc (collectively called OSKM), that brought mouse and human fibroblasts back the developmental stage-towards pluripotent state (referred to as iPSCs). Further studies proved that these reprogrammed cells were molecularly and functionally equivalent to ES cells, including their capacity to form teratomas upon injection in mice and ability to give rise to cells of all three germ layers (76, 77). This work showed that only a small group of TFs are able to override complex developmental processes, similar to MyoD experiments.
Direct reprogramming

A next big step in reprogramming field was the finding that reprogramming can occur between distantly related somatic cells from different germ layers. It was first demonstrated by reprogramming mouse fibroblasts (representing a mesodermal lineage) to functional induced neurons (iNs) (representing an ectodermal lineage) by forced expression of three TFs Brn2, Ascl1 and Myt1l (collectively called BAM) (78). In the following years, other groups confirmed this concept by deriving functional cardiomyocyte-like and hepatocyte-like cells (79, 80). In addition, not only mouse but also human fibroblasts were converted to iNs (81, 82). Further refinements of the reprogramming protocols and screening for lineage specific TFs, led to generation of specific subtypes of neuronal cells such as dopaminergic neurons (83-85), spinal motor neurons (86), cholinergic neurons (87), serotonergic neurons (88, 89) and striatal medium spiny neurons (90). These iNs were derived by combining pro-neural TFs with subtype specific TFs. Recently, small molecules were used not only to improve efficiency of iN cell conversion (91-93), also to convert human and mouse fibroblasts into functional neurons (94, 95). However, cortical pyramidal neurons have so far not been generated by direct conversion.

Transcription factor programming

Two main limitations observed over the years in the reprogramming field are the rather lengthy period of neuronal maturation from human PSCs as well as low yield of neuronal cells derived by direct reprogramming approach (30, 96). These limitations are rather important considering a potential use of such cells in disease modeling or regenerative medicine. This issue has recently been addressed by improved protocols with accelerated neuronal differentiation by either using TFs (97, 98) or small molecules (99). This method is based on the logic of direct conversion, using lineage-specific TFs to drive differentiation, but applying them to PSCs rather than to somatic cells such as fibroblasts. Transcription factor programming of human ES cells efficiently gives rise to functional excitatory (98) and inhibitory (97) neurons. These human ES cell-derived induced neurons (hES-iNs) exhibit neuronal morphology and gene expression profile, are able to produce action potentials and establish synaptic connections, and survive transplantation into neonatal mouse brain. However, even though the excitatory hES-iNs possess a homogenous gene expression profile resembling that of excitatory forebrain neurons, it is unclear whether they represent a cell population with specific cortical layer and area identity.
Aims of the thesis

A general aim of the thesis was to test different cell types as a source to generate subtype specific cortical neurons using reprogramming technology and to explore the magnitude of their capability of integrating into human neuronal circuits. The specific aims of projects are:

I. To derive cortical neurons by direct conversion of human fetal lung fibroblasts and to characterize their functional properties and ability to integrate into human neuronal networks (paper I);

II. To develop strategies for efficient production of functional human cortical projection neurons with specific layer identity using TF programming of ES cells (paper II);

III. To assess the degree of morphological and functional integration of a newly generated iPSC-derived cortical neurons after ex vivo transplantation onto adult human cortical organotypic slices (paper III).
Experimental procedures

Animals and surgical procedures (paper III)

Adult male (225-250g) Sprague-Dawley (SD) rats (Charles River), housed in standard caging under a 12-hour light/dark cycle with ad libitum access to food and water, were used. All procedures were conducted in accordance with the European Union Directive (2010/63/EU) and were approved by the ethical committee for the use of laboratory animals at Lund University and the Swedish Board of Agriculture (Dnr. M68-16).

Focal ischemic injury in cerebral cortex was induced as described previously (56, 100, 101). Briefly, animals were anaesthetized with isoflurane (3.0% induction; 1.5% maintenance) mixed with air and locally injected with Marcaine for pain relief, and the temporal bone was exposed. A craniotomy of 3 mm was made, the dura matter was carefully opened, and the cortical branch of middle cerebral artery was exposed and permanently ligated by suture. Both common carotid arteries were isolated and temporarily ligated during 30 min. After releasing common carotid arteries, surgical wounds were closed and rats were injected with 1.5 ml Ringer’s solution, returned to their cages and put on a heating pad. During the first week after distal middle cerebral artery occlusion (dMCAO), rats were provided with high calories gel diet (DietGelTM Boost, Clear H2O) if required and injected subcutaneously with Ringer’s solution in case of dehydration.

Intracortical implantation of cortically committed NSPCs, transduced with lentivirus carrying GFP, was performed stereotaxically 48h after dMCAO. On the day of surgery, cortically committed GFP+ NSPCs were resuspended to a final concentration of 100,000 cells/µl in cytocon buffer. A volume of 1.5 µl was injected at two sites at the following coordinates (from bregma and brain surface): anterior/posterior +1.5 mm, medial/lateral -1.5 mm, dorsal/ventral -2.0 mm and anterior/posterior +0.5 mm, medial/lateral -1.5 mm, dorsal/ventral -2.5 mm. Tooth-bar was set at -3.3 mm. Rats were injected subcutaneously with 10 mg/kg Cyclosporine A every day during the first month after transplantation and every other day during the second month.
Cell Culture

**Derivation of human lung fibroblasts (paper I)**

Human fetal tissue was obtained from Lund and Malmö University Hospitals according to guidelines approved by the Lund-Malmö Ethical Committee. Developmental stage of the fetuses was determined by crown-to-rump length and careful evaluation of external features of the fetuses and internal features of the nervous system. Tissue was microdissected under a stereo-microscope (Leica, Germany) in ice-cold hibernation medium. Human fetal lung fibroblasts (henceforth referred to as HEFL) were isolated from dead aborted human fetuses aged 7-9 week post-conception. After removal of the central nervous system and spinal ganglia, the trachea was exposed and resected at the bifurcation. Lungs were then transferred to a clean petri dish, washed several times with cold hibernation medium and sub-dissected with another set of sterile tools. Great care was taken to remove the residual bronchi. The tissue was transferred several times to a clean petri dish containing sterile cold hibernation medium to remove contaminating cells. The sub-dissected pulmonary tissue was digested with 0.25% trypsin (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) in DMEM 4.5g/L glucose supplemented with 2mM glutamax and 10% fetal bovine serum (FBS) (all from Life technologies) (herein indicated as human fibroblast expansion medium, hFEM) and were passaged after reaching confluence with 0.25% trypsin. The HEFL cell line was used for neuronal differentiation protocols after passage 3 to avoid contamination from neural tissue and other cell types (78, 85).

**Derivation of human fetal primary cortical and striatal cells (papers I and II)**

Primary cortical (hCtx) and striatal (hStr) cells were derived from cerebral cortex and striatum, respectively, of aborted human fetuses (7.2 and 11 weeks of age) according to guidelines approved by Lund/Malmö Ethical Committee. The tissue was carefully dissected, minced into small pieces and then triturated with pipette tip into a single cell suspension. The cells were either used directly for sorting experiments (fresh fetal human cortical cells, hereafter called hFCtxF cells (paper II) or hCtx (paper I)) or washed with culture medium and plated onto poly-D-lysine (PDL) (Sigma-Aldrich)/Fibronectin (Life Technologies) (both 10µg/mL)-coated glass coverslips at a density of 20 000 cells per cm2 and maintained in culture medium (cultured human fetal cortical cells, hereafter called hFCtxC cells (paper II)). Cells were fixed with 4% paraformaldehyde (PFA) after a week for morphological analysis.
Derivation of human adult cortical cells (paper II)

For the primary adult human cortical cell (hACtxC) cultures, the tissue samples were submerged in Hibernation medium (Thermo Fisher Scientific, MA, USA) immediately after surgery and transferred to a cell culture laboratory. The meninges were removed under a dissection stereomicroscope (Leica, Germany) and the tissue was cut into small pieces with a sterile surgical blade under a laminar flow bench. The tissue was then processed using a kit for dissociation of adult brain tissue (Neural Tissue Dissociation Kit, Miltenyi, Germany) according to the manufacturer’s instructions. A single cell suspension was obtained and plated onto PDL- (Sigma Aldrich, USA) and human fibronectin- (Thermo Fisher Scientific, USA) coated culture flasks (Nunc, Thermo Fisher Scientific, USA), glass/plastic chamberslides and glass/plastic Petri dishes (all from Ibidi, Germany). The cells were cultured in Neurobasal medium supplemented with B27 (1:50) and Glutamax (1:100) (Thermo Fisher Scientific) and recombinant human factors such as: Brain Derived Neurotrophic Factor (hBDNF), Glial cell Derived Neurotrophic Factor (GDNF) and Ciliary Neurotrophic Factor (CNTF) (10 ng/ml) (all from Peprotech, UK).

Derivation of iPSC and NSPC lines (paper III)

Human iPSC-derived NSPCs were produced as described previously (54, 55, 102, 103) with some modifications. Briefly, human dermal fibroblasts from a healthy adult donor were subjected to sendai virus transduction with the reprogramming factors Oct4, Sox2, KLF4 and c-MYC (CytoTuneTM iPS 2.0 Sendai Reprogramming kit, Invitrogen) and split into plates with mouse embryonic fibroblasts. Colonies were then picked and expanded to establish iPSC lines in feeder-free conditions using mTeSR medium (Invitrogen). On day 0, iPSCs were split using dispase (0.5 mg/ml) in order to collect the whole colonies. Colonies from 3 wells of a 6 well plate were gently resuspended in embryoid body (EB) medium (Dulbecco’s modified Eagle medium/F12 (DMEM/F12), 10% KSR, 2-Mercaptoethanol (1:1000), non-essential amino acids (NMEAA) (1:100), Glutamine (1:100)) with Rock inhibitor (1:1000), 3 mM Dorsomorphin (Sigma-Aldrich) and 10 mM SB431542 (Sigma-Aldrich) and plated into ultra low-attachment 10cm culture dishes to generate EBs. The EB medium with freshly dissolved Rock inhibitor, Dorsomorphin and SB431542 was changed daily. On day 5, EBs were collected and plated on poly-ornithine-laminin-coated 6 well plates in EB medium with 3 mM Dorsomorphin and 10 mM SB431542. On day 6, medium was changed to N2 medium (DMEM-F12 (without Hapes, +Glutamine), N2 (1:100), Glucose (1.6 g/l)) supplemented with 1 mM Dorsomorphin and 10 ng/ml bFGF. Six days later, neural rosettes appeared in culture and were carefully picked and grown in suspension in N2 medium with 20 ng/ml bFGF. On day 14, neural rosette spheroids were collected and dissociated with trypsin. The small clumps obtained were grown in adhesion on poly-ornithine-laminin-coated dishes in the presence of 10 ng/ml FGF2,
10 ng/ml EGF (both from Peprotech) and B27 (1:1000, Invitrogen). The iPSC-derived NSPC line was routinely cultured and expanded on 0.1 mg/ml poly-L-ornithine and 10 mg/ml laminin (both from Sigma)-coated plates into the same media supplemented with FGF, EGF and B27 and passaged at a ratio of 1:2 to 1:3 every second to third day using trypsin (Sigma).

**Human ES cell culture (paper II)**

Human ES cells H1 (WA01) from WiCell Research Institute (Wicell, WI) were cultured in feeder-free conditions on MATRIGEL™ (BD Biosciences)-coated 6-well plates in mTeSRTM1 medium (StemCell Technologies) that was changed daily. Cells were dissociated with Accutase (ThermoFisher Scientific) after reaching 80% confluence and replated in mTeSRTM1 medium. Human H1 ES cells were kept in culture and used for all induction experiments between passages 43 to 51.

**Derivation of mouse glia cells (papers I and II)**

Mouse glia cells were cultured from the forebrain of newborn wildtype CD1 mice as described (104). Briefly, newborn mice (P3-P5) were decapitated with sterile scissors, forebrain pieces of newborn mice (P3-P5) were cut by a sterile surgical blade in small pieces subsequently digested with dissociation medium (DM) (0.7 mg/ml hyaluronidase, 0.2 mg/ml kynurenic acid, 1.33 mg/ml trypsin (Sigma) in Hank’s balanced salt solution (HBSS) (Life Technologies)) for up to 30 min. Cell dissociation was facilitated by harsh trituration to avoid contamination of neurons. Cell suspension was then plated onto T75 flasks coated with poly-L-lysine (PLL) (Sigma) in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (Life Technologies). After reaching confluence, mouse glia cells were dissociated with trypsin and passaged at least 3 times to remove potential trace amounts of neurons before co-culture experiments.

**Induction of cortical neurons**

All lentiviral vectors were handled in a class II biosafety laboratory and a MOI of 2 for each vector was used for all viral transductions. Lentiviral particles with the VSVG capsid were prepared according to protocol from Dull et al. (105).

*Paper I.* The pLD-puro-2A-M2rtTA-TcVA (106) (Addgene plasmid # 24592; henceforth referred to as puro-rtTA) was used for selecting cells with puromycin (2µg/ml) for rtTA expression and used for subsequent reprogramming experiments. The human consensus coding sequences (CCDS) of Fezf2, Ctip2 (also known as BCL11B), NeuroG2 (henceforth referred to as Ngn2) and NeuroD1 (henceforth
referred to as Nd1) were synthesized (Genscript, CA) and cloned into BamHI/PmeI site of pBOB-TRE-WPRE. FUW-TetO-Brn2 and FUW-TetO-Myt1l (Addgene plasmids #27151 and 27152, respectively (78)) were used along with the above-mentioned vectors in nine different combinations. In addition, cells were transduced with pLemir9-124 (Addgene plasmid #31779) to further enhance the conversion efficiency of BMF condition. HEFL cells selected with puromycin for rtTA expression were grown to 90% confluency and passaged. Upon passage, cells were transduced with the respective pool of transcription factor, expressing lentiviral vectors and plated to 6 well plates with coverslips or T75 flasks. Three days later, doxycycline was added to induce expression of the transcription factors and thereby generate cortical neurons from fibroblasts (iCtx cells), and after another 3 days the medium was changed to neuronal induction medium (iCtx medium: Neurobasal, 2% B27 without vitamin A, 0.5mM Glutamine and 100U/ml Pen/Strep). For small molecule (SM) condition (single cell qPCR and co-culture with adult human cortex organotypic slice culture experiments), iCtx medium was supplemented with growth factors at the following concentrations: 10 ng/mL BDNF, 2 ng/mL GDNF, 10 ng/mL NT3 (Peprotech) and 0.5 mM db-cAMP (Sigma). The SMs CHIR99021, SB431542 (Sigma), Noggin (Peprotech) and LDN-193189 (Axon), were added to the media along with growth factors at the same concentrations as previously reported (91). Cells were cultured in iCtx medium until 25-38 days after adding doxycycline (days post induction; dpi). Doxycycline was maintained throughout the differentiation protocol and medium was changed every 3 days. Cells were additionally transduced with pHG-hSynI-GFP lentiviral vector (SynI-GFP; kind gift of Dr. Cecilia Lundberg, Lund University) 72 h before electrophysiological analysis in some experiments.

**Paper II.** The human consensus coding sequences (CCDS) of NEUROGENIN2 (henceforth referred to as NGN2), FEZF2 and SATB2 were synthesized (Genscript, CA) and cloned into EcoRI/BamHI site of tetO-FUW lentiviral vectors carrying resistance genes for blasticidin (Addgene plasmid #97330) and puromycin (Addgene plasmid #97329). The rtTA expressing lentiviral vector (Addgene plasmid #20342) was used along with vectors mentioned above to induce cortical fate in H1 ES cells. Cells were additionally transduced with FUW-tetO-GFP lentiviral vector before co-culture with adult human cortex organotypic slices.

On day -2, H1 cells were passaged using Accutase and 5x105 cells re-plated on MATRIGEL-coated 6-well plates in mTeSRTM1 medium with Rock Inhibitor (10 µM, Y-27632, StemCell Technologies). The day after (day -1), medium was replaced with fresh mTeSRTM1 containing polybrene (4 µg/ml, Sigma-Aldrich) and 1µL of each virus was added per well. One day after infection (day 0), medium was replaced with induction medium (DMEM/F12 (Life Technologies), supplemented with N2 (100x, Invitrogen). Doxycycline (2.5 µg /ml, Sigma) was added on day 0 to induce TetO gene expression, and kept in the medium until the end of experiment. On day 1, a 6-day puromycin (1.25 µg/ml) and blasticidin (1.25 µg/ml, Invitrogen)
selection period was started. On day 7, cells were dissociated with Accutase and replated onto poly-L-Ornithine (10 µg/ml, Sigma) – Laminin (10µg/ml, Invitrogen) coated glass coverslips in 24 well plates, along with mouse glia in maturation medium (Neurobasal, Life Technologies) supplemented with B27 (50x, Invitrogen) and Glutamax (250x, Invitrogen), containing BDNF (10 µg/ml, PeproTech). 5-Fluoro-2'-deoxyuridine (5-FUdR) (2 mg/ml) was added to the medium to inhibit glia proliferation on day 2 of co-culture. FBS (2.5%) was added on day 7 of the co-culture to support glia viability. 50 % of medium was replaced every 2-3 days, and cells were kept in co-culture for up to 8 weeks.

Paper III. Differentiation of NSPCs to cortical neurons was carried out as previously described (57). Briefly, growth factors (FGF, EGF) and B27 were omitted and NSPCs were cultured at low density in differentiation-defined medium (DDM) containing DMEM/F12 with glutamine (Sigma) and supplemented with N2 (1x), NMEAA (0.1 mM), sodium pyruvate (1 mM), bovine serum albumin (500 mg/ml) and 2-mercaptopethanol (0.1 mM), in the presence of bone morphogenetic protein 4 (BMP4) (10 ng/ml), wingless-type MMTV integration site family, member 3A (Wnt3A) (10 ng/ml) and cyclopamine (1 mM) for 8 days. Neural progenitors were then dissociated on day 9 using trypsin and plated on poly-L-ornithine/laminin-coated glass coverslips in BrainPhys/DDM (1:1) medium supplemented with B27 (1:50 without vitamin A, Invitrogen). For transplantation into stroke-injured rat brains or on human organotypic slices, NSPCs were transduced with lentiviral vector carrying green fluorescent protein (GFP) under constitutive promoter, cortically primed for 7 days, collected and resuspended into cytocon buffer or matrigel (BD Biosciences) respectively at a density of 100 000 cells/µl.

Derivation of human organotypic slice cultures

Adult human cortical tissue was obtained with informed consent from patients undergoing elective surgery for temporal lobe epilepsy (3 females and 2 males, aged 29 – 52 (Paper I); 3 females and 1 male, aged 27– 49 (Paper II); 2 females and 1 male, aged 27 – 49 years (Paper III)) according to guidelines approved by the Regional Ethical Committee, Lund (Dnr. H15 642/2008). The surgically resected tissue was immediately kept in ice-cold modified human artificial cerebrospinal fluid (mhACSF) and then glued to the slicing stage within the chamber of a Vibratome (Leica VT1200S) filled with ice-cold mhACSF. Adult human cortical slices (hACtx: Paper I; hACtxS: Paper II) of 300 um thickness were cut and kept in 24-well plates containing ice-cold mhACSF with one slice per well until sectioning was completed. Slices were then transferred to cell culture inserts Millicell cell (PICM03050, Millipore) (Paper I) or inserts containing Alvetex scaffold membranes (Reinnervate) in 6-well plates filled with slice culture medium (BrainPhys medium, Stemcell) supplemented with 2% B27,
Glutamax (1:200), Gentamycin (50 ug/ml) (Life Technologies)) and incubated in 5% CO2 at 37°C. Medium was changed once a week.

Co-culture of in vitro derived cortical neurons with adult human cortical organotypic slice

**Paper I.** The slices were transduced with SynI-RFP lentivirus and electrophysiological analysis was performed on RFP+ neurons. Non-transduced slice cultures were used for co-cultures with iCtx cells. The miR124.T-GFP reporter and SynI-RFP transduced 10 dpi HEFL and iCtx cells (BMF combination) were transplanted on top of the slices at two weeks after the start of culturing and then co-cultured for another three weeks before they were fixed in 4% PFA and assessed by immunocytochemistry. SynI-GFP+ iCtx cells, co-cultured with hACtx slices for three weeks, were used for electrophysiological recordings. Tracing experiment was performed on hACtx slices with lentivirus FUW-TetO-GFP at day 4-6 in culture. hACtx slices were washed with slice culture medium at least twice before co-culture with iCtx cells. We used a polycistronic lentiviral tracing vector (107) and plated newly formed iCtx cells (15 days after Dox application) on top of the slices that were kept 2 weeks in culture. At the end of additional three weeks in culture, slices were subjected to ΔG-rabies virus and 72h later fixed and stained.

**Paper II.** FUW-TetO-GFP transduced hES-iNs were plated on top of the slices at 5 days after the start of transgene induction, and co-cultured for another four weeks before they were either fixed in 4% PFA and assessed by immunohistochemistry, or used for electrophysiological recordings and subsequently fixed and stained.

**Paper III.** Cortically committed GFP+ NSPCs were detached at day 8 of differentiation and resuspended into 50 ul of pure matrigel previously thawed on ice. To avoid matrigel solidification during the transplantation procedure, all pipette tips and glass capillaries used, as well as the matrigel-GFP+ NSPC mix, were kept on ice. Human organotypic slices were kept in culture for at least one week before transplantation of GFP+ NSPCs. To facilitate the transplantation procedure, the medium was removed from the well, and the slice was left on the membrane of the insert. Around 10 ul of matrigel-GFP+ NSPC mix were collected into a cold glass capillary and injected in small drops stabbing the slice in various sites. After the matrigel was solidified (30 min), the medium was carefully added in the well. The medium was changed once a week and co-culture was kept for 4 to 8 weeks before electrophysiology recordings or fixation.
RNA extraction and qPCR

*Papers I and III.* Cells grown in 6 well plates were washed with ice-cold phosphate-buffered saline (PBS) twice after removal of culture media. Then, 350 µL of RLT buffer were used to lyse the cells in the plate, which were snap frozen on dry ice and stored at -80°C until extraction of RNA. RNA was extracted with RNeasy Micro kit (Qiagen) according to manufacturer’s instructions. RNA, extracted from human fetal cortical samples, dissected from aborted human fetuses at 6, 7, 8.5 and 11 weeks post-conception, was used as controls. cDNA was synthesized using iScript Advanced cDNA synthesis kit (Bio-Rad, CA). The cDNA was pre-amplified with TaqMan PreAmp Master Mix (Applied Biosystems, CA) according to manufacturer’s instructions with TaqMan assays (Applied Biosystems) that would be used for qPCR (Table 1). The pre-amplified cDNA was diluted 1:20 for qPCR reactions on iQ5 thermal cycler (Bio-Rad). Ct values of genes were normalized (ΔCt) with geometric mean of Ct values for GAPDH and βActin. Relative expression (ΔΔCt) to tau (MAPT) within each sample was plotted on Prism (GraphPad).

**Table 1.**
List of primers/probes used in qPCR analysis

<table>
<thead>
<tr>
<th>Target</th>
<th>Expression / Function in</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX2</td>
<td>Early NSPCs</td>
<td>Hs01053049_s1</td>
</tr>
<tr>
<td>NESTIN</td>
<td>Early NSPCs</td>
<td>Hs04187831_g1</td>
</tr>
<tr>
<td>DACH1</td>
<td>Early NSPCs</td>
<td>Hs00362088_m1</td>
</tr>
<tr>
<td>PLZF</td>
<td>Early NSPCs</td>
<td>Hs00957433_m1</td>
</tr>
<tr>
<td>PAX6</td>
<td>Early NSPCs</td>
<td>Hs00240871_m1</td>
</tr>
<tr>
<td>GFAP</td>
<td>Radial glia/astrocytes</td>
<td>Hs00909233_m1</td>
</tr>
<tr>
<td>P75</td>
<td>Neuronal crest</td>
<td>Hs00609976_m1</td>
</tr>
<tr>
<td>OCT4</td>
<td>Pluripotency</td>
<td>Hs01654807_s1</td>
</tr>
</tbody>
</table>

For single cell qPCR analysis (*papers I and II*), candidate genes related to cortical layer or region identity and neuronal function were curated from multiple sources (1, 3, 108). UBC, YWHAZ and TBP were included as housekeeping genes. COL1A1 was included to identify fibroblasts (*paper I*). Control genes normally expressed in dopaminergic, noradrenergic, serotonergic and hindbrain neurons were also included. A complete list of TaqMan assays is shown in Table 2.
<table>
<thead>
<tr>
<th>Target</th>
<th>Alias</th>
<th>Expression / Function in</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASCL1</td>
<td>MASH1</td>
<td>Diencephalon</td>
<td>Hs04187546_g1</td>
</tr>
<tr>
<td>MAP2</td>
<td></td>
<td>pan-neuronal</td>
<td>Hs00258900_m1</td>
</tr>
<tr>
<td>TUBB3</td>
<td>TUBULIN, beta</td>
<td>pan-neuronal</td>
<td>Hs00964963_g1</td>
</tr>
<tr>
<td>MAPT</td>
<td>Tau</td>
<td>Pan-neuronal</td>
<td>Hs00902194_m1</td>
</tr>
<tr>
<td>MYT1L</td>
<td></td>
<td>Pan-neuronal</td>
<td>Hs00903951_m1</td>
</tr>
<tr>
<td>DCX</td>
<td></td>
<td>Neuroblasts</td>
<td>Hs00167057_m1</td>
</tr>
<tr>
<td>SOX1</td>
<td></td>
<td>neural progenitors</td>
<td>Hs01057642_s1</td>
</tr>
<tr>
<td>SP8</td>
<td>BTD</td>
<td>neural progenitors</td>
<td>Hs01941366_s1</td>
</tr>
<tr>
<td>PAX6</td>
<td></td>
<td>neural progenitors</td>
<td>Hs00240871_m1</td>
</tr>
<tr>
<td>NEUROD1</td>
<td></td>
<td>telencephalon</td>
<td>Hs00159598_m1</td>
</tr>
<tr>
<td>NEUROG2</td>
<td></td>
<td>neural progenitors</td>
<td>Hs00702774_s1</td>
</tr>
<tr>
<td>COL1A1</td>
<td>OL4</td>
<td>Fibroblasts</td>
<td>Hs00164004_m1</td>
</tr>
<tr>
<td>CUX2</td>
<td>CDP2</td>
<td>Progenitor cells and upper-layer neurons</td>
<td>Hs00390035_m1</td>
</tr>
<tr>
<td>FOXG1</td>
<td></td>
<td>telencephalon</td>
<td>Hs01850784_s1</td>
</tr>
<tr>
<td>EMX2</td>
<td></td>
<td>neural progenitors</td>
<td>Hs00244574_m1</td>
</tr>
<tr>
<td>NR2F1</td>
<td>COUPTF1</td>
<td>temporal lobe (8-12 pcw)</td>
<td>Hs01354342_mH</td>
</tr>
<tr>
<td>CUX1</td>
<td>CUTL1</td>
<td>progenitor cells and upper-layer neurons</td>
<td>Hs00738851_m1</td>
</tr>
<tr>
<td>LIMCH1</td>
<td></td>
<td>layer 1-2 CPN</td>
<td>Hs00405524_m1</td>
</tr>
<tr>
<td>NECTIN3</td>
<td></td>
<td>layer 1-2 CPN</td>
<td>Hs00210045_m1</td>
</tr>
<tr>
<td>INHBA</td>
<td></td>
<td>layer 1-3 CPN</td>
<td>Hs00170103_m1</td>
</tr>
<tr>
<td>LMO4</td>
<td></td>
<td>layers 2/3 and 5, CPN</td>
<td>Hs01086790_m1</td>
</tr>
<tr>
<td>CHN2</td>
<td></td>
<td>layer 3 CPN</td>
<td>Hs00906969_m1</td>
</tr>
<tr>
<td>LPL</td>
<td></td>
<td>layer 4 CPN</td>
<td>Hs00173425_m1</td>
</tr>
<tr>
<td>EAG2</td>
<td>KCNH5</td>
<td>layer 4 (upper) CPN</td>
<td>Hs00544949_m1</td>
</tr>
<tr>
<td>RORB</td>
<td></td>
<td>layer 4 (lower) CPN</td>
<td>Hs00199445_m1</td>
</tr>
<tr>
<td>POU3F2</td>
<td>BRN2</td>
<td>Progenitor cells and upper-layer neurons</td>
<td>Hs00271595_s1</td>
</tr>
<tr>
<td>SATB2</td>
<td></td>
<td>upper layers and layer 5 &amp; neocortex</td>
<td>Hs01546828_m1</td>
</tr>
<tr>
<td>POU3F2</td>
<td>BRAIN2</td>
<td>Progenitor cells and upper-layer neurons</td>
<td>Hs00271595_s1</td>
</tr>
<tr>
<td>ETV1</td>
<td></td>
<td>layer 5 cortical-striatal PN</td>
<td>Hs00951951_m1</td>
</tr>
<tr>
<td>BHLHE22</td>
<td>BHLHB5</td>
<td>cortical-spinal/motor neurons</td>
<td>Hs01084964_s1</td>
</tr>
<tr>
<td>DIAPH3</td>
<td></td>
<td>cortical-spinal/motor neurons</td>
<td>Hs01107330_m1</td>
</tr>
<tr>
<td>CNTN6</td>
<td></td>
<td>layer 5b cortical-spinal/motor neurons</td>
<td>Hs00207291_m1</td>
</tr>
<tr>
<td>PCP4</td>
<td></td>
<td>layer 5b cortical-spinal/motor neurons</td>
<td>Hs01113638_m1</td>
</tr>
<tr>
<td>S100A10</td>
<td></td>
<td>layer 5</td>
<td>Hs00741221_m1</td>
</tr>
<tr>
<td>SOX5</td>
<td></td>
<td>cortical-spinal/motor neurons</td>
<td>Hs00753050_s1</td>
</tr>
<tr>
<td>OTX1</td>
<td></td>
<td>Cortical-spinal/motor neurons</td>
<td>Hs00951099_m1</td>
</tr>
<tr>
<td>CRYM</td>
<td></td>
<td>deep-layer subcerebral PN</td>
<td>Hs00157121_m1</td>
</tr>
<tr>
<td>FEZF2</td>
<td></td>
<td>telencephalon</td>
<td>Hs01115572_g1</td>
</tr>
<tr>
<td>BCL11B</td>
<td>CTIP2</td>
<td>deep-layer neurons</td>
<td>Hs00256257_m1</td>
</tr>
<tr>
<td>TBR1</td>
<td></td>
<td>layer 1 and deep-layer neurons &amp; telencephalon (8-12 pcw)</td>
<td>Hs00232429_m1</td>
</tr>
<tr>
<td>TLE4</td>
<td></td>
<td>deep-layer &amp; CthPN</td>
<td>Hs00419101_m1</td>
</tr>
<tr>
<td>FOXP2</td>
<td></td>
<td>layer 6 CthPN</td>
<td>Hs01074133_m1</td>
</tr>
<tr>
<td>FOG2</td>
<td>ZFPM2</td>
<td>layer 6 CthPN</td>
<td>Hs01101779_m1</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Gene Name</td>
<td>Function</td>
<td>GenBank Accession</td>
</tr>
<tr>
<td>------------</td>
<td>-----------</td>
<td>----------</td>
<td>------------------</td>
</tr>
<tr>
<td>PLEXIND1</td>
<td>PLXND1</td>
<td>layer 5 CPN</td>
<td>Hs00892417_m1</td>
</tr>
<tr>
<td>DKK3</td>
<td>layer 6 CPN</td>
<td>Hs00247429_m1</td>
<td></td>
</tr>
<tr>
<td>NR4A2</td>
<td>NURR1</td>
<td>dopaminergic/midbrain</td>
<td>Hs00428691_m1</td>
</tr>
<tr>
<td>TH</td>
<td>dopaminergic/midbrain</td>
<td>Hs00165941_m1</td>
<td></td>
</tr>
<tr>
<td>TPH1</td>
<td>serotonergic</td>
<td>Hs00188220_m1</td>
<td></td>
</tr>
<tr>
<td>HTR2C</td>
<td>serotonergic &amp; layer 5</td>
<td>Hs00168365_m1</td>
<td></td>
</tr>
<tr>
<td>LBX1</td>
<td>hindbrain</td>
<td>Hs00198080_m1</td>
<td></td>
</tr>
<tr>
<td>FOXO1</td>
<td>layer 5b &amp; hindbrain</td>
<td>Hs01054576_m1</td>
<td></td>
</tr>
<tr>
<td>DBH</td>
<td>adrenergic</td>
<td>Hs01089840_m1</td>
<td></td>
</tr>
<tr>
<td>SLC17A7</td>
<td>VGlut1</td>
<td>glutamate transport</td>
<td>Hs00220404_m1</td>
</tr>
<tr>
<td>SLC17aA6</td>
<td>VGlut2</td>
<td>glutamate transport</td>
<td>Hs00220439_m1</td>
</tr>
<tr>
<td>GAD1</td>
<td>GAD67</td>
<td>GABA synthesis</td>
<td>Hs01065893_m1</td>
</tr>
<tr>
<td>GAD2</td>
<td>GAD65</td>
<td>GABA synthesis</td>
<td>Hs00609534_m1</td>
</tr>
<tr>
<td>UBC</td>
<td>UBIQUITINC</td>
<td>control</td>
<td>Hs00824723_m1</td>
</tr>
<tr>
<td>YWHAZ</td>
<td></td>
<td>control</td>
<td>Hs03044281_g1</td>
</tr>
</tbody>
</table>

pcw – post conception week

Single-cell suspension was generated using accutase dissociation followed by labeling for NCAM and CD44. Cells were sorted on a FACSARiaI cell sorter (BD Biosciences). Gates were set to include only live (Draq7 (Abcam) negative) cell population. CD44+/NCAM+/RFP+ single cells were sorted into a 96-well PCR plate containing a lysis buffer (0.4% NP40, deoxynucleoside triphosphates, dithiothreitol, and RNase OUT (Invitrogen)) and snap frozen. On thawing, CellsDirect reaction mix containing SSIII/PlatinumTaq (CellsDirect One-Step RT_qPCR kit, no ROX, Invitrogen) and 48 TaqMan assays to a final dilution of 0.05× each were added to the cell lysate for RT-PCR pre-amplification. Zero-, 10- and 50-cell controls were included. RT–PCR pre-amplification cycling conditions were: 50 °C, 60 min; 95 °C, 2 min; 25×(95 °C, 15 s; 60 °C, 4 min). Individual TaqMan assays were combined with assay loading reagent (Fluidigm) before being pipetted onto a Biomark chip (Fluidigm). The cDNA from the 96-well PCR plate was diluted with water 1 in 5, combined with sample loading reagent (Fluidigm) and loaded onto the same Biomark chip. This was run on a Biomark analyser system (Fluidigm). The arrays were read in a Biomark genetic analysis system (Fluidigm) and the data exported into Microsoft Excel for downstream analysis. The amplification curves were quality-controlled and the data filtered according to no-reverse-transcription control reactions and to exclude Ct>25. Detection thresholds (109) were automatically generated using a baseline linear correction model and a quality threshold of 0.65.

The data was analyzed in Singular (Fluidigm, CA) package in R for hierarchical clustering analysis and single cell expression visualizer SCExV (110) for principal component analysis. A well was defined as containing a cell that had been successfully reverse transcribed if there were detectable levels (Ct < 25) of at least two out of three housekeeping genes. Genes detected in no template controls were excluded from further analysis.
We used 3 independent viral transduction experiments in order to generate induced neuronal cells and collect samples for bulk qPCR experiments. For single cell qPCR we sorted cells from 2 independent viral transduction experiments and used 4 (paper I) or 6 (paper II) chips of 48x48 (Fluidigm) to run the analysis.

**Immunocytochemistry**

Cell cultures were washed with PBS and fixed in 4% PFA for 20 min. The coverslips were washed three times with PBS, permeabilized with 0.025% TritonX-100 in PBS for 10 min, and then blocked with 5% normal donkey serum for 45 min at room temperature. Primary antibodies (Table 2), diluted in blocking solution were applied overnight at 4°C. For SATB2 and BRAIN2 immunostaining, antigen retrieval with 10mM Citrate buffer (pH 6.0) was performed. After three rinses with PBS, Alexa488, Cy3 and Cy5 conjugated donkey or goat secondary antibodies (1:500, Jackson Immunoresearch, PA) against the respective primary antibodies diluted in blocking solution were applied for 1.5 h. Streptavidin-conjugated Alexa647 was used for labeling cells filled with biocytin from electrophysiology experiments. Cell nuclei were counterstained with Hoechst33342 and mounted with PVA-Dabco.

Acute rat brain slices were fixed overnight at 4°C in 4% PFA and rinsed three times in 0.1 M KPBS. Then they were incubated in blocking solution for 1 h (5% NDS and 0.25% Triton X-100 in KPBS). The rest of the procedure follows that outlined above for cell cultures. The list of primary antibodies used can be found in the Table 3.
Table 3.
List of primary antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP2</td>
<td>Mouse</td>
<td>1:500</td>
<td>Sigma</td>
</tr>
<tr>
<td>MAP2</td>
<td>Chicken</td>
<td>1:5000</td>
<td>Abcam</td>
</tr>
<tr>
<td>βIII-tubulin</td>
<td>Mouse</td>
<td>1:500</td>
<td>Covance, NJ</td>
</tr>
<tr>
<td>βIII-tubulin</td>
<td>Mouse</td>
<td>1:2000</td>
<td>Sigma</td>
</tr>
<tr>
<td>βIII-tubulin</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>Covance, NJ</td>
</tr>
<tr>
<td>RFP</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>PSD95</td>
<td>Mouse</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
<tr>
<td>Tbr1</td>
<td>Rabbit</td>
<td>1:300</td>
<td>Abcam</td>
</tr>
<tr>
<td>TBR1</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>ProteinTech</td>
</tr>
<tr>
<td>TBR1</td>
<td>Chicken</td>
<td>1:1000</td>
<td>Millipore</td>
</tr>
<tr>
<td>CTIP2</td>
<td>Rat</td>
<td>1:500</td>
<td>Abcam</td>
</tr>
<tr>
<td>SATB2</td>
<td>Mouse</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
<tr>
<td>SMI 311</td>
<td>Mouse</td>
<td>1:400</td>
<td>Covance, NJ</td>
</tr>
<tr>
<td>GFAP</td>
<td>Goat</td>
<td>1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rabbit</td>
<td>1:400</td>
<td>Zymed</td>
</tr>
<tr>
<td>GFAP</td>
<td>Mouse</td>
<td>1:400</td>
<td>Sigma</td>
</tr>
<tr>
<td>GFP</td>
<td>Chicken</td>
<td>1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>BRAIN2</td>
<td>Goat</td>
<td>1:400</td>
<td>SantaCruz</td>
</tr>
<tr>
<td>Oct4</td>
<td>Mouse</td>
<td>1:50</td>
<td>SantaCruz</td>
</tr>
<tr>
<td>Nanog</td>
<td>Rabbit</td>
<td>1:150</td>
<td>Abcam</td>
</tr>
<tr>
<td>GFAP</td>
<td>Chicken</td>
<td>1:3000</td>
<td>Merk Millipore</td>
</tr>
<tr>
<td>Sox2</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Merk Millipore</td>
</tr>
<tr>
<td>Zo-1</td>
<td>Mouse</td>
<td>1:200</td>
<td>Lifetech</td>
</tr>
<tr>
<td>Pax6</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Covance</td>
</tr>
<tr>
<td>NeuN</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>Abcam</td>
</tr>
<tr>
<td>S100β</td>
<td>Rabbit</td>
<td>1:400</td>
<td>DAKO</td>
</tr>
<tr>
<td>Nestin</td>
<td>Mouse</td>
<td>1:500</td>
<td>Millipore</td>
</tr>
<tr>
<td>Synl</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Millipore</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Chicken</td>
<td>1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>STEM101</td>
<td>Mouse</td>
<td>1:200</td>
<td>Stem Cell inc</td>
</tr>
</tbody>
</table>

For the adult human slice cultures and their co-cultures with induced cells, slices were fixed in 4% PFA overnight and then rinsed three times in KPBS for 15 min. The slices were then permeabilized in KPBS with 0.02% bovine serum albumin (BSA) and 1% Triton X-100 at 4°C over night, blocked in KPBS with 0.2% Triton X-100 and 1% BSA and 5% NDS or normal goat serum (NGS) at 4°C over night. After blocking step, slices were incubated for 48 h with primary antibodies and then for an additional 48 h with respective secondary antibodies in blocking solution at 4 °C. The slices were washed three times in KPBS and incubated in Hoechst 33342 for 15 min at room temperature. Slices were mounted on glass slides with PVA-Dabco after rinsing with deionised water.
Microscopy

Images were obtained on BX61 epifluorescence (Olympus, Japan) and LSM780 confocal (Zeiss, Germany) microscopes. Optical sections were made and z-stacks were rendered for 3D reconstruction using Imaris (Bitplane, Switzerland) (Paper I).

Immunoelectron microscopy (paper III)

Adult human organotypic slice cultures were fixed with 2% PFA and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, cryoprotected, freeze thawed in liquid nitrogen, and incubated overnight in primary goat anti-GFP antibody (1:500, Novus Biologicals) at +4 °C. Slices were then incubated at room temperature for 2 h with biotinylated rabbit anti–goat secondary antibody (1:200, DakoCytomation), and avidin biotin peroxidase complex (ABC) (Vector Laboratories) followed by 3,3-diaminobenzidine tetrachloride (DAB) and 0.015% hydrogen peroxide. Following DAB reaction, slices were processed for electron microscopy. Immunostained slices were postfixed in 1% osmium tetroxide in 0.1M phosphate buffer (PB), dehydrated in a graded series of ethanol and propylene oxide, and flat-embedded in Epon. For the identification of GFP/DAB-labeled synaptic contacts, ultrathin sections were cut with a diamond knife and then counterstained with lead citrate and uranyl acetate. Ultrathin sections were mounted on grids, examined and photographed using a transmission electron microscope JEM-100CX (JEOL, Japan). Synapses were defined by the presence of at least two to three synaptic vesicles in presynaptic terminal, a postsynaptic density in postsynaptic structure and synaptic cleft.

Conversion efficiency (paper I)

In vitro quantification was done in at least 20 regions of interest from three different coverslips per condition in an epifluorescence microscope. Total number of MAP2/βIII-tubulin’ cells with neuronal morphology was quantified at 25-30 dpi. Conversion efficiency was calculated as previously described (78, 82). 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 a number of plated cells during transduction to get the percentage of starting population of cells that acquired neuron-like characteristics.
**Immunoreactivity assay (paper I)**

Assessment of MAP2/βIII-tubulin immunoreactivity was done using cellSens Dimension (Olympus, Japan) imaging software, to determine the neurite density. Twenty 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 µm^2). 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 µm^2/cell) to obtain a measure of neurite density.

**Pyramidal morphology index (papers I and II)**

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 (111) in CellSens imaging software (Olympus, Japan). To determine the PMI, at least 25 induced cells of each type (BMF-induced iCtx or N, NF and NS-induced hES-iNs), as well as cultured fetal and adult human cortical neurons 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 multipolar cells that had more than two processes.

**Cell soma size (papers I and II)**

The cell soma size was measured from 25 cells randomly chosen from 3 coverslips of induced cells of each type (BMF-induced iCtx or N, NF and NS-induced hES-iNs), as well as cultured fetal and adult human cortical neurons in CellSens imaging software. Images were obtained using 20X objective and the outline of the cell body was delineated manually. The area corresponding to the cell soma was calculated by the software and used for analyzing the average cell soma size.

**Neuronal morphology tracing (papers I and II)**

Neuronal morphology was assessed for 3 representative induced cells of each type (BMF-induced iCtx or N, NF and NS-induced hES-iNs), as well as cultured fetal and adult human cortical neurons using simple neurite tracer plugin in Fiji (112). The outlined areas were filled out and then converted to 8-bit monochrome image in Fiji.
Electrophysiology

Whole-cell patch-clamp recordings were performed with a HEKA double patch clamp EPC10 amplifier using PatchMaster for data acquisition. Cells were grown on coverslips and transferred to the recording chamber. The coverslip was constantly perfused (1ml/min) with carbogenated artificial cerebrospinal fluid (aCSF, in mM: 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 25 glucose, (11 glucose for recordings in hACtx slices), pH 7.2-7.4, 295-300 mOsm) at 34oC. Recording pipettes were filled with intracellular solution containing (in mM): 122.5 KGlu, 12.5 KCl, 10.0 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 EPSCs, and 135 CsCl, 10 HEPES, 10 NaCl, 2 MgATP, and 0.3 NaGTP for recordings of evoked IPSCs and sPSC. Intracellular solutions had pH of 7.2-7.4, osmolarity of 285-295 mOsm, and resistance of 2.5-9.5 MΩ. 2-4 mg/ml biocytin were added to the internal solution prior recording for post hoc identification of the recorded cell. 5 mM QX314 was added to the internal solution before recording of sPSCs. Voltage values were not corrected for the liquid junction potential, which was 13.82 mV, 15.55 mV and 5.10 mV for KGlu-, CsGlu- and CsCl-based internal solutions, respectively. Only cells with a series resistance below 30 MΩ were included in the analysis (BMF-induced cells: Rseries = 15.0 ± 1.2 MΩ, n = 29).

Acute human cortical (hCtx) brain slices (Paper III) were prepared as previously described (113). Briefly, human cortical tissue resected during hippocampectomy was collected and sliced in a solution containing (in mM): 200 sucrose, 21 NaHCO₃, 10 glucose, 3 KCl, 1.25 NaH₂PO₄, 1.6 CaCl₂, 2 MgCl₂ and 2 MgSO₄, pH ~7.4. During recordings, acute hCtx slices and organotypic hCtx brain slices were perfused with carbogenated human ACSF (hACSF, in mM: 129 NaCl, 21 NaHCO₃, 10 glucose, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄ and 1.6 CaCl₂, pH ~7.4).

Voltage- and current-clamp recordings were used for 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 1 µM tetrodotoxin (TTX) and 10 mM tetroethylammonium (TEA), respectively was determined. A series of current steps (0- 200, 400 or 1000 pA in 10, 20 or 50 pA steps) lasting 500 ms were performed from a membrane potential of ~-70mV (current was injected when needed to keep the membrane potential ~-70mV) to determine the cell’s ability to generate action potentials (APs). EPSCs and IPSCs were evoked by puff application (0.5-0.75 bar) of 100 mM glutamate or 100 mM GABA lasting 0.5-1s using a pneumatic drug ejection system (PDES-02DE-2). Spontaneous postsynaptic currents (sPSCs) were recorded from a holding potential of -70 mV. AMPA and NMDA receptors were blocked by 5 µM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) and 50 µM D-(-)-2-Amino-5-
phosphonopentanoic acid (D-APV), respectively. GABA receptors were blocked by 0.1 mM picrotoxin (Ptx). Data were analyzed offline with Fit-Master, IgorPro 6.3 and NeuroMatic v2.8b.

**Data representation and statistics**

Statistical analysis was performed using GraphPad Prism (GraphPad software, La Jolla, CA), by One-way ANOVA followed by Tukey’s multiple comparisons test or Uncorrected Fisher’s LSD, Kruskal-Wallis test followed by Dunn’s multiple comparison test (Paper I), or one sample t-test or Mann-Whitney test (Paper I and III). Significance was set at p < 0.05. Data were presented as mean ± SEM.
Results

Derivation of functional excitatory cortical neurons from human fibroblasts (*paper I*)

Defining a combination of TFs instructive of cortical fate in human fibroblasts

For this purpose we chose nine different combinations of TFs that are cortical layer- and development-specific including Brn2 (B), Myt1l (M) along with Fezf2 (F), Ctip2 (C), NeuroG2 (Ngn2), and NeuroD1 (Nd). We transduced human fetal lung fibroblasts (HEFL) with lentiviral vectors carrying the different combinations of TFs and characterized newly formed cells 25-30 days post induction (dpi) by their morphology, electrophysiological recordings and cortical marker expression. We found that all combinations of TFs gave rise to neuronal marker MAP2 positive cells with neuronal morphology, firing one or more action potentials. We singled out BRN2 as an important component in HEFL conversion to neurons as the majority (62-89%) of MAP2⁺ cells generated multiple APs in the presence of this TF. We also found that BMNd-, BMF-, and BMC-induced cells predominantly expressed the deep-layer projection neuronal markers SOX5, FEZF2, CTIP2, OTX1, and TBR1 and callosal projection neuronal marker SATB2, as evidenced by qPCR experiments. Importantly, BMF-induced neuronal cells (henceforth referred to as iCtx) expressed some of projection neuronal markers, such as SOX5, CTIP2 and OTX1, at comparatively higher levels and had electrophysiological properties similar to those of human fetal cortical neurons (hCtx). Therefore, we chose BMF as a potential TF combination for deriving iCtx cells from HEFL and characterized them in more detail.

Single cell analysis reveals common molecular features between iCtx cells and human fetal primary cortical neurons

We analyzed 61 iCtx cells derived using BMF combination by single cell qPCR and compared their molecular profile with hCtx cells. The principal component analysis (PCA) identified three main populations of iCtx cells and one of those populations (13.1% of iCtx cells) shared a gene expression profile with hCtx cells. In addition, iCtx
cells expressed some of cortical markers such as EMX2, PAX6, FOXG1 and RORB at levels comparable to those in hCtx cells. Other two populations of iCtx cells grouped either with HEFL cells or with both HEFL and hCtx cells. This suggests that a part of iCtx cells failed to reprogram (iCtx cells grouping with HEFL) or were only partially reprogrammed (iCtx cells grouping with HEFL and hCtx).

**Human iCtx cells mature to functional neurons and form synaptic contacts with human cortical neurons in vitro and in organotypic slice cultures**

Next, we co-cultured BMC-induced iCtx cells with hCtx cells for 3 weeks to promote their maturation. We found no differences of iCtx cell morphology and soma size, two hallmark morphological characteristics of cortical projection neurons, when compared to hCtx cells. More importantly, we found by immunocytochemistry that some iCtx cells expressed the callosal projection (CP) neuron marker SATB2 and the deep-layer projection neuronal markers CTIP2 and TBR1. These data indicate that iCtx cells adopt a phenotype resembling that of developing human cortical projection neurons.

We then tested in whole-cell patch-clamp recordings whether iCtx cells expressed functional glutamate and GABA receptors. We found glutamate-evoked excitatory postsynaptic current (EPSC) in 88% of iCtx cells generated by activation of NMDA or AMPA receptors, as evidenced by D-APV and NBQX inhibition respectively. Moreover, a similar proportion of iCtx cells (75%) had GABAA–evoked inhibitory postsynaptic current (IPSC) events that could be blocked by picrotoxin (Ptx). In addition, we found fast decaying, glutamatergic-like spontaneous postsynaptic currents (sPSCs) in 38% of iCtx cells that could be abolished by Ptx, D-APC and NBQX. Therefore, we provide evidence that iCtx cells develop to functionally mature neurons that form synaptic connections with hCtx cells.

To assess whether iCtx cells could integrate into adult human cortical circuitry, we used human adult cortex (hACtx) organotypic slice cultures obtained from epileptic patients undergoing hippocampal resection surgery. First, we found that hACtx slices were viable and resembled the in vivo conditions even after extended time in culture (4-6 weeks), as evidenced by maintaining the characteristic cell morphology, expression of pan-neuronal markers (Fox3 and SC121) and generation of at least one AP in whole-cell patch-clamp recordings. Next, we co-cultured iCtx cells with hACtx slices (kept for up to 2 weeks in culture) for 3 weeks and found that iCtx cells had survived, integrated morphologically, and extended neurites throughout the adult human cortical tissue. In addition, iCtx cells contained a variety of spines with different size and shape and were functional as they had the capability to generate APs. Finally, we used retrograde monosynaptic tracing by a modified rabies virus (ΔG-rabies) (114, 115) to explore if the transplanted iCtx cells receive afferent synaptic input from host neurons in the organotypic slice cultures. In order to visualize host cells, we transfected hACtx slices...
with FUW-TetO-GFP (tissue cells expressing cytoplasmic GFP (GFP\textsuperscript{cyto})) lentivirus. We then transplanted TVA receptor-expressing iCtx cells on the organotypic slices. Three weeks after \textit{ex vivo} transplantation, iCtx cells were selectively infected with \textDelta G-rabies virus carrying SynI-mCherry gene, and three days later fixed and immunostained. We found many mCerry\textsuperscript{+/+} GFP\textsuperscript{−} cyto host neurons and mCerry\textsuperscript{+/−}/GFP\textsuperscript{−} cyto neurons that could be either host or iCtx cells. This suggests that both of these groups of neurons had formed synapses on iCtx cells. Taken together, our findings indicate that \textit{ex vivo} transplanted iCtx cells received afferent synapses from adult human cortical neurons.

Derivation of functional excitatory cortical neurons from human embryonic stem cells (\textit{paper II})

\textbf{Three TF combinations direct human ES cells to cortical neurons morphologically resembling adult human cortical cells}

We followed a standard protocol of TF programming described by Zhang and colleagues (98) in order to specify the fate of ES cells. We combined NGN2, known to rapidly drive human ES cells towards excitatory neuronal fate, with either SATB2 or FEZF2. We delivered the TFs by doxycycline (Dox)-inducible lentiviral vectors carrying TFs coupled to resistance genes, and plated newly formed hES-iNs onto mouse glia after 7 days of antibiotic selection for long-term culture. All combinations of TFs had produced neuron-like cells with mature neuronal morphology expressing pan-neuronal marker MAP2 at two weeks. When we compared morphometric features of hES-iNs co-cultured with mouse astrocytes for 8 weeks with cultured fetal (hFCtx\textsuperscript{C}) and adult human cortical (hACtx\textsuperscript{C}) neurons. We found that all three combinations gave rise to hES-iNs cells with soma size and pyramidal morphology index (PMI) (111) higher than that of hFCtx\textsuperscript{C} but similar to hACtx\textsuperscript{C} cells. Also, most hES-iNs had pyramidal shape and rich harborization resembling that of hACtx\textsuperscript{C} cells more than hFCtx\textsuperscript{C} neurons. This suggested that the hES-iNs were more mature than hFCtx\textsuperscript{C} cells. In addition, immunocytochemistry analysis revealed a different proportion of MAP2\textsuperscript{+} hES-iNs expressing upper layer callosal projection neuron (CPN) marker SATB2 between TF combinations: NS (around 15\% SATB2\textsuperscript{+} cells), NF (9\% SATB2\textsuperscript{+} cells) and N (4\% SATB2\textsuperscript{+} cells). Some NF-derived hES-iNs expressed the deep layer marker TBR1 (2\%) and some the upper layer marker BRAIN2 (4\%), but not N- or NS-derived hES-iNs.
Three TF combinations induce similar molecular and functional phenotype in human ES cells

We analyzed 189 hES-iNs (N: 51; NF: 43; NS: 40) by single cell qPCR using Fluidigm platform and compared their molecular profile with hFCtxF (55 cells) cells. We found that all cells expressed pan-neuronal markers MAP2 or beta-III-Tubulin and the majority of them were glutamatergic, as revealed by vGLUT2 expression (hFCtxF: 79%; N: 67%; NF: 55% and S: 15%). Importantly, we detected expression of neural progenitor markers such as PAX6, NEUROD1 and EMX2 in hFCtxF cells but not in hES-iNs. Surprisingly, we found expression of both upper and deep layer cortical markers in single hES-iNs and hFCtxF cells. Upper layer CPN markers such as CUX1, LMO4, NECTIN3, and BRAIN2 were expressed by most hFCtxF cells and hES-iNs with CUX1 and NECTIN3 expression being at relatively higher levels in hES-iNs than the hFCtxF cells. We detected expression of deep-layer cortico-spinal PN marker SOX5 in all analyzed cell populations, but cortico-striatal PN marker ET1 and the subcerebral PN markers FEZF2, CTIP2 (BCL11B) and TBR1 only in hFCtxF cells, and not hES-iNs. In contrast, a low relative expression level of deep-layer CPN marker DKK3 was detected in a small portion (26%) of hFCtxF cells whereas it was at relatively high expression level in most N- and NS-derived hES-iNs (78% and 65%, respectively), and 23% of NF-derived hES-iNs. These findings indicated that the tested TF combinations gave rise to excitatory cortical neurons with a mature neuronal gene expression pattern, surpassing that of hFCtxF cells.

Maturation and integration of cortical neurons generated by three TF combinations in vitro and after grafting onto organotypic slice cultures of adult human cortex

Whole-cell patch-clamp recordings were performed from hES-iNs co-cultured with mouse glia cells for 4 and 8 weeks. We found that the resting membrane potential (V_{rest}) of NF- and NS-derived hES-iNs reached a plateau already after 4 weeks of culturing as compared to N-derived hES-iNs, which suggests faster maturation of NF- and NS-derived hES-iNs. Also, hES-iNs from all three TF combinations, were able to generate multiple APs upon current injection, indicating that these cells become functional neurons after 4 weeks of culturing. Moreover, we detected spontaneous activity of hES-iNs after 8 weeks in culture, providing evidence for their capacity to establish functional afferent synaptic inputs.

Next, wanted to explore whether hES-iNs can integrate into human neural networks. For this purpose, we transplanted hES-iNs onto organotypic slice cultures of adult human cortical tissue, obtained from epileptic patients. We kept hES-iNs in co-culture with organotypic slices for 4 weeks and then assessed them by immunohistochemistry and electrophysiological analysis. We found that many hES-iNs, derived by all three
TF combinations, survived and extended neurites not only throughout the slice surface area but could also be found deeper within the tissue as revealed by confocal microscopy. Some grafted cells expressed deep-layer marker TBR1 (NF-derived hES-iNs), whereas other cells were positive for SATB2 (N- and NS-derived hES-iNs). Whole-cell patch-clamp recordings revealed that hES-iNs could fire multiple APs and had spontaneous activity after 32-36 days in co-culture with organotypic slices. Taken together, these finding provide the first evidence that TF programmed cells can survive and integrate in the adult human brain.

Human iPSC-derived cortical neurons integrate into adult human cortical neural circuitry (paper III)

Differentiation of human iPSC-derived NSPCs to functional, cortical-like neurons in vitro and after transplantation into stroke-injured rat cortex

We generated a new iPSC-derived NSPC cell line by optimizing the developed protocol (54) and combined it with dual SMAD inhibition for more efficient neural conversion (102, 103). We confirmed the identity of our NSPCs by immunocytochemistry and qPCR for expression of early NSPC markers such as Sox2, Nestin, Dach1, PLZF and Zo-1 and absence of glial marker GFAP, the pluripotency marker Oct4 and the neural crest marker p75. These characteristics were in line with those previously described for the lt-NES cell line (55). We committed the newly generated NSPC line to cortical neurons as described by Tornero and co-workers (57). Immunocytochemistry analysis revealed that at 4 weeks of in vitro differentiation, the cell cultures consisted of populations of immature glia (S100β+), neurons (beta III-Tubulin+) and Nestin positive cells. However, after additional 4 weeks in culture (8 weeks time-point), cells expressed mature neuronal markers MAP2 and NeuN, and synaptic marker Synapsin I, indicating overall maturation of the culture. We confirmed the cortical identity of the neuronal population by TBR1 staining, as previously used for cortically fated neurons generated from human iPSC-derived lt-NES cells (57).

Next, we performed whole-cell patch-clamp recordings on cortically fated NSPCs (ctx-NSPCs) at 4 and 10 weeks in vitro and found basic electrophysiological characteristics similar at both time-points and comparable to our previous findings (57). The ctx-NSPCs were able to produce a few APs already at 4 weeks and generated trains of APs at 10 weeks. In addition to increased number of APs, the current needed to induce maximum number of APs shifted, and the shape of APs altered (i.e., AP amplitude, rise time and ½ amplitude width), providing strong evidence that the ctx-NSPCs had developed into more mature neurons after 10 weeks in culture. Moreover, we observed that 1 out of 8 analyzed ctx-NSPCs had spontaneous activity after 10 weeks of differentiation, providing additional evidence that these cells become functional neurons and are capable of forming synaptic connections in vitro.
Finally, we transplanted cortically fated NSPCs into stroke-injured rat cortex. Eight weeks after transplantation, we found that the electrophysiological characteristics of ctx-NSPCs were similar to those observed for ctx-NSPCs in vitro after 10 weeks of differentiation. Moreover, grafter ctx-NSPCs were able to fire multiple APs upon current injection, 8 weeks after implantation. Also, most of AP characteristics, such as AP threshold, amplitude, rise time and AHP were lower than those detected in ctx-NSPCs differentiated in vitro. Taken together, these results demonstrate that ctx-NSPCs can differentiate to mature functional neurons in vivo after grafting into stroke-injured rat cortex.

Grafted human iPSC-derived cortical neurons establish afferent inputs from the adult human cortical neurons in human organotypic cortical slices

We transplanted GFP+ ctx-NSPCs onto the human organotypic slice cultures and assessed their ability to integrate into adult human cortical circuitry 4 and 8 weeks after transplantation. We found that both neurons and astrocytes within the slices were preserved after an extensive in vitro culture, evidenced by positive NeuN, GFAP and Vimentin immunostainings. Also, the transplanted ctx-NSPCs survived long-term co-culture with adult human organotypic slices and had complex morphology, extending neurites throughout the whole organotypic slice area.

Immune-electron microscopy (iEM) showed that the grafted ctx-NSPCs received axodendritic or axospinous inputs from GFP– host axon terminals. Newly formed synaptic contacts had typical features of synapses such as clustering of synaptic vesicles close to the presynaptic membrane, synaptic cleft and postsynaptic membrane. At 4 weeks after transplantation, GFP+ spines of ctx-NSPC-derived neurons made contact with multiple GFP– axon terminals, whereas at 8 weeks after transplantation only with a single. The synaptic inputs, observed at this time-point were mature axodendritic (96.2%) or axosomatic (3.8%) synaptic contacts. The iEM analysis revealed that axodendritic contacts had a characteristic ultrastructure of asymmetric excitatory/glutamatergic synapses. These data show that the transplanted ctx-NSPC-derived neurons established afferent inputs from the adult human cortical neurons.

The whole-cell patch-clamp recordings revealed that ctx-NSPC-derived cells were able to fire multiple APs already at 4 weeks, and the main AP characteristics (AP threshold, AP amplitude, AP rise time, etc.) remained similar at 4 and 8 weeks after ex vivo transplantation. More importantly, we detected spontaneous post-synaptic currents in grafted cells at both time-points. These findings provide further evidence that the human ctx-NSPCs differentiate to mature functional neurons and receive synaptic inputs from neighboring cells after transplantation onto adult human cortical tissue.
Discussion

In this thesis, we report three main findings. First, combination of three transcription factors, BRAIN2, MYT1L, and FEZF2, have the ability to directly convert human fibroblasts to functional excitatory cortical neurons. A subpopulation of those cells display a molecular signature closely resembling that of human fetal primary cortical neurons and have the capacity for synaptic integration into human neural networks. Second, TF programming can direct human ES cells towards cortical neurons, morphologically resembling adult human cortical neurons, expressing cortical PN markers, and with mature electrophysiological properties. Grafting onto organotypic slice cultures suggests that these hES-iNs are also capable of integrating into adult human cortical neural circuitry. Third, a new human iPSC-derived NSPC line, committed to cortical neurons, differentiate into functional cells with the ability to form synaptic connections in vitro as well as after transplantation into stroke-injured rat cortex. The iPSC-derived NSPCs are also capable of forming functional afferent synapses with adult cortical neurons after ex vivo transplantation onto adult human cortical slices, therefore, integrate into adult human cortical neural circuitry.

Instructing cortical fate in somatic and pluripotent cells

Forced expression of lineage-specific TFs has previously been used to directly convert somatic cells to specific subtypes of neurons, including dopaminergic (83, 85), serotonergic (88, 89), spinal motor (86), cholinergic (87), and striatal medium spiny neurons (90), but not cortical pyramidal neurons. Although several studies have reported generation of excitatory (VGLUT1+) neurons regardless of the reprogramming cocktail used (78, 82, 91, 116), it is not clear whether these cells represent a specific subtype of excitatory neurons. We show, for the first time, direct conversion of human fibroblasts to cortical neurons. For this purpose we chose TFs based on current knowledge of cortical development in order to specifically generate induced cortical (iCtx) neurons. Among nine different combinations of TFs tested, we singled out BMF as a potential candidate to drive cortical fate. This combination differs from ABM (78), which was initially used to derive neurons from mouse fibroblasts in the seminal study of Vierbuchen and co-workers, with respect to ASCL1 and another factor, namely FEZF2. This factor has been reported to be necessary and sufficient for cortical
progenitors to generate deep-layer neurons and is specifically expressed in layer V neurons throughout life (18, 117). Also, it has been shown to reprogram layer IV neurons to layer V identity in vivo (118, 119). Thus, FEZF2 is a key TF that could potentially override the default program in precursor cells to generate cortical pyramidal neurons, especially of the deep-layer subtype.

Besides direct reprogramming, transcription factor programming has recently emerged as a novel strategy to rapidly and efficiently generate neural cells of excitatory (98) and inhibitory (97) fate. We wanted to further develop these strategies and explore, for the first time, if this more rapid and efficient method using three different combinations of TFs NGN2, FEZF2 and SATB2 could be used to produce specific subtypes of human ES cell-derived cortical neurons (hES-iNs). Previously, it has been shown that NGN2 can guide ES cells to undefined excitatory neuronal phenotype (98). We combined NGN2 with either FEZF2 or SATB2, the most prominent TFs involved in upper and deep layer PN specification during cortical development. However, we observed only subtle differences in gene expression and marker expression pattern between hES-iNs derived by three TF combinations. These findings were surprising since FEZF2 has a strong role in deep layer cortical neuron formation during development as mentioned above. In contrast, SATB2 is a main regulator of upper layer cortical identity and is crucial for specifying callosal neuronal fate (19, 20). Several potential explanations for these results should be considered: First, given that cell differentiation in the TF programming follows a similar, only accelerated temporal order as observed in ES cell differentiation in vitro, it is conceivable that the timing of TF overexpression may have been incorrect. Second, TF levels may have not been optimal and further fine-tuning might be required. During development, the expression of key TFs such as FEZF2 and SATB2 is overlapping in cortical neurons presenting different levels of their expression, i.e., FEZF2 is expressed at high levels by SCPNs and at lower levels by CThPNs whereas SATB2 is expressed at a higher level in CPNs and lower level in CFuPNs (3). Third, even if part of cortical neurogenesis is controlled cell-autonomously through TF expression, a number of extracellular cues are also involved (12). Therefore, it is possible that specific combination of morphogens and growth factors would facilitate the formation of defined subtypes of cortical neurons from ES cells.

With recently emerged techniques for cellular reprogramming, direct differentiation of ES cells (and later on iPSCs) to specific neuronal subtypes relevant for cell replacement therapy remains a golden standard in the field. Therefore, we have in this thesis used the established protocols with minor modifications to produce a new iPSC-derived NSPC line (55, 102, 103). This line can then be primed to cortical identity by the presence of Wnt3A, BMP4 and cyclopamine (WBC) (57), expressing canonical markers of cortical neurons such as TBR1, CTIP2 and CUX1. In analogy, we show in this thesis that our new NSPC line can be primed to form cortical neurons in vitro using the same protocol.
Direct comparison of derived cells with \textit{bona fide} cortical neurons

When attempting to produce a specific subtype of neurons, it is of crucial importance to determine the composition of generated cell population and to what extent these cells resemble their \textit{in vivo} counterparts. Such direct comparison of derived cells with \textit{bona fide} neurons is largely missing in cellular reprogramming studies. In this thesis, we have compared phenotype of our iCtx and hES-iNs with human fetal and adult cortical cells. Single cell analysis revealed that we produced a heterogeneous population of iCtx cells from HEFL cells including non-reprogrammed and partially reprogrammed cells. However, we found that 13.1\% of iCtx cells were similar to human fetal cortical neurons (hCtx cells) in their gene expression pattern. This is in line with Handel and colleagues’ findings on iPSC-derived cortical neurons at the single-cell level (120). In contrast, we found that neural progenitor markers were not expressed by hES-iNs in single cell analysis as compared with hCtx cells, suggesting that hES-iNs had advanced further into maturation. This data supports our findings in cell morphometric measurements of hES-iNs, such as soma size, PMI and cell morphology, which closer resembled human adult cortical neurons rather than hCtx cells. Importantly, we found that hCtx expressed markers for both upper and deep layer cortical neurons in single cell gene expression assay. This agrees with recent data by Zahr and co-workers (121) where they found that mouse neural precursors are transcriptionally primed to make diverse cortical neuron subtypes, and the neuronal specification occurs at a post-transcriptional level. In analogy, our single cell data may reflect that the hES-iNs that we generated by three different combinations of TFs, are at early stage of specification being transcriptionally primed to make cortical neurons of diverse subtypes.

Electrophysiological properties of functional \textit{in vitro} derived cortical neurons

In addition to presenting specific morphological and molecular features, it is a defining characteristic of neurons to receive and generate synaptic signals, which are essential to the normal function of CNS. The translational success of reprogrammed neurons depends on the ability to recapitulate \textit{in vitro} the complexity of functional human brain circuits (122). We have evaluated in detail electrical properties of generated cortical neurons \textit{in vitro} and found that cells from all three derivation protocols were capable of firing multiple action potentials and had electrophysiological characteristics of mature neurons after 4-8 weeks in culture. We also found spontaneous activity in these
cells *in vitro*, indicating their capability of forming synaptic contacts. It has to be noted, thought, that it is largely accepted that many standard *in vitro* protocols for derivation of neuronal cells do not generate cultures that exhibit robust synaptic properties (123). Therefore, these protocols often employ techniques to promote synaptic formation such as co-culture with primary rodent glia or neurons (124). In this thesis, we have used hCtx and mouse glial cells to promote synaptic maturation of iCtx and hES-iNs, respectively, but not ctx-NSPCs. However, we have previously showed that GFAP⁺ cells spontaneously appear at later time point (35 days) of iPSC-derived NSPC differentiation and comprise up to 30% of the total number of cells in such cultures (57). Similarly, we found GFAP⁺ cells in our ctx-NSPC cultures at 8 weeks of differentiation. It is conceivable that these GFAP cells contribute to synaptic maturation of ctx-NSPCs *in vitro*.

**Integration of *in vitro* derived cortical neurons into human neuronal networks**

An important step in clinical translation will be to explore whether grafted human *in vitro* derived cortical neurons can integrate into human brain tissue. Clinical trials are planned in order to use different type of stem cells to address this question (26, 125). However, the design of these studies heavily rely on results obtained from animal models. It remains elusive to what extent results obtained in animal models can be directly translated to the human brain, calling for strategies allowing validation or even primary investigation in live human CNS tissue (126). The use of organotypic slice cultures of human brain tissue is an attractive alternative to explore the functional and morphological integration of transplanted neurons into human neuronal network. This model system represents a cellular network that developed physiologically and has preserved three-dimensional architecture, synaptic connectivity and microenvironment (127). It is important, though, to note that resected human tissue is subjected to severe injury response, involving proliferation of reactive cells and progressive neurodegeneration (128, 129). Nonetheless, recent studies show that it is possible to ameliorate such response by optimizing slice culture medium which enables to partially preserve long-term neuronal viability and robust electrophysiological single cell and network function (126, 130). In this thesis, we have utilized adult human cortex organotypic slices to establish co-cultures with induced cortical cells derived from human fibroblasts, human ES cells or iPSC-derived NSPCs to further explore the possibility of these cells to integrate into adult human neuronal circuits. We showed that cells derived by all three methods were able to form neurons when transplanted on the slices, as indicated by their morphology and extension of neurites within the slice. More importantly, we could show that grafted human fibroblast- and iPSC-derived
ctx-NSPCs could receive afferent synaptic inputs from adult human cortical neurons using rabies virus retrograde tracing and immunoelectron microscopy, respectively. It is important to note, that even though we have provided here evidence for human-to-human cell engraftment, such a model system can only reveal cell integration into local neuronal network. Whether grafted cells connect with other brain structures or have an impact on behaviour outcome remains to be assessed in animal models. This is also true for studies of transplanted cell integration mechanisms, which are likely to be limited in organotypic slice culture systems due to the absence of components of the vascular and immune systems. Nevertheless, the results obtained in this thesis provide evidence that adult human organotypic slice culture is a valuable tool, with the potential to bridge the gap between cell culture, animal models and clinical translation.
Concluding remarks

In this thesis we show the production of cortical neurons from different type of starting cells: human fibroblasts, human ES cells and human iPSC-derived NSPCs. We have evaluated the phenotype of generated cortical neurons by combining morphological, functional, and molecular signature analyses rather than relying mainly on conversion efficiency and functionality data as performed in previous studies. Regardless the starting cell context and derivation protocol, we have produced in all studies neuronal cells pyramidal in shape, expressing key cortical markers and functional *in vitro*. More importantly, we showed that these cells are capable of forming synaptic connections with adult human cortical neurons (Figure 2). One remaining challenge now is to further develop these protocols to produce specific subtypes of cortical neurons in more homogeneous populations. It is a daunting task because there is a shortage of knowledge in the field for unique molecular markers of layer and region. Nonetheless, considering recent advances in mouse (131, 132) and human (133, 134) cortical cell taxonomy, this may be soon feasible.

Another remaining challenge is to assess whether cells derived in this thesis are capable of projecting to correct brain regions *in vivo*. Axonal connectivity to specific targets is a key, defining feature of many classes of neurons in the central nervous system and prime predictor of functional integration (96). Previously, we showed that cortically primed human iPSC-derived NSPCs, similar to ctx-NSPCs derived here, receive functional synaptic inputs from the host brain areas with projection pattern closely resembling that in the intact brain (58). By providing the first evidence that cortical neurons derived here integrate in adult host neural networks also in a human-to-human grafting situation, this thesis represents an early but important step in the clinical translation of neuronal replacement to promote recovery in the injured brain.
Figure 2. Schematic summary of studies included in this thesis.

Each row of the scheme illustrates main highlights of each paper. We used different starting cells, such as HEFL (paper I), hESCs (paper II) and iPSC-derived NSPCs (paper III) to derive cortical neurons. Three TFs, BMF, facilitated direct reprogramming of HEFLs to cortical neurons that had spontaneous activity in electrophysiological recordings, expressed cortical markers such as SATB2, BRAIN2 and TBR1, and formed synapses with both fetal and adult human cortical neurons. Similarly, three different combinations of TFs (N, NF and NS) drove hESCs to cortical fate. Generated cells had spontaneous activity, expressed SATB2, BRAIN2 and TBR1, and formed synapses with adult human cortical cells. Finally, a new human iPSC-derived NSPC line, committed to cortical neurons, differentiated into functional cells with the ability to form synaptic connections in vitro. Also, the scheme shows that the iPSC-derived NSPCs were capable of forming functional afferent synapses with adult cortical neurons after ex vivo transplantation onto adult human cortical slices, therefore, integrated into adult human cortical neural circuitry. HEFL- human embryonic fetal lung fibroblast, hESC- human embryonic stem cell, NSPC- neural stem/progenitor cell, ctx-NSPC- cortically fated NSPC, TF- transcription factor, iCtx- induced cortical cell, hES-iN- hESC-derived induced neuron. Drawing by Bengt Mattsson.
I thank my main supervisor Zaal Kokaia, for giving me the opportunity to do PhD in his laboratory and for excellent supervision during this time. I also thank my co-supervisor Henrik Ahlenius for guidance in the projects that I took on and fruitful collaboration. A special thanks to my informal co-supervisor Olle Lindvall, for the patience and for sharing his invaluable skills on scientific writing. In addition, I thank my former co-supervisor Johan Jakobsson, for valuable advises regarding molecular biology.

I thank my past and current colleagues, Emanuela Monni, Sibel Aktay, Tania Singh, Ruimin Ge, Somsak Wattananit, Daniel Tornero, Karthikeyan Devaraju, Linda Jansson, Marita Grønning Hansen, Sara Palma, Cecilia Laterza, Jemal Tatarishvili, Camilla Ekenstierna, Tamar Memanishvili, Masao Hirota, Teona Roschupkina, Jonas Fritze, Isaac Canals Montferrer, Aurelie Ginisty, Ella Quist, Johan Bengzon, Tania Ramos, Andreas Bruzelius, Francesca Romana Stefani, Katarina Turesson, Christina Parknäs, Märtta Wiren, Hanna Barjosef, My Andersson, Bengt Mattsson, Claire Mckay, all members of Lund Stem Cell Center and many others that were unintentionally left out of this list, for all the support, lively discussions regarding science and life beyond.

I thank my husband Nils for bringing Tudde, Tilda and Mitzi into my life, and for putting the meaning into the following words: “Den som väntar på något gott väntar aldrig för länge”.

Finally, I thank my family members, mother Janina, father Kazimieras and sister Audronė, for the endless support during the PhD period, believing in my choices, all the advises given and kind words shared, for being my strength, my joy and my home throughout life’s curvy roads.
References


