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Efficient neuronal reprogramming into corticofugal projection neurons at embryonic and postnatal stages in the mouse

Torsten Felske

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THÈSE DE DOCTORAT

Reprogrammation efficace en neurones de
projection corticofuges aux stades
embryonnaires et postnatals chez la souris

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Institut de Biologie Valrose (IBV)

**Présentée en vue de l'obtention
du grade de docteur en** Interaction
moléculaire et cellulaire **de**
l'Université Côte d'Azur

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Reprogrammation efficace en neurones de projection
corticofuges aux stades embryonnaires et postnatals
chez la souris

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Titre : Reprogrammation efficace en neurones de projection corticofuges aux stades embryonnaires et postnatals chez la souris

Résumé

Le néocortex des mammifères est tangentiellement organisé en aires fonctionnelles et radialement divisé en six couches de neurones qui présentent chacune des projections, des morphologies et des patterns d'expression distinctes. Alors que les neurones de projection des couches supérieures projettent leurs axones dans d'autres couches du cortex ou vers l'hémisphère controlatéral, tel que les neurones calloseux, la plupart des neurones des couches inférieures, nommés neurones corticofuges, innervent des structures sous-corticales à travers la voie pyramidale. La perte de neurones corticofuges peut conduire à des désordres neurologiques sévères, tel que la sclérose latérale amyotrophique ou d'autres maladies et/ou lésions impliquées dans le tractus cortico-spinal. Étant donné que le cerveau adulte des mammifères ne dispose pas d'une capacité de régénération efficace et que, par conséquent, la perte de neurones devient permanente, des nouvelles méthodes de remplacement de neurones sont indispensables.

Des études récentes ont révélé une plasticité surprenante des neurones post-mitotiques, qui peuvent être convertis en types cellulaires d'une lignée neuronale différente. Cette approche, appelée reprogrammation directe, contourne un état de pluripotence intermédiaire, est assez rapide et susceptible de conserver une signature épigénétique. L'expression forcée de facteurs de transcriptions, connu pour agir comme principaux régulateurs du destin cellulaire pendant le développement, est la meilleure stratégie pour convertir un type cellulaire en un autre. Cependant, il est encore difficile de comprendre dans quelle mesure des neurones, générés par reprogrammation directe, acquièrent les caractéristiques moléculaires du type cellulaire souhaité, jusqu'au stade de la spécification précise du sous-type. Le facteur de transcription FEZF2 est connu pour son rôle dans la spécification des neurones de projections sous-corticales de la couche 5, et une surexpression ectopique de *Fezf2* peut convertir les couches supérieures ou les neurones striataux en neurones corticofuges de projection, même si cela est à faible efficacité.

Durant ma thèse, j'ai utilisé *Fezf2* et le co-adaptateur nucléaire *Lmo4* pour reprogrammer efficacement les neurones calloseux de la couche supérieure en neurones corticofuges de la couche inférieure, à la fois, au stade embryonnaire et postnatale. Les cellules reprogrammées avec succès régulent négativement les marqueurs de la couche supérieure, alors que les marqueurs de la couche inférieure sont surexprimés considérablement. De plus, en utilisant des méthodes de traçage avancées, les cellules reprogrammées ont la capacité de projeter vers des cibles sous-corticales, tels que le thalamus, le peduncule cérébrale et la moelle épinière. Ces données démontrent un rôle synergique inattendu de *Lmo4* avec *Fezf2* dans la reprogrammation neuronale et révèlent un cocktail efficace pour la conversion de cellules neuronales en sous-types de neurones corticofuges de projection.

Mots clés : Reprogrammation, *Lmo4*, *Fezf2*, Néocortex, Neurones corticofuges de projection

Title: Efficient neuronal reprogramming in corticofugal projection neurons at embryonic and postnatal stages in the mouse

Abstract

The mammalian neocortex is tangentially organized into functional areas and radially subdivided into six layers of neuronal populations with distinct projections, morphology and expression patterns. While neurons in the upper layers project within the neocortex or towards the contralateral hemisphere, such as callosal projection neurons, most lower layer neurons, named corticofugal projection neurons, innervate subcerebral targets via the corticospinal tract. Injuries or loss of these neurons can lead to severe neurological disorders, such as Amyotrophic Lateral Sclerosis or other diseases and/or lesions implicating the corticospinal tract. Since the adult mammalian brain lacks a significant regenerative capacity and thus, damage or loss of neurons is permanent, methods for restoring neurons are urgently needed.

Recent studies revealed an unexpected plasticity of post-mitotic neurons, which can be converted into cell-types of other neuronal lineages. This approach, called direct neuronal reprogramming, bypasses an intermediate pluripotent state. Direct reprogramming is fast, likely to keep epigenetic hallmarks and it can be conducted *in vivo*. Forced expression of transcription factors, known to act as master regulators of cell fate during development, is one of the best strategies for directly converting one cell-type into another one. However, it is still not clear to which extent neurons, generated by direct reprogramming, acquire authentic molecular signatures of the desired cell type, down to the point of precise subtype specification. The transcription factor FEZF2 is known for its role in cell fate specification of layer 5 subcerebral projection neurons and high ectopic *Fezf2* expression can convert upper layer or striatal neurons into a corticofugal fate, even if at low efficiency.

During my PhD thesis, I used *Fezf2* and the nuclear co-adaptor *Lmo4* to efficiently reprogram upper layer projection neurons into corticofugal lower layer projection neurons, at both, embryonic and postnatal stages. The successful reprogrammed cells downregulated upper layer markers, while lower layer markers were drastically increased. Additionally, by using advanced tracing methods, we showed that reprogrammed neurons projected towards subcerebral targets, including the thalamus, cerebral peduncle and spinal cord. These data demonstrate an unexpected synergistic role of *Lmo4* with *Fezf2* in neuronal reprogramming and reveals an effective cocktail for the conversion of neuronal cells into corticofugal neuronal subtypes.

Keywords: Reprogramming, *Lmo4*, *Fezf2*, Neocortex, Corticofugal projection neurons

Table of Contents

List of Abbreviations.....	11
INTRODUCTION	14
I. The Mammalian Neocortex: Cytoarchitecture and Development	15
A. Cytoarchitecture of the neocortex.....	15
1. Cellular components.....	15
2. Tangential organisation of the neocortex	16
3. Radial organisation of the neocortex	16
4. Connections of the neocortex	20
B. Development of the neocortex.....	25
1. Corticogenesis.....	25
2. Development of the callosal projection neurons (CPNs).....	27
3. Development of the CST	29
4. Development of CTA.....	31
5. Development of TCA.....	32
C. Genetic regulation of PNs position and identity	32
1. Positional information in progenitors.....	32
2. Genetic regulation of PN diversity.....	35
3. Layer-specific molecular markers in the neocortex.....	40
a. Upper layer CPN marker <i>CUX1</i>	40
b. SCPN marker <i>CTIP2</i>	41
c. CThPN marker <i>FOG2 (Friends of GATA2)</i>	42
d. CFuPN marker <i>PCP4</i>	43
e. CThPN marker <i>DARPP32</i>	43
4. Areal specialization of post-mitotic projection neurons.....	44
a. <i>BHLHB5</i>	45
b. <i>LMO4</i>	45
c. <i>PBX1</i>	46
d. Other post-mitotic area regulators	47
5. The motor area as “output” region	47
<i>Fezf2</i>	48
a. <i>Fez</i> expression during embryogenesis and early postnatal stages	49
b. <i>Fez</i> genes during development of the olfactory system and diencephalon	51
c. <i>Fez</i> genes play unique and redundant roles in forebrain neurogenesis.....	51
<i>Lmo4</i>	52
a. Expression pattern of <i>Lmo4</i>	56

<i>b. Lmo4 in cortical subtype specification</i>	56
II. Cell reprogramming	58
1. General Introduction.....	58
2. Nuclear reprogramming	59
3. Cell fusion reprogramming	60
4. Cell reprogramming by cell extract treatment.....	60
5. Natural occurring de-differentiation and trans-differentiation	61
6. Reprogramming to induced Pluripotent Stem Cells (iPSCs)	62
7. Direct reprogramming.....	64
8. Direct neuronal reprogramming <i>in vivo</i>	72
<i>a. Direct neuronal reprogramming in vivo in the injured brain</i>	73
<i>b. Direct neuronal reprogramming in vivo under physiological conditions</i>	75
<i>c. Data leading to the thesis work</i>	78
III. Aims of the thesis	79
MATERIAL AND METHODS.....	80
I. Animals.....	81
II. Plasmids.....	81
1. Insert.....	81
2. Vector	83
3. Inducible pCAG-Fezf2-IRES-GFP	85
4. Inducible pCAG-IRES-GFP	85
III. <i>In utero</i> electroporation.....	87
IV. Tamoxifen injection.....	89
V. Cell Dissociation	90
VI. Cell sorting.....	90
VII. RNA Extraction	91
VIII. Intracardiac Perfusion	91
IX. Embedding and Slicing	91
X. Immunofluorescence	91
XI. Microscopy.....	93
XII. Analysis.....	93
RESULTS.....	94
Section I.: Reprogramming upper layer neurons in the embryonic mouse brain	95
A. Ectopic expression of <i>Lmo4</i> induces lower layer molecular markers in post-mitotic upper layer neurons at low efficiency	95
B. Ectopic expression of <i>Lmo4</i> in UL neurons does not alter their axonal projection.....	98

C. <i>Lmo4</i> in combination with <i>Fezf2</i> efficiently induces lower layer markers in UL neurons	100
D. <i>cFezf2</i> & <i>cLmo4</i> or single <i>cFezf2</i> ectopic expression alter axonal projection of post-mitotic UL neurons towards corticofugal targets	104
E. The motor area is a more instructive environment for CFuPN-reprogramming.....	106
F. Reprogrammed post-mitotic UL neurons maintain stable regarding their altered molecular identity	109
Section II.: Reprogramming upper layer neurons at postnatal stages	112
A. Postnatal induction at P3 of single <i>Fezf2</i> or <i>Fezf2</i> & <i>Lmo4</i> alters molecular identity of UL neurons.....	112
C.1. Postnatal induction of single <i>iFezf2</i> or <i>iFezf2</i> & <i>iLmo4</i> drives UL neurons to change their axonal projections towards corticofugal targets	118
D. Postnatal induction at P7 of <i>iFezf2</i> & <i>iLmo4</i> still drives UL neurons to express lower layer marker PCP4.....	120
D.1. UL neurons receiving expression of single <i>iFezf2</i> or <i>iFezf2</i> & <i>Lmo4</i> at P7 show aberrant projections to corticofugal targets.....	122
E. Ectopic expression of single <i>iFezf2</i> or <i>iFezf2</i> & <i>Lmo4</i> in late postmitotic UL neurons show corticofugal projections	124
Section III.	126
RNA extraction from UL neurons electroporated with <i>cGFP</i> , <i>cFezf2</i> or <i>cFezf2</i> and <i>cLmo4</i> to screen for downstream targets <i>via</i> RNA-sequencing	126
DISCUSSION	128
I. Induction of <i>Lmo4</i> alone fails to efficiently convert post-mitotic UL neurons	129
II. <i>Lmo4</i> synergize with <i>Fezf2</i> to trigger efficient reprogramming of post-mitotic UL neurons into CFuPNs	131
III. <i>Fezf2</i> & <i>Lmo4</i> trigger significant higher numbers of UL neurons to express CTIP2 and PCP4 compared to single <i>Fezf2</i>	133
IV. <i>Fezf2</i> & <i>Lmo4</i> overexpression drive UL neurons to re-route their axonal projections towards corticofugal targets	135
V. The cellular environment of the motor cortex has an impact on reprogramming efficiency of <i>Fezf2</i> & <i>Lmo4</i>	137
VI. Reprogrammed post-mitotic UL neurons maintain expression of CFuPN markers at juvenile-adult stages.....	138
VII. Postnatal UL neurons retain the capacity to convert their molecular identity	139
1. Addition of inducible <i>Ctip2</i> to inducible <i>Fezf2</i> & <i>Lmo4</i>	140
2. Addition of inducible <i>PBX1</i> to inducible <i>Fezf2</i> & <i>Lmo4</i>	140
VIII. UL neurons retain their ability to change their axonal projections towards corticofugal targets from P3 to P21	141
1. <i>Fezf2</i> & <i>Lmo4</i> induction at P7 and P10 still enable partial re-routing of UL neuron axons	142
2. <i>Fezf2</i> & <i>Lmo4</i> induction at P21	142

IX. RNA extraction of early post-mitotic electroporated UL neurons	142
CONCLUSION.....	144
PERSPECTIVES	146
I. To further characterize reprogrammed UL neurons	147
II. To investigate reprogramming in other cell-types.....	147
III. To increase reprogramming efficiency at postnatal stages	147
IV. To apply possible downstream targets of FEZF2 revealed by <i>mRNA-sequencing</i>	148
References	149

List of Figures

Figure 1: Projection neuron diversity in the cerebral cortex	19
Figure 2: Long-range axonal projections define two classes of corticostriatal projection neuron....	24
Figure 3: Neocortical projections neurons are generated in an “inside-out” fashion by diverse progenitor types in the VZ and SVZ	26
Figure 4: CPN development and diversity.....	28
Figure 5: Mechanisms and time course of mouse corticospinal tract (CST) development	30
Figure 6: CST projections to the spinal cord in rodents.....	31
Figure 7: Transcription factors in the VZ establish an area identity fate map	34
Figure 8: Competing molecular programs direct differentiation of newly-postmitotic projection neurons into one of three broad subtype identities	39
Figure 9: IHC of CTIP2 protein expression in M and S1 of WT mice at P8	48
Figure 10: The Fez family of transcription factors.	49
Figure 11: Fezf1 and Fezf2 expression during embryogenesis.....	50
Figure 12: Fezf1 and Fezf2 functions during forebrain neurogenesis.	52
Figure 13: Schematic representation of the human LMOs and LDB1.....	54
Figure 14: Schematic representation of LMOs in gene transcriptional regulation.	54
Figure 15: Schematic model of the putative mechanism by which Lmo4 de-represses Ctip2 expression	55
Figure 16: The Cook Islands model applied to neuronal reprogramming	71
Figure 17: Ectopic expression of Lmo4 induces lower layer markers at low efficiency in post-mitotic upper layer neurons	97
Figure 18: Ectopic expression of Lmo4 in UL neurons does not alter their axonal projection	99
Figure 19: Ectopic Expression of single Fezf2 or Fezf2 in combination with Lmo4 drives post-mitotic UL neurons to acquire molecular features characteristic of CFuPN.	102
Figure 20: cFezf2 & cLmo4 or single cFezf2 ectopic expression alter axonal projection of post-mitotic UL neurons towards corticofugal targets.....	105
Figure 21: Ectopic expression of cFezf2 & cLmo4 in UL neurons of the motor area induces higher levels of corticofugal identity markers CTIP2 and DARPP2 compared to somatosensory area.	107
Figure 22: Reprogrammed post-mitotic UL neurons maintain stable regarding their altered molecular identity.....	112
Figure 23: A. Verification of iLmo4 and iFezf2 induction	114
Figure 24: Postnatal induction at P3 of single Fezf2 or Fezf2 & Lmo4 alters molecular identity of UL neurons	115
Figure 25: Postnatal induction of single iFezf2 or iFezf2 & iLmo4 combined with iCtip2 or iPBX1 does not increase reprogramming efficiency	117
Figure 26: Postnatal induction of single iFezf2 or iFezf2 & iLmo4 drives UL neurons to change their axonal projections towards corticofugal targets.....	119
Figure 27: Postnatal induction at P7 of iFezf2 & iLmo4 still drives UL neurons to express lower layer marker PCP4.....	121
Figure 28: UL neurons receiving expression of single iFezf2 or iFezf2 & Lmo4 at P7 show aberrant projections to corticofugal targets.....	123
Figure 29: Ectopic expression of single iFezf2 or iFezf2 & Lmo4 in increasingly differentiating UL neurons show corticofugal projections	125
Figure 30: RNA extraction of UL neurons electroporated with cGFP, cFezf2 or cFezf2 and cLmo4 to screen for downstream targets via RNA-sequencing	127

List of Abbreviations

A1: Primary auditory

AC: Anterior commissure

Ascl1: Achaete-Scute Family BHLH
Transcription Factor 1

bHLH: basic Helix-Loop-Helix

Bhlhb5: Basic Helix-Loop-Helix Family Member
E22

BRCA1: Breast Cancer 1

C. Elegans: Caenorhabditis Elegans

Cas9: CRISPR associated protein 9

CC: Corpus callosum

Cdh10: Cadherin 10

CFuPN: Corticofugal projection neuron

CGE: Caudal ganglionic eminence

cKO: conditional Knock-out

Clim1: Ldb2

Clim2: Ldb1

CNS: Central nervous system

Coup-TFI: Chicken ovalbumin upstream
promoter transcription factor 1

CP: Cortical plate

CPD: Cerebral Peduncle

CPN: Commissural projection neurons

Crb: Cerebellum

CRISPR: Clustered Regularly Interspaced Short
Palindromic Repeats

Crym: Crystallin Mu

CSMN: Corticospinal motor neuron

CST: Corticospinal Tract

CStr: Corticostriatal

CTA: Corticothalamic Axon

CThPN: Corticothalamic projection neuron

Ctip2: B-cell lymphoma/leukemia 11B

Cux1: Cut Like Homeobox 1

Cux2: Cut Like Homeobox 2

Darpp32: Protein Phosphatase 1 Regulatory
Inhibitor Subunit 1B

DCC: DCC Netrin 1 Receptor

dLGN: dorsal lateral geniculate nucleus

DNA: Deoxyribonucleic acid

Dnmt1: DNA methyltransferase 1

E: Embryonic day

Egf: Epidermal growth factor

Eh1: Engrailed homology 1

Emx2: Empty spiracles homolog 2

Ep: Electroporation

Er81: ETV1

ERK: Extracellular Signal-regulated Kinase

Erm: Earmuff

ER α : Estrogen Receptor alpha

ESC: Embryonic Stem Cell

FACS: Fluorescence-activated cell sorting

Fezf2: FEZ Family Zinc Finger 2

FGF17: Fibroblast growth factor 17

FGF8: Fibroblast growth factor 8

Fog2: ZFPM2

FoxP2: Forkhead box protein P2

GABA: c-aminobutyric acid

GFAP: Glial Fibrillary Acidic Protein

GFP: Green Fluorescent Protein

GLIS1: Glis Family Zinc Finger 1

GW: Glial Wedge

HDAC: Histone deacetylase

Hes5: Hes Family BHLH Transcription Factor 5

IC: Internal Capsule

IGG: Indusium Griseum Glia

iN: induced Neuron

IN: Interneuron

IP: Intermediate progenitor

IPC: Intermediate precursor cell

iPSC: induced Pluripotent Stem Cell

IQ: Amino acids Isoleucine and Glutamine

ISH: In Situ Hybridization

IT: Intratelencephalic

kDa: kilo Dalton

Klf4: Kruppel-like factor 4

KO: Knock-out

L: Layer

Ldb1: LIM domain binding protein 1

Ldb2: LIM domain binding protein 2

Lmo4: LIM domain only 4

Lmx1a: LIM Homeobox Transcription Factor 1 Alpha

M1: Primary motor

MAPK: Mitogen-activated Protein Kinase

Mbd3: Methyl-CpG Binding Domain Protein 3

MC_c: caudal motor cortex

MC_r: rostral motor cortex

MGE: Medial ganglionic eminence

MGv: Medial geniculate nucleus

miRNA: micro RNA

MOE: Main Olfactory Epithelium

mRNA: messenger RNA

MSN: Medium spiny neuron

MTA1: Metastase Associated 1

MyoD: Myoblast Determination Protein

MZ: Marginal zone

MZG: Midline Zipper Glia

NCAM: Neural Cell Adhesion Molecule

NeuN: Fox-3

NeuroD1: Neurogenic Differentiation 1

Nkx-2: Nk2 Homeobox 2

NSC: Neural Stem Cells

Nurr1: Nuclear receptor related protein 1

OB: Olfactory bulb

Oct3/4: Octamer-binding transcription factor^{3/4}

oRG: outer Radial glial cell

OSKM: Oct3/4, Sox2, Klf4 and c-Myc

OT: Optic tectum

P: Postnatal day

Pax6: Paired box 6

Pbx1: Pre-B-cell Leukemia Homeodomain 1

PCP4: Purkinje-cell protein 4

PN: Projection neuron

PO: Pons

POA: Preoptic area

PP: Preplate

PSPB: Pallial/Subpallial Boundary

PT: Pyramidal tract

RGC: Radial glial cell

RNA: Ribonucleic acid

Robo: Roundabout Guidance Receptor
Ror β : RAR-related orphan receptor beta
RTN: Reticular nucleus
S1: Primary somatosensory
SAP: Subapical progenitor
Satb2: Special AT-rich Sequence Binding Protein 2
SC: Spinal cord
SCPN: Subcerebral projection neuron
SCS: Subcallosal Slings
SEMA3A: Semaphorin 3A
SEMA3C: Semaphorin 3C
Sey: *small eye* hypomorphic mutant
SFRP2: Secreted frizzled-related protein 2
Slit: Slit Guidance Ligand
Sox2: SRY-Box 2
Sox5: SRY-Box 5
SP: Subplate
Sp8: Trans-acting transcription factor 8
STN: Subthalamic nucleus
SVZ: Subventricular zone
Tbr1: T-Box Brain Protein 1
TCA: Thalamocortical afferents
TF: Transcription factor
Th: Thalamus
Tle4: Transducin Like Enhancer Of Split 4
UNC5H3: Unc-5 Netrin Receptor C
UV: Ultraviolet light
V1: Primary visual
VL: Ventrolateral nucleus
VNO: Vomeronasal Organ
VP: Ventroposterior nucleus
VPM: Ventral posteromedial nucleus
VZ: Ventricular zone
Wnt: Vertebrate orthologs of drosophila wingless/integrated
YAC: Yeast artificial chromosome

INTRODUCTION

During evolution, one part of the brain experienced a drastic development in complexity and size and is one of the major reasons for the evolutionary success of mammals: the cerebral cortex. It covers the outer portion of the cerebrum and is anatomically comprised of the archicortex, the paleocortex and the neocortex – its largest component (O’Leary, Chou and Sahara, 2007). The neocortex is the evolutionary newest part of the cerebral cortex and its cytoarchitecture is a distinguishing feature of mammals; in humans, it covers up to 80% of the total volume (Rakic, 2009; Kaas, 2011). The neocortex represents the major and critical organ for sensory input processing, movement, memory and language and is additionally responsible for higher cognitive functions, such as abstract thinking and self-awareness (Kaas, 2011). Due to these intrinsic features it is a major organ of interest for fundamental and medical research, but still represents a major challenge because of its high complexity. Therefore, gaining a better understanding on the molecular, cellular and systemic level is crucial to unravel its mystery and thus, this work on the mouse neocortex is intended to contribute to it.

I. The Mammalian Neocortex: Cytoarchitecture and Development

A. Cytoarchitecture of the neocortex

1. Cellular components

The neocortex is composed of neuronal cells and glia. Glutamatergic projection neurons (PNs) make up almost 80% of the total neuronal population in the neocortex, they are generating the neocortical output and are responsible for the connection of the neocortex to subcortical, subcerebral or intracortical targets. The other major population is composed of interneurons (INs), which make up around 20% of the neuronal population (Lodato and Arlotta, 2015). They are largely inhibitory and modulate the activity of neuronal circuits by release of γ -aminobutyric acid (GABA). INs share a common feature of connecting within the neocortex via short-range projections even though they appear to be a very heterogenous class with diverse morphology, connectivity, biochemistry and physiological properties (Lim *et al.*, 2018). Non-neuronal cells are the third category of cells that are present in the neocortex and are

named glia. Their role besides supporting neuronal circuits is very various. Three main subcategories of glial cells exist: Microglia, Oligodendrocytes and Astrocytes. Microglia are known as the ‘immunocompetent’ cells with phagocytic abilities. They cover a huge part of the brain parenchyma and not only survey the brain tissue for any damage, but are also implicated in the maintenance of the diverse cell populations that comprise the CNS (Schafer and Stevens, 2015). Oligodendrocytes are the myelin-producing cells insulating axons to allow fast axon potential propagation. Some oligodendrocytes are not producing myelin and may have other functions, as they are present in the grey matter of the cerebral cortex (Kang *et al.*, 2013). Astrocytes are the most abundant glial cell population in the neocortex, their function in the neuronal circuit is various and ranges from water and ion homeostasis, participation in synapse signalling or maintenance of the blood brain barrier (Jäkel and Dimou, 2017).

The investigated cell type in this thesis is the excitatory projection neuron and therefore the following part of the introduction will be focused on this cell type.

2. Tangential organisation of the neocortex

The neocortex is tangentially organised into functional areas, each having a unique cytoarchitecture, distinct connections and specific functions. For the neuroscientist Brodmann, “each area represents an organ” of the adult brain (Brodmann, 1909), implying that each area is well distinguished from another one by rather distinct cytoarchitecture borders and has a unique function within the brain. Four major areas can be distinguished in all eutherian mammals (Kaas, 2011) and are defined as primary areas due to their function as first “receiver” of sensory inputs – auditory area A1, visual area V1 and somatosensory area S1 – or as first “executor”: the motor area M1. The sensory areas are processing information from the cochlea (A1), the retina (V1) and the body (S1), while M1 controls voluntary movement of different body parts (O’Leary, Chou and Sahara, 2007).

3. Radial organisation of the neocortex

Tangentially, the neocortex is subdivided into functional areas, whereas in its radial dimension the neocortex is organised into six layers (see **Fig. 1**). Each layer is composed of a different

pool of neurons and, each layer varies in its cellular composition, its projections and its thickness depending on their areal identity (Lodato and Arlotta, 2015).

Layer (L)1 represents the first sheet just underneath the pial surface (the inner most membrane that envelopes the cortex) and mainly consists of axons, dendrites and sparse cells – named Cajal-Retzius cells (Kaas, 2011). The following layers are grossly classified by their projections within the central nervous system (CNS, see **Fig. 1**): L2-3 are mainly composed of neurons which extend their axons to the contralateral cortical hemisphere, named callosal projection neurons (CPNs) and associative projection neurons, projecting within the neocortex. Most of the CPN axons cross the midline through the corpus callosum (CC), which represents the major “bridge” between the two cortical hemispheres. Some CPNs send their axons through the anterior commissure (AC), which represents a second “bridge” with fewer cortical axonal projections, connecting both hemispheres (Greig *et al.*, 2013). In fewer numbers, CPNs are also residing in L5 and L6. Neurons in L4 are the main recipient of thalamocortical afferents and the majority of L4 neurons project within one cortical hemisphere to L2/3 (Lodato and Arlotta, 2015).

PNs in L5 and L6 project away from the cortex to distinct subcortical or subcerebral structures. These projection neurons are named corticofugal projection neurons (CFuPNs) and can be further subdivided into subcerebral projection neurons (SCPNs) - residing in L5 – and, corticothalamic projection neurons (CThPNs), in L6. SCPNs are projecting towards distinct subcerebral targets in function of their areal location: from V1 to the superior colliculus (corticotectal PNs), from S1 to the brainstem (mostly pons, cortico-pontine PNs) and from M1 to the spinal cord (Corticospinal motor neurons, CSMNs). CThPNs project to specific thalamic nuclei, in a topographic manner, meaning that CThPNs from distinct areas project to specific nuclei in the thalamus (Molyneaux *et al.*, 2007; Greig *et al.*, 2013; Lodato and Arlotta, 2015).

This is a rough classification and gives only a general idea of neuronal population differences within layers. For example, associative projection neurons are present in each layer and extend their axons within a single cortical hemisphere (Molyneaux *et al.*, 2007). Additionally, some neurons send projections to multiple targets and therefore can be classified in several classes. Among them are CPNs, projecting to both the contralateral hemisphere and ipsilaterally within the cortex, whereas intrathelencephalic corticostriatal PNs, extend callosal axons to the ipsilateral but also to the contralateral striatum (Shepherd, 2013). SCPN often

project to multiple targets, as they send collaterals to diverse corticofugal targets (Shepherd, 2014). Also, some SCPN extend backward projections, sending axons to subcerebral targets and within the cortex (Molyneaux *et al.*, 2007; Greig *et al.*, 2013; Lodato and Arlotta, 2015).

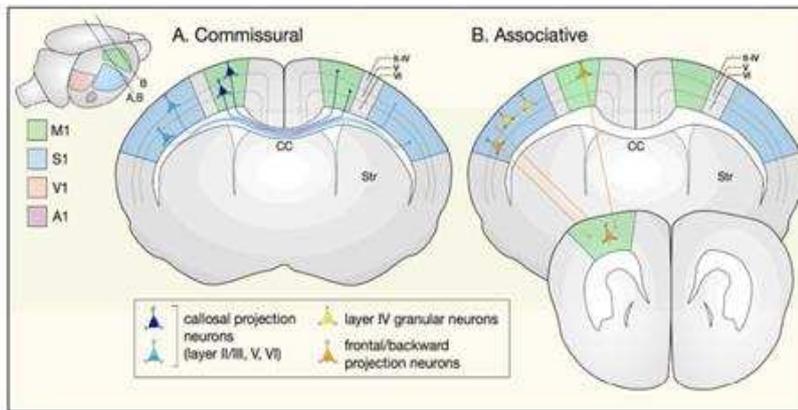
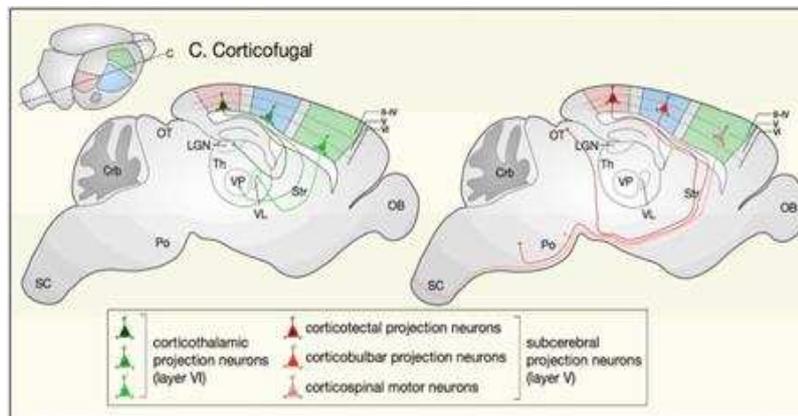


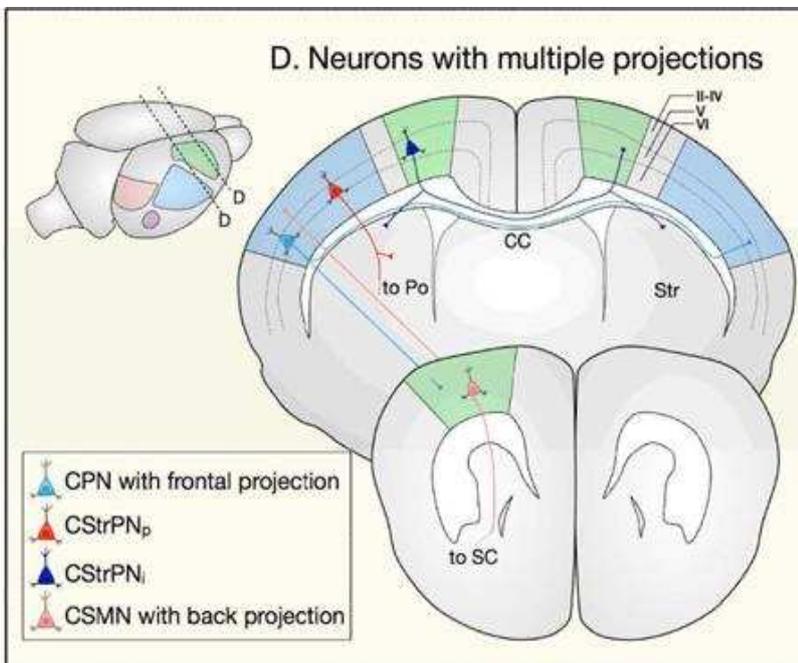
Figure 1: Projection neuron diversity in the cerebral cortex

Schematic presentation showing origin of PNs and their respective axonal projections.

A. Commissural projection neurons project to the contralateral cerebral hemisphere. Most cross the midline through the corpus callosum (callosal projection neurons, CPN), while a smaller population crosses through the anterior commissure. CPN reside primarily in upper layer 2 - 3, with fewer in lower layers 5 and 6 and extend axons to mirror-image locations in the same functional area of the contralateral hemisphere.



B. Associative projection neurons are present in all layers of the neocortex, projecting within a single cerebral hemisphere. This population includes short-distance intrahemispheric projection neurons, which extend axons within a single cortical column or nearby cortical columns and long-distance intrahemispheric projection neurons, which extend axons to adjacent or distant cortical areas (such as forward and backward projection neurons).



C. Corticofugal projection neurons (CFuPNs) project outside the cortex to subcortical targets including corticothalamic projection neurons (CThPNs), which reside in layer 6, and subcerebral projection neurons (SCPNs), which reside in layer 5.

D. Neurons that send projection to multiple targets can sometimes be classified into more than one category. Examples include CPN with frontal projections, which extend axons to the contralateral hemisphere and to ipsilateral frontal cortex. SCPN with backward projections, which extend axons to subcerebral targets and to ipsilateral caudal cortex. Intratelencephalic corticostriatal projection neurons, which extend projections to contralateral hemisphere and to ipsilateral striatum.

CC Corpus Callosum, Crb cerebellum, LGN lateral geniculate nucleus of thalamus, OB olfactory bulb, OT optic tectum, PO pons, SC spinal cord, Th thalamus, VL ventral lateral nucleus of thalamus, VP ventral posterior nucleus of thalamus. Taken from Greig et al. (2013).

4. Connections of the neocortex

Axonal trajectories are important messenger propagation routes to ensure communication between higher cognitive upper layer neurons and executive lower layer neurons. PNs can be characterized by their axonal projections, revealing their functional role in the cortex. For simplification, associative PNs and callosal PNs are grouped together as intratelencephalic (IT) PNs, as they project to the contralateral hemisphere or within the telencephalon (cortex and striatum) or both (bilateral IT neurons). Names of SCPNs and CThPNs are not changed.

a. Cortico-cortical projections

Inputs on cortical neurons originate mainly from other cortical neurons of the same hemisphere (associative projection neurons) or from neurons projecting from the contralateral hemisphere. The axonal pathway from one hemisphere to its contralateral region through the CC is a relatively recent feature in cortical evolution and is unique to placental mammals (Aboitiz, Morales and Montiel, 2003). Other interhemispheric commissures exist and are more ancient, the hippocampal commissure and the AC, but the CC is by far the largest and the only one which serves solely the communication between the two neocortical hemispheres (Aboitiz, Morales and Montiel, 2003). Axons in the CC arise mainly from CPNs which are abundantly located in L2 – 3 (around 80% in rodents), in L5 (around 20%) and some in L6 (Fame, MacDonald and Macklis, 2011). All CPNs extend an axon to the contralateral hemisphere, but they can also innervate local cortical neurons within the hemisphere and/or the striatum. In fact, mainly lower layer CPNs possess dual projections to the contralateral side and ipsilaterally within the forebrain, while upper layer CPNs project to cortical neurons in L2 – 3, to stellate cells in L4 and they also send collaterals to L5 and L6 cortical neurons (See Fig. 2, Petreanu *et al.*, 2007). Thus, CPN function is mainly related to integration and connection of neuronal circuits within the neocortex.

b. Corticofugal projections

The sub-classification of CFuPNs is complex regarding their connectivity and is not yet completely understood. The complexity arrives from the fact that many CFuPNs target several

structures via branches along their trajectory. This is especially the case for L5 SCPNs, which can project to the midbrain, striatum, thalamus, and subthalamic nuclei, *en route* to their dorsal-most destinations in the brainstem and the spinal cord (Shepherd, 2013). Additionally, SCPNs also form ipsilateral connections within the cortex, but do not cross through commissures to the contralateral hemisphere (Shepherd, 2013). Less complex projections are found of L6 CThPNs, which are projecting to the thalamus only. Here, the major trajectories of CFuPNs will be presented.

c. The corticospinal tract (CST)

The control of voluntary movements in mammals is based on sophisticated motor and sensory communication in the CNS. Axonal trajectory of the CST enables fast and controlled motor execution that has been carried out by corticospinal motor neurons (CSMNs) in the neocortex. The functions of the CST comprise control of afferent inputs, spinal reflexes and motor neuron activity (Lemon and Griffiths, 2005). Axons in the CST originate from L5 SCPNs in the primary motor and somatosensory cortex and in less extent from parietal, cingulate, visual and prefrontal regions (Miller, 1987; Akintunde and Buxton, 1992; Tennant *et al.*, 2011; Kamiyama *et al.*, 2015). The canonical path of the CST in mammals starts from axonal projections of L5 SCPNs in the neocortex, which then travel into the internal capsule (IC), further on to the cerebral peduncle (CP), into the brainstem and some reach the spinal cord (see **Fig. 2**). The axonal bundles of the CST remain at the ventral position until reaching the medulla in the brainstem. In rodents, 80 – 95% CST axons cross the midline at the junction between the brainstem and spinal cord, and pass from a ventral to a dorsal position, forming the pyramidal decussation (Welniarz, Dusart and Roze, 2017; Armand, 1982; Schreyer and Jones, 1982; Rouiller *et al.*, 1991; Joosten *et al.*, 1992). Therefore, the majority of the axon trajectory continues in the contralateral part of the SC and terminates in the ventral part of the dorsal funiculus, located between the dorsal horn and the midline (Kuypers, 1964). The remaining 5 – 20% uncrossed CST project in the ventral funiculus. However, both crossed CST and uncrossed CST originate from the same cortical regions (Galea and Darian-Smith, 1994; Brösamle and Schwab, 1997; Lacroix *et al.*, 2004), but control different muscles: the crossed CST is involved in fine movements of distal extremities, whereas the uncrossed CST targets

proximal or axial musculature (Welniarz, Dusart and Roze, 2017). The terminations of crossed CST are located in the dorsal and intermediate horns (grey matter) of the contralateral spinal cord, uncrossed CST terminations on the ipsilateral side show the same pattern (Welniarz, Dusart and Roze, 2017). Interestingly, CSMNs of adult rodents do not form direct connections with motor neurons in the spinal cord (Alstermark and Ogawa, 2004), whereas higher primates have monosynaptic connections between CSMNs and motor neurons, allowing fine hand dexterity control (Lemon, 2008). However, this monosynaptic connection is present in juvenile mice and is eliminated by signalling molecules and is therefore lost in the adult mouse (Gu *et al.*, 2017). Thus, the CST connection to motor neurons in the spinal cord might be formed by polysynaptic transmission via interneurons and propriospinal neurons (Lemon, 2008). Overall, L5 SCPNs extend their axons within the CST and project to several corticofugal targets *via* branches or collaterals of their axons, *en route* to their dorsal-most locations, the brainstem and the spinal cord.

d. Cortico-striatal connections

The striatum of the mammalian cerebrum is a critical component of motor and reward systems, including motor and action planning, motivation and reward perception (Yager *et al.*, 2015). It receives glutamatergic input from the cortex and dopaminergic input from basal ganglia structures. Connectivity between the cortex and the striatum is directional, meaning that cortical projections form mono-synaptic input, whereas neurons in the striatum communicate indirectly with the cortex via polysynaptic downstream circuits (Shepherd, 2013). The cortical neurons innervating the striatum originate from L5 IT or SCPNs and L6 IT neurons. Moreover, cortico-striatal projections can either originate from L5 IT neurons or SCPNs, but not from both (Shepherd, 2013). L5/6 IT neurons projecting to the striatum have the unique attribute of being both corticofugal and callosal, because they project to the striatum and they additionally project to the contralateral hemisphere (Macklis *et al.*, 2012). Interestingly, the striatum is special in receiving both L5/6 IT (bilateral) and SCPN (ipsilateral) inputs, differently from other cerebral areas. In the striatum, L5/6 IT neurons and SCPNs make synaptic contacts primarily with spines of striatal projection neurons, also called medium spiny neurons (MSNs, Reiner, 2010), which is by far the most abundant neuron type in the striatum

(Yager *et al.*, 2015). MSNs themselves project to basal ganglia structures such as globus pallidus and substantia nigra pars reticulata. Taken together, the cortical input the striatum receives originates from several types of PNs, which includes L5 and L6 IT neurons and SCPNs.

e. Connections between cortex and thalamus

The thalamus is a prominent structure in the diencephalon and is the major receiver and relay of sensory inputs, which propagates in a topographic manner to the cortex. The thalamus and the cortex are strongly interconnected and both are important for proper development of one another and represent an integrated processing unit, which regulates thalamic transmission of peripherally derived data for cortical processing (Leyva-Díaz and López-Bendito, 2013). The cortex innervates the thalamus, by corticothalamic axonal (CTA) projection and the thalamus projects to targets in the cortex, by thalamocortical axonal (TCA) projection.

The cortical innervation of the thalamus originates from CThPNs of L6 and to a smaller extent of SCPNs in L5. But, the projection patterns of L6 CThPNs differ from L5 SCPNs. For instance, L6 CThPNs project to all thalamic nuclei (including both first-order and higher-order relays), whereas SCPNs only project to higher-order thalamic nuclei (Hoerder-Suabedissen *et al.*, 2018). First-order relays get their driving input from subcortical sources and form a reciprocal feedback pattern with CThPNs. Moreover, CTA of CThPNs innervate thalamic nuclei, which in turn send TCA to the same cortical region in L6 from where the input originated, depending on their area identity (Briggs and Usrey, 2008). These projections from CThPNs modulate how sensory information is relayed to the cortex (Olsen *et al.*, 2012; Lam and Sherman, 2013; Crandall, Cruikshank and Connors, 2015). Most of TCA terminate in L4, thereby innervating local circuit neurons, named stellate cells. In addition, also L1 and L2 – 3 receive TCA input (Caviness and Frost, 1980). Thus, the neocortical primary areas are connected via CThPNs with the thalamic nuclei in the following way: M1 is connected with the ventrolateral (VL) nucleus, S1 with the ventroposterior nucleus (VP), V1 with the dorsal lateral geniculate nucleus (dLGN) and A1 with the ventral part of the medial geniculate nucleus (MGv, Guillery, 1967; Hoogland, Welker and Van der Loos, 2004; O’Leary, Chou and Sahara, 2007).

In contrast, higher-order relays get their driving input from SCPNs (Usrey and Sherman, 2018). SCPNs from S1 innervate posterior medial nucleus, V1 SCPNs innervate pulvinar and A1 SCPNs project to dorsal division of medial geniculate nucleus (Usrey and Sherman, 2018). These projection patterns imply that L6 CThPNs provide a more modulatory input to the thalamus and SCPNs provide driving inputs (Usrey and Sherman, 2018). Thus, thalamic nuclei receive input from L6 CThPNs and L5 SCPNs. CThPNs project to the thalamus and receive reciprocal thalamic afferents. SCPNs project solely to higher-order thalamic nuclei, providing cortical input. Overall, PNs of the neocortex can be classified by their axonal projections, which in turn indicates their functional role. Upper layer IT neurons project within the cortex while some lower layer IT neurons also project to the striatum. Moreover, lower layer SCPNs project to multiple subcerebral targets and CThPNs solely innervate thalamic nuclei (see Fig. 2).

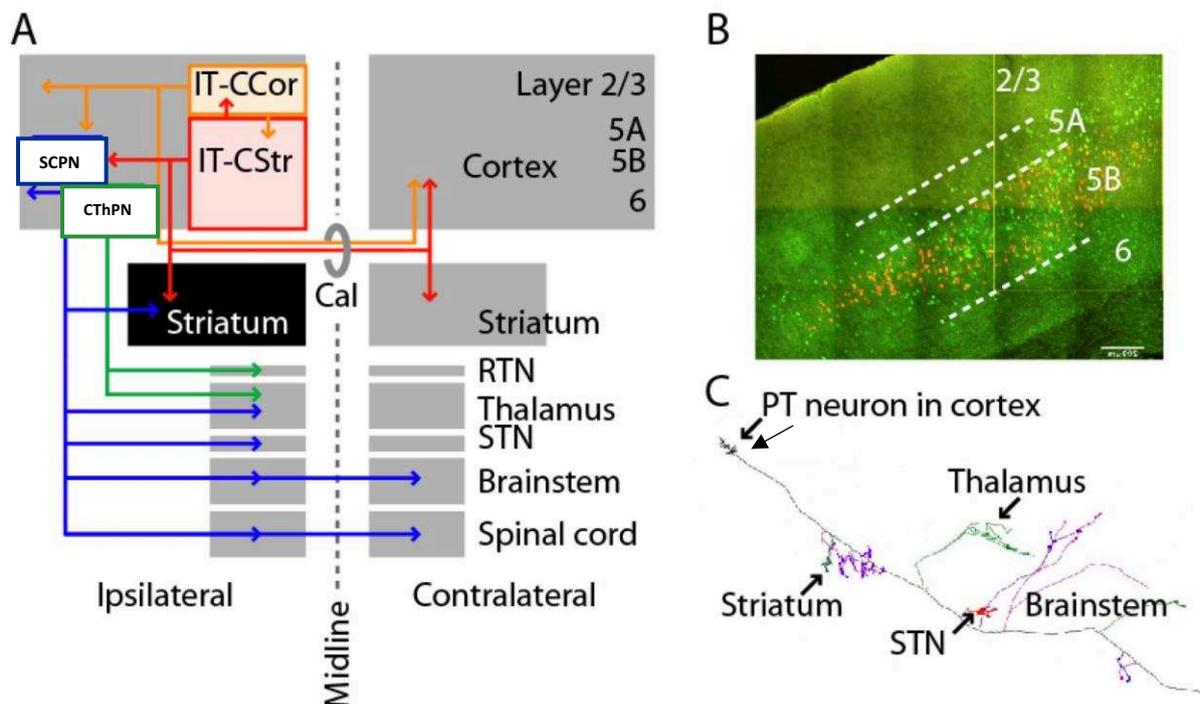


Figure 2: Long-range axonal projections define two classes of corticostriatal projection neuron

A. SCPNs (blue) project to ipsilateral striatum, thalamus, subthalamic nucleus (STN), and many brainstem and spinal cord regions. Intratelencephalic (IT) neurons project ipsi- or bilaterally (via corpus callosum, Cal), within the cerebral hemispheres to cortex (IT-CCor, orange), and many of these also to striatum (IT-CStr, red). The ipsilateral striatum (black) is unique in receiving Cstr input from both IT neurons and SCPNs. Layer 6 corticothalamic neurons (CT, green) project only to thalamus and its reticular nucleus (RTN). **B.** Retrogradely labelled corticospinal CSMNs and callosally projecting IT-CStr neurons in mouse motor cortex. IT neurons (green) and SCPNs (orange) are intermingled in layer 5B, but not double-labeled. **C.** A single SCPN's axon is multiprojectional, sending branches to many subcortical areas. Taken and modified from Shepherd (2013).

B. Development of the neocortex

1. Corticogenesis

Early in development, the telencephalic wall is composed of neuroepithelial cells. They divide symmetrically, thereby generating two more neuroepithelial cells, intended for expansion purposes. These epithelial cells are multipotent and represent the first neuronal population in the neocortex (Haubensak *et al.*, 2004; Greig *et al.*, 2013; Tan and Shi, 2013). Starting at E9.5, neuroepithelial cells give rise to radial glial cells (RGCs). While RGCs start to proliferate symmetrically (for self-renewal purposes), some expand onto the lateral ventricle, establishing the germinative or ventricular zone (VZ). At later stages, RGCs undergo a final asymmetric division and give rise to some excitatory PNs, some RGCs in turn, give rise to outer RGCs (very few in rodents) and intermediate progenitors (IPs), forming the subventricular zone (SVZ). IPs act as transit-amplifying cells, undergoing asymmetric division to self-renew, limited proliferative divisions, and more often dividing symmetrically to produce two neurons (Haubensak *et al.*, 2004; Noctor *et al.*, 2004; Wu *et al.*, 2005; Sessa *et al.*, 2008). A fourth class of progenitors, the short neural precursors, reside in the VZ, producing post-mitotic neurons directly within the VZ (Gal, 2006; Stancik *et al.*, 2010). Neocortical progenitors begin to produce excitatory PNs around E10.5 (Angevine and Sidman, 1961; Rakic, 1974). Earliest-born cortical neurons migrate past the VZ and form the preplate (PP), which is then split into two structures: the marginal zone (MZ) and the subplate (SP). The marginal zone will give rise to L1 neurons. Between these two structures, the cortical plate (CP) will develop and give rise to the other layers of the neocortex. The first newborn neurons migrate into the CP *via* somal-translocation, whereas later newborn neurons move along a glial scaffold *via* glia-guided neuronal migration. Throughout the rest of corticogenesis, newly born neurons migrate into the CP, organizing themselves in an “inside-out” fashion (see **Fig. 3**). This implies that early-born neurons form lower layers 6 and 5, and late-born neurons populate upper layers 4 – 2 (Greig *et al.*, 2013). After neurogenesis is complete, neural progenitors transit to a gliogenic mode, generating astrocytes and oligodendrocytes.

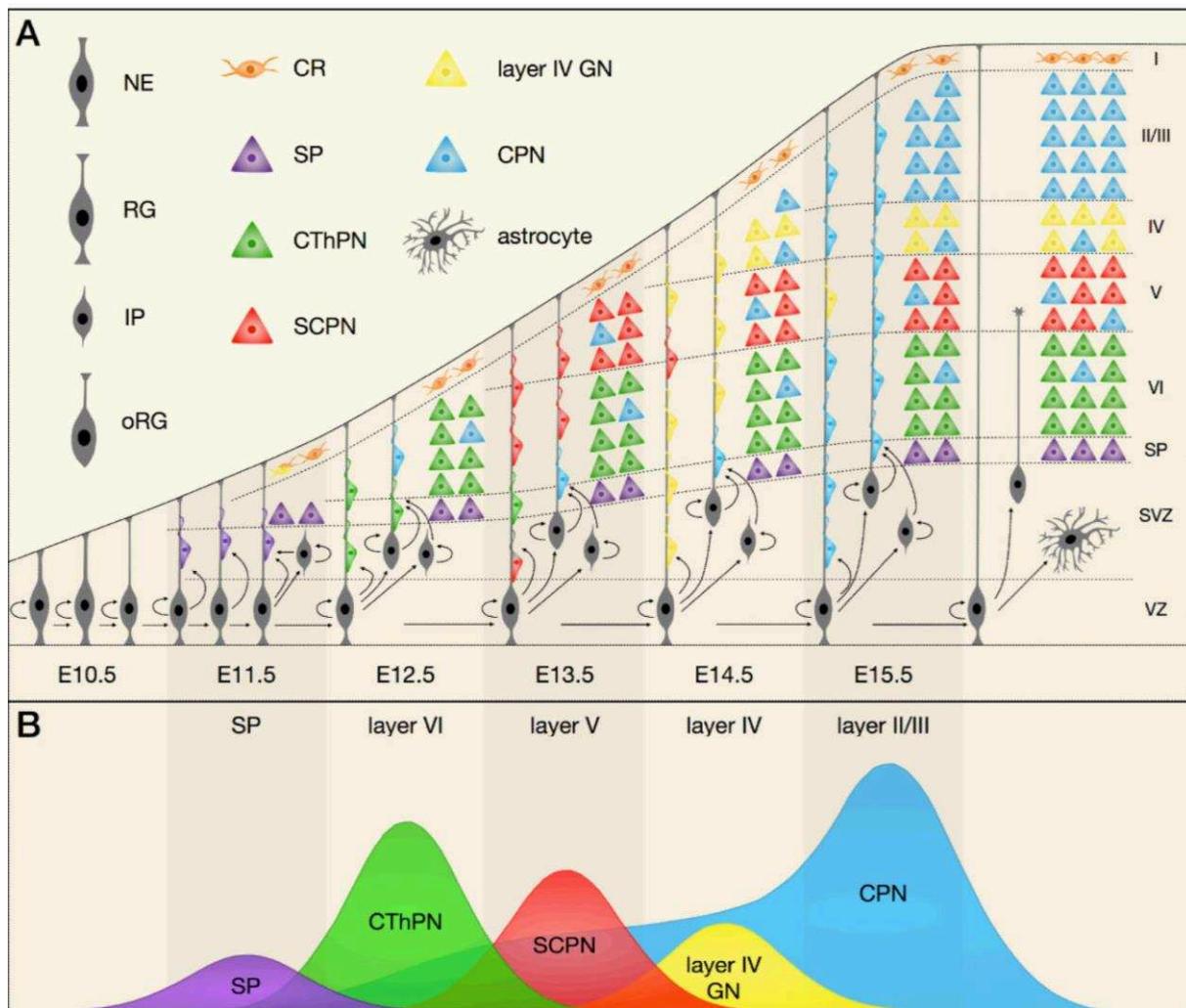


Figure 3: Neocortical projections neurons are generated in an “inside-out” fashion by diverse progenitor types in the VZ and SVZ

This schematic depicts the sequential generation of neocortical projection neuron subtypes and their migration to appropriate layers over the course of mouse embryonic development

A. Radial glia (RG) cells in the VZ begin to produce projection neurons around E11.5. At the same time, RG generate intermediate progenitors (IP) and outer radial glia (oRG), which establish the SVZ and act as transit-amplifying cells to increase neuronal production. After neurogenesis is complete, neural progenitor transition to a gliogenic mode, generating astrocytes and oligodendrocytes. Cajal-Retzius (CR) cells primarily migrate into neocortical layer I from non-cortical locations, while other PNs are born in the neocortical VZ/SVZ and migrate along radial glial process to reach their final laminar destinations.

B. Distinct PN sub-types are born in sequential waves over the course of neurogenesis. The peak birth of SP neurons occurs around E11.5 with the peak birth of CThPNs and SCPNs occurring at E12.5 and E13.5, respectively. Layer IV granular neurons (GN) are born around E14.5. Some CPNs are born starting at E12.5 and those CPN born concurrently with CThPNs and SCPNs also migrate to deep layers. Most CPNs are born between E14.5 and E16.5, and these late-born CPNs migrate to superficial cortical layers. Peak sizes are proportional to the approximate number of neurons of each sub-type born on each day. Ne, neuroepithelial cell, WM, white matter. Taken from Greig et al. (2013).

2. Development of the callosal projection neurons (CPNs)

The birthdate of CPNs is in line with their future location in the radial column of the neocortex, such as CPNs of L6 are born at E12.5 together with CThPNs, L5 CPNs at E13.5 and L2-3 CPNs from E15.5 to E17.5 (Molyneaux *et al.*, 2007). As newborn CPNs are migrating to the CP, they already start to send axons towards premature zones of the developing CC. In *ex vivo* experiments, Semaphorin 3 has been shown to repel axons away from the cortical marginal zone (Polleux *et al.*, 1998) and guide premature CPN axons towards the midline (Zhao *et al.*, 2011). Parallel to the birth of CPNs, the two telencephalic hemispheres start to fuse with help of glial cells (Lindwall, Fothergill and Richards, 2007). A transient zone of a 'bridge-like subcallosal sling', which is formed by local neurons and glial cells, builds up a first window for CPNs axons to cross. Thanks to guidance cues, repelling or attracting from glial cells, first CPNs axons are crossing the midline (see **Fig. 4**). Since newborn CPNs in the cingulate cortex (most dorsomedial structure of the neocortex) are closest to the midline, they are referred to as pioneer axons crossing the hemisphere bridge (at E17, Rash and Richards, 2001) and they help guiding later-arriving CPN axons (approximately 1 day later) in providing a structural framework (Norris and Kalil, 1991).

Due to the different birthdates, axons of lower layer CPNs cross the midline before axons of upper layer CPNs. When reaching the contralateral side, CPN axons turn dorsally and extend into the neocortex towards homotopic targets (Fame, MacDonald and Macklis, 2011). Important signalling molecules responsible for proper guidance of CPN axons are members of the Slit/Robo, Wnt, Netrin and Ephrin families. The specific mechanisms of the callosal axon guidance to their homotopic regions of the contralateral side are largely unknown. However, callosal fibers that fail to cross the midline due to wrong guidance cues or absence of molecular factors remain ipsilateral and form Probst bundles (Fame, MacDonald and Macklis, 2011).

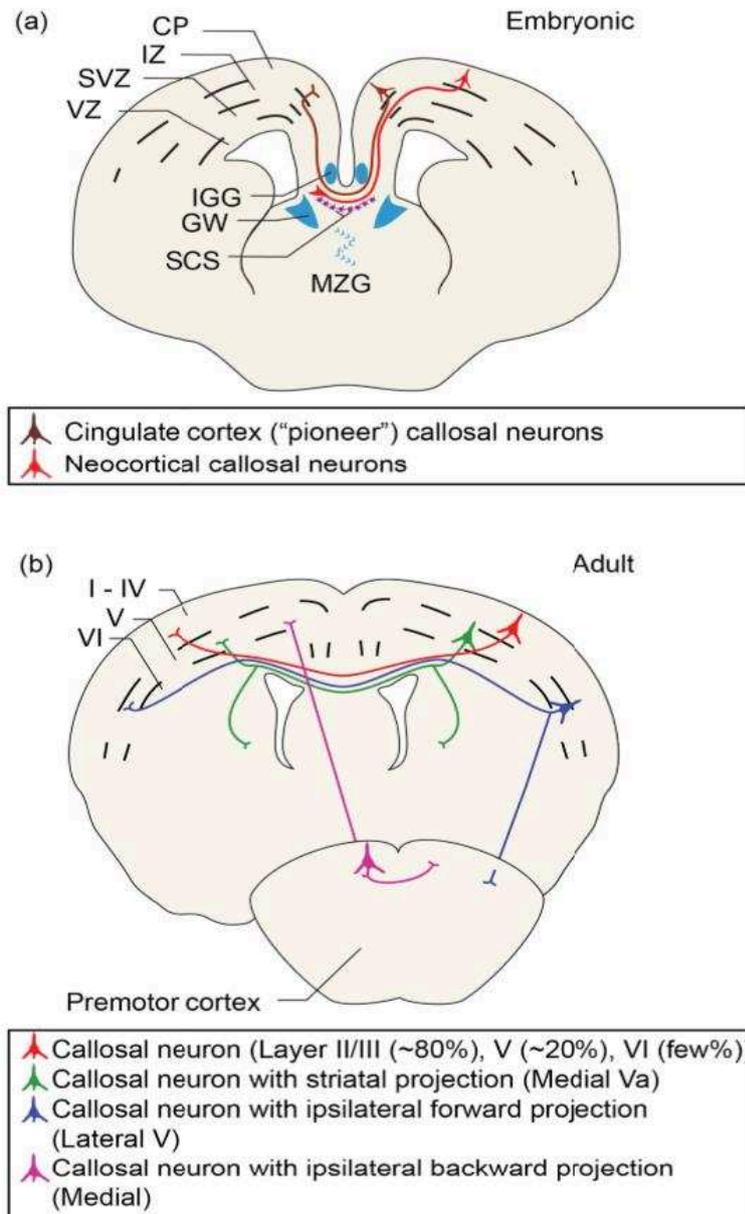


Figure 4: CPN development and diversity

This schematic representation shows callosal projection development at embryonic stages and shows characteristic callosal connections in the adult mouse brain

A During development, callosal axons (red) turn toward the midline. Multiple glial populations (blue) and mixed neuronal/glial populations (purple) play critical roles in CPN axon guidance and midline crossing. Pioneering axons (brown) from neurons of the cingulate cortex begin the process of midline crossing. This schematic represents process that occur across multiple embryonic times during mouse CPN development. Abbreviations: CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; IGG, indusium griseum glia; GW, glial wedge; SCS, subcallosal sling; MZG, midline zipper glia.

B At least four major types of adult CPN can be classified based on projection patterns. These include: single projections to the contralateral cortex (red); dual projections to the contralateral cortex and ipsilateral or contralateral striatum (green); dual projections to the contralateral cortex and ipsilateral premotor cortex (blue); or dual projections to the contralateral cortex and ipsilateral sensorimotor cortex (purple). Taken from Fame, MacDonald and Macklis (2011).

3. Development of the CST

Axons of the CST originate from SCPNs, residing in lower layer 5B of the cortex. Axons of SCPNs, once generated, have to travel a long distance towards the spinal cord and do so by early outgrowth of the cortex. At E14, CST axons are already reaching the IC and at E17, CST axons are present in the brainstem. At P0, CST cross the midline after the pyramidal decussation and reach the spinal cord at postnatal day (P) 2 (see **Fig. 5**).

Initially, corticofugal projections, including axons from SCPNs and CThPNs, grow out of the cortex and make a lateral turn away from the midline, thus diverging from callosal projections (Welniarz, Dusart and Roze, 2017). This long trajectory implies proper guidance by extrinsic signals. Guidance cues accompany pioneer axonal projections and ensure they reach their targets. After CST axons have left the cortex, Semaphorin family members SEMA3A and SEMA3C together with NETRIN-1, attract CST axons away from the midline (Srivatsa *et al.*, 2014). After CST axons have passed the pallial/subpallial boundary (PSPB) - a molecular and cellular border between pallial and subpallial structures - they make a medial turn to enter the IC, attracted by Slit guidance ligand 1 and 2 (SLIT1/2) and ROBO1/2 receptors (Bagri *et al.*, 2002; Lopez-Bendito *et al.*, 2007). Passing the IC, CST axons, with the help of NKX-2, enter the brainstem in the caudal medulla reaching the pyramidal decussation (Welniarz, Dusart and Roze, 2017). At the level of the pyramidal decussation, CST axons are starting to cross the midline. Several molecules are involved in the corticospinal midline crossing of CST. NETRIN-1, DCC and UNC5H3 are crucial factors, as loss of these molecules in animal models leads to a failure of most CST axons to cross the midline. The exact mechanisms are still not known and it is uncertain how the small proportion of CST axons are instructed not to cross the midline (Welniarz, Dusart and Roze, 2017). What is known is that once the CST cross the midline, they are prevented from re-crossing by repellent protein EPHRIN-B3, secreted from the midline (Kullander *et al.*, 2001). However, a small fraction of CST axons does not cross the midline at the pyramidal decussation and instead project to the ipsilateral spinal cord (Welniarz, Dusart and Roze, 2017 **see Fig. 6**).

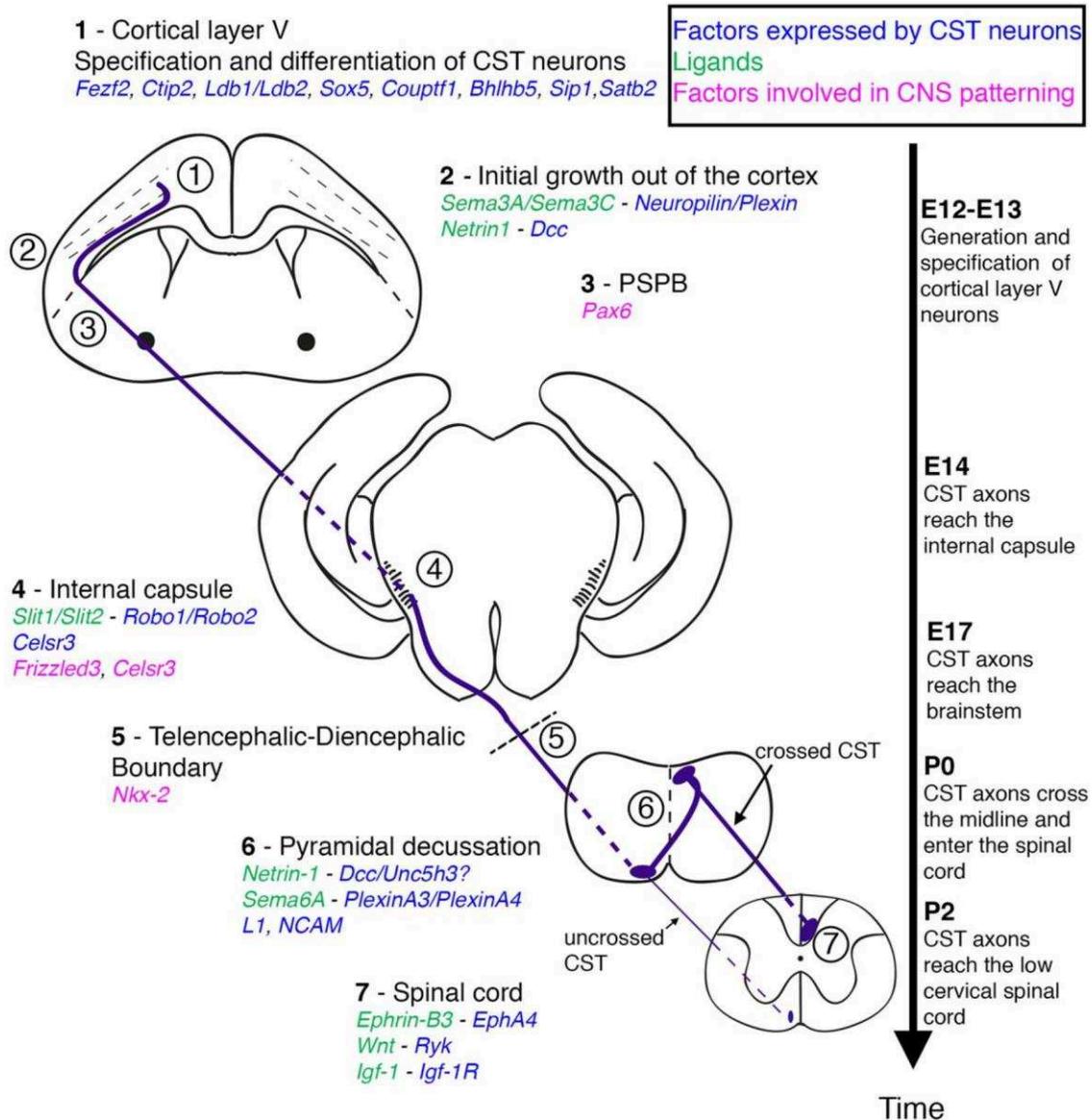


Figure 5: Mechanisms and time course of mouse corticospinal tract (CST) development

The left side of the figure shows the trajectory of the mouse CST from the cortex to the spinal cord. The genes involved in CST development are indicated at the corresponding level. The right side of the figure indicates the time course of CST development.

CST, Corticospinal tract; PSPB, pallial/subpallial boundary. Taken from Welniarz, Dusart and Roze (2017).

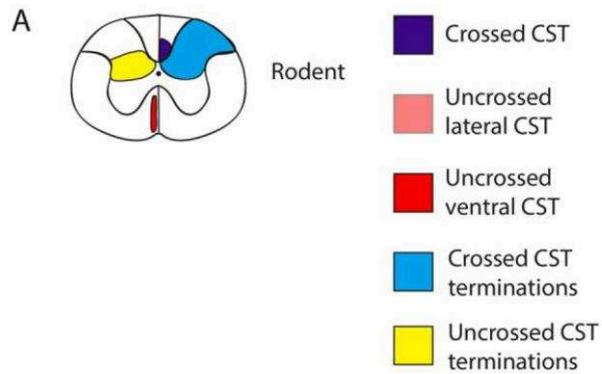


Figure 6: CST projections to the spinal cord in rodents

A The crossed CST (dark blue) is located in the most ventral part of the dorsal funiculus, and the uncrossed CST (red) runs in the ventral funiculus. Taken and modified from Welniarz, Dusart and Roze (2017).

4. Development of CTA

Newborn post-mitotic cortical neurons directly start to extend their axons before even leaving the IZ. Characteristic for corticofugal axons, they are attracted by Sema3C expressed in the IZ (Bagnard *et al.*, 1998; Skalióra, 1998). Furthermore, the SEMA3 gradient within the IZ guides CTA towards the lateral side of the cortex (Bagnard *et al.*, 1998). Then, CTA pass through the IZ and reach the lateral part of the IC at E13 – E15.5 (Jacobs *et al.*, 2007). At E15.5, CTA cross the PSPB (Carney *et al.*, 2009). At this point, ventrolateral oriented CTA tend to turn medially in order to reach the IC in the subpallium (Agmon *et al.*, 1995). As it is the case in the development of the CC, also pioneer axons of the very first-born CThPNs in the subplate act as pioneer axons to guide later-arriving CTAs. Guidance cues of the Slit and Robo family ensure CTAs remain in the IC until they reach the diencephalon-telencephalon boundary (DTB, Leyva-Díaz and López-Bendito, 2013). At the DTB, CTA enter the premature thalamus and meet cells of the perireticular thalamic nucleus and the thalamic reticular nucleus (RTN), at E16 (Jacobs *et al.*, 2007). Then, CTA finally reach the thalamus (E18.5 – P2.5) and start to innervate their specific nuclei, a process which can take several days (Jacobs *et al.*, 2007). Therefore, the CTA form proper connections at early postnatal stages, for instance, the first CTA to reach the dLGN appear at P4.5 (Jacobs *et al.*, 2007).

5. Development of TCA

The reciprocal connection between the cortex and the thalamus starts between E12 – E18 (Leyva-Díaz and López-Bendito, 2013). TCA follow a canonical pattern in order to reach the cortex. They run rostrally to the telencephalon, make a sharp turn at the DTB and follow through the striatum to reach the developing cortex (Leyva-Díaz and López-Bendito, 2013). TCA are already present in the IC at E13. Interestingly, they even reach the cortex before their appropriate target neurons are even born. Just before birth of the embryo, TCA grow into the CP and form branches and synapses in their appropriate layer (Leyva-Díaz and López-Bendito, 2013).

In summary, the development of the neocortex is organized in an “inside-outside” fashion, where PNs first populate lower layers and upper layers are populated at later embryonic stages. While PNs migrate to their destined locations, they already start to send axons to their appropriate targets and thus, establish first major connections at early postnatal stages.

C. Genetic regulation of PNs position and identity

1. Positional information in progenitors

The establishment of areas is driven by an interplay between intrinsic and extrinsic mechanisms throughout the development of the neocortex (see **Fig. 7**). First, broad patterning of areas is initiated by a complex interaction between morphogens and molecular signals secreted from opposing patterning centres of the neocortical primordium (Grove and Fukuchi-Shimogori, 2003; Alfano and Studer, 2013). Beginning at E9.5 in mice, fibroblast growth factors (FGF) 8 and 17 are secreted from the commissural plate, Wnt and Bmp signalling molecules are diffused from the cortical hem and SFRP2 and some Egf signalling molecules derive from the cortical antihem (Greig *et al.*, 2013). Their spatial- and time-dependent secretion establishes a gradient expression of TFs in the VZ, which act in a complementary manner, thereby shaping boundaries in the developing telencephalon. Namely, transcription factors PAX6 (paired box 6), EMX2 (empty spiracles homolog 2), SP8 (trans-acting transcription factor 8) and COUP-TFI (chicken ovalbumin upstream promoter transcription factors 1) are expressed in progenitors of the VZ. (Bishop, Goudreau and O’Leary, 2000; Zhou, Tsai and Tsai,

2001; Sahara *et al.*, 2007), the starting point of corticogenesis. While *Pax6* is expressed in a high rostralateral to low caudomedial manner, *Emx2* is expressed in a low rostralateral to high caudomedial manner. Moreover, *Sp8* is expressed in a high rostromedial to low caudolateral manner, whereas *Coup-TFI* is expressed in a high caudolateral to low rostromedial manner. Progenitors located at different medio-lateral and rostro-caudal coordinates express distinct levels of these TFs, which establishes a fate map of cortical areas in the VZ. Loss-of-function and gain-of-function studies revealed the areal identity establishment driven by these TFs. Here, *Fgf8* has been shown to function as key organizer of area identity. Upon overexpression of *Fgf8*, rostromedial areas of the cortex are expanded caudally (Fukuchi-Shimogori and Grove, 2001; Suzuki-Hirano *et al.*, 2010). In contrast, reduced *Fgf8* expression in hypomorphic mutants causes caudal areas of the cortex to expand rostrally (Garel, 2003).

Strong caudal expression of *Emx2* and *Coup-Tf1* promotes specification of sensory areas. In *Nestin-Emx2* transgenic mice, *Emx2* is expressed more uniformly throughout the VZ, leading to an increase in the size of visual cortex, and a concomitant size decrease and rostralateral shift of somatosensory and motor areas. In contrast, upon absence of one allele of *Emx2*, motor areas expand, and sensory areas shift caudomedially (Hamasaki *et al.*, 2004). Similarly, in *Coup-tf1*-conditional null mice, motor areas expand dramatically to occupy a large portion of cortex, while sensory areas are displaced to a narrow occipital band that contains compressed, but properly-configured, sensory representations (Armentano *et al.*, 2007; Greig *et al.*, 2013). Rostrally, expression of *Pax6* and *Sp8* drives specification of motor identity. Both *Sp8* and *Pax6* conditional *null* mice, as well as *Pax6^{sey/sey}* (“small eye”) hypomorphic mutants, exhibit a drastic loss of motor areas (Bishop, Goudreau and O’Leary, 2000; Muzio *et al.*, 2002; Zembrzycki *et al.*, 2007). Gain- and loss-of-function *in utero* electroporation experiments, however, independently support a role for *Sp8* in cortical area identity, both by cell-autonomous repression of *Coup-tf1* in neocortical progenitors and indirectly by induction of *Fgf8* (Sahara *et al.*, 2007; Borello *et al.*, 2014).

These complementary expression patterns allow coordination of areas in any tangential dimension and form a first rough area pattern, known as the ‘protomap’ (Rakic, 1988). As mentioned above, thalamocortical afferents reach the cortical primordium starting from E12. The developmental program of the thalamus and the neocortex is occurring temporally in

parallel and both structures are influencing each other during ontogeny (Moreno-Juan *et al.*, 2017; Antón-Bolaños, Espinosa and López-Bendito, 2018). Their reciprocal interplay is crucial for proper development of both structures and disruption in the developmental program of one partner can have consequences for the other one. For example, mice with a reduced somatosensory cortex (artificially induced via miss-expression of *Pax6* in cortical progenitors) have a miniaturized body map representation which can also be observed in its corresponding thalamic nuclei (Zembrzycki *et al.*, 2013). On the contrary, ablation of TCA inputs to the developing V1 is leading to a loss of V1 identity, which is not anymore distinguishable from higher order visual areas (Chou *et al.*, 2013). At postnatal stages, TCA innervations are responsible for refining the initial ‘protomap’, influencing the size and identity of specific cortical areas (Antón-Bolaños, Espinosa and López-Bendito, 2018).

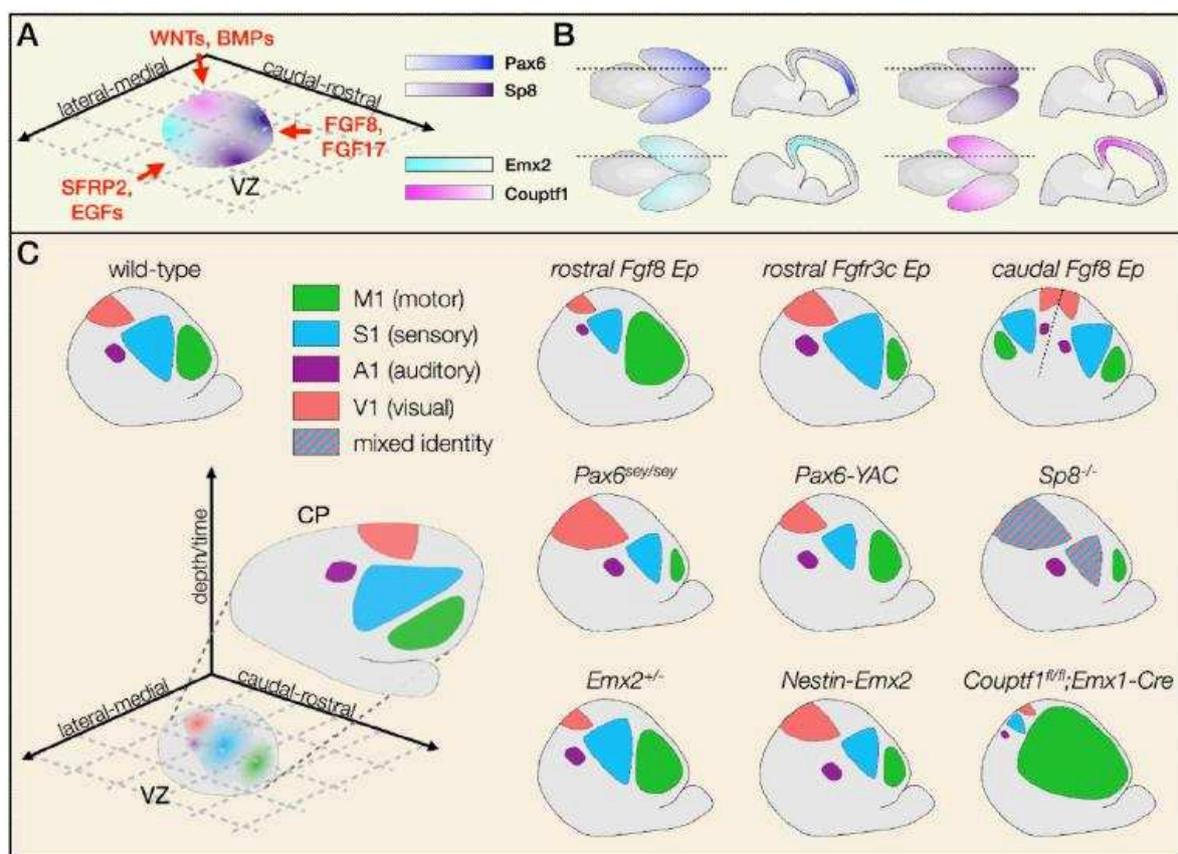


Figure 7: Transcription factors in the VZ establish an area identity fate map

A. Arealization of the cerebral cortex is initiated by diffusible morphogens and signaling molecules secreted from opposing sides of the neocortical periphery (left panel). These signals induce expression of complementary and orthogonal transcription factor gradients such as *Pax6/Emx2* and *Sp8/Coup-TFI*, seen in a schematized flatmount view of the ventricular zone (VZ)

B. *Pax6* is expressed most highly rostrally, in opposition to *Emx2*, which is expressed most highly caudally. Similarly, *Sp8* is expressed most highly rostromedially, in opposition to *Coup-TF1*, which is expressed most highly caudolaterally. Gradients are shown in wholemount (left) and sagittal (right) views for each.

C. Progenitors located at different medio-lateral and rostro-caudal coordinates express specific levels of these transcription factors, which combinatorially establish a fate map of cortical areas in the ventricular zone. This fate map is later translated into a definitive area map in the cortical plate (CP), shown in flatmount view (left panel). Manipulation of morphogen signaling or VZ transcription factor expression results in dramatic changes in the size and position of cortical areas (right panel). Hatching indicates mixed area identity. A1, primary auditory cortex; Ep, electroporation; M1, primary motor cortex; S1, primary somatosensory cortex; sey/sey, small eye hypomorphic mutant; V1, primary visual cortex; YAC, yeast artificial chromosome. Taken from Greig et al. (2013).

2. Genetic regulation of PN diversity

In the beginning of corticogenesis, undifferentiated neurons share molecular sub-type identities. With proceeding developmental stages, neurons start to differentiate and sub-type identities become more distinct from each other. The sub-type identities of post-mitotic PNs are established by cross-repressive molecular controls. Molecular boundaries for example exist between CFuPN and CPN subpopulations. Furthermore, among CFuPNs, molecular controls diverge SCPN and CThPN subpopulations (see **Fig. 8**).

a. Molecular fate specification of CThPNs

Corticofugal projection neurons are among the first class of neurons to be generated during cortical neurogenesis (E12.5-E13.5) and are populating L6 and L5.

Tbr1

Important for the post-mitotic specification of CThPNs is the transcription factor T-Box Brain Protein 1 (TBR1). Its expression starts in the first post-mitotic neurons, the preplate neurons, followed by subplate neurons. Studies using genetic manipulations revealed that *Tbr1* is necessary for the differentiation of L6 neurons. In *Tbr1*^{-/-} mice, early-born neurons (E11.5) fail to express a subset of L6 marker such as Transducin Like Enhancer Of Split 4 (TLE4) and Forkhead box protein P2 (FOXP2). Instead these cells express markers associated with L5 neurons, such as B-cell lymphoma/leukemia 11B (CTIP2) and FEZ Family Zinc Finger 2 (FEZF2, McKenna et al., 2011). On the other hand, ectopic expression of *Ctip2* and *Fezf2* in L6 neurons

represses the connectivity of CThPNs to the thalamus and instead cells send their axons to subcerebral targets, characteristic of L5 neurons (McKenna *et al.*, 2011). Chromatin immunoprecipitation assays revealed that TBR1 directly binds *Fezf2* and inhibits its expression *in vivo*, whereas *Ctip2* does not seem to be directly regulated by TBR1, as overexpression of *Tbr1* in dissociated cells (E12.5 and E13.5) does not change the expression of *Ctip2*. In a complementary manner, *Fezf2*-deficient mice show an aberrant expansion of TBR1 expression in L5 at the expense of SCPNs. (McKenna *et al.*, 2011).

Sox5

Another TF involved in the specification of CThPNs is SRY-Box 5 (SOX5). Its genetic inactivation in mice shows a similar phenotype as in *Tbr1*^{-/-} mice (Kwan *et al.*, 2008; Lai *et al.*, 2008). SOX5 is expressed at high levels in the CP and SP during the specification of CFuPNs. Its expression is restricted to post-mitotic cells and is present in L6 TBR1-positive post-mitotic neurons but not present in progenitors of the SVZ (Lai *et al.*, 2008). Genetic inactivation of *Sox5* in mice alters the molecular identity of L6 neurons leading to a failure of these neurons to express TBR1 or the CThPN marker Zinc Finger Protein ZFPM2 (also named FOG2), but instead the SCPNs markers CTIP2 and FEZF2 are expressed (Kwan *et al.*, 2008). In contrast to *Tbr1*^{-/-} cortices, the corticothalamic projections of L6 neurons are not affected in *Sox5*^{-/-} mutant mice. Instead, an additional subcerebral projection tract appears, which runs within the external capsule and reaches the cerebral peduncle and the pons (Lai *et al.*, 2008).

These studies suggest a reciprocal TF mechanism, where SOX5 acts upstream of TBR1, specifying post-mitotic CThPNs. Both TFs can inhibit the expression *Fezf2*, thereby indirectly inhibiting the expression of *Ctip2* and thus, specification of SCPNs.

b. Molecular fate specification of SCPNs

Fezf2 and Ctip2

FEZF2 is expressed in a subset of committed progenitors during CFuPN generation and remains expressed in post-mitotic SCPN and CThPNs (Eckler and Chen, 2014). In the absence of FEZF2, *Ctip2* expression in L5 is downregulated, whereas *Tbr1* is upregulated in putative L5 neurons

(see **Fig. 8**). The low-level CTIP2-expression in L6 is not affected though, suggesting a precise layer-specific or time-specific regulation of the repressing machinery of TFs. Interestingly, not only SCPN lineage specification is perturbed in *Fezf2*^{-/-} mice, also expression of CThPNs markers such as FOXP2 and Protein Phosphatase 1 Regulatory Inhibitor Subunit 1B (DARPP32), which are decreased in L6 neurons, revealing a function of FEZF2 in CThPN specification (Chen *et al.*, 2008; McKenna *et al.*, 2011). Together with changes in molecular identity, axonal projections are also affected, as typical SCPN projections in *Fezf2*^{-/-} cortices are disturbed. More specifically, L5 projections to the tectum, pons and spinal cord are reduced and fail to reach their final targets, while more axons tend to project to the thalamus (Molyneaux *et al.*, 2005; Chen *et al.*, 2008). On the other hand, overexpression of *Fezf2* in progenitors at E13.5 induces *Ctip2* expression at similar levels as in L5 neurons, indicating that FEZF2 is acting upstream of *Ctip2* (Guo *et al.*, 2013). As overexpression of *Fezf2* leads to an induction of *Ctip2*, *Fezf2* absence results in downregulation of *Ctip2* in L5 (Hirata *et al.*, 2004). While *Fezf2*-overexpressed cells show a clear migration deficit - they remain in a heterotopic layer beneath the corpus callosum - they anyhow differentiate and project to subcerebral targets (Molyneaux *et al.*, 2005). Interestingly, in *Ctip2*^{-/-} mice, L5 neurons fail to reach the spinal cord, indicating that *Ctip2* is critical for SCPNs to extend projections to the SC (Arlotta *et al.*, 2005).

Taken together, *Sox5* seems to act upstream of *Tbr1* as determinant of CThPNs *via* inhibition of SCPN determinant *Fezf2* and indirectly *Ctip2*. On the contrary, *Fezf2* is inhibiting *Tbr1* and promotes *Ctip2* expression to regulate SCPN specification. Additionally, *Fezf2* seems to be involved in CThPN specification.

c. COUP-TFI

Another transcription factor implicated in the post-mitotic lineage specification during cortical neurogenesis is COUP-TFI. Interestingly, *Coup-Tf1*^{-/-} mice show superficial CThPNs, which express abnormal high levels of *Ctip2* and *Fezf2* (see **Fig. 8**). Also, *Lmo4* expression (highly expressed in motor cortex) is abnormally upregulated in L5 of the somatosensory cortex (Alfano *et al.*, 2014). Surprisingly, the expression pattern of TBR1 is not altered. This observation adds up to the function of COUP-TFI as sensory area identity determinant not only

in progenitors, but also in post-mitotic neurons (Tomassy *et al.*, 2010). Thus, COUP-TFI acts as an area- and laminar-specific regulator, suggesting that the two processes are tightly linked.

d. Fate specification of CPN

Satb2

The peak of CPN generation during cortical neurogenesis appears around E14.5 and the majority of newborn CPN neurons migrate to upper L2-4. Some CPNs are also generated at earlier stages, together with CFuPNs, thus, sharing a developmental boundary. However, their axonal projections are diverging as soon as they exit the CP. The transcription factor special AT-rich sequence binding protein 2 (SATB2) has been found to be critical for CPN specification. SATB2 is expressed in a subset of neurons in L2-5 during differentiation and can be found in CPNs as well as in associative projection neurons (Alcama *et al.*, 2008; Chen *et al.*, 2008; Leone *et al.*, 2015). Genetic inactivation studies, using *Satb2*-deficient mice, revealed that CPN identity markers such as Cadherin 10 (CDH10) or Cut Like Homeobox 1 (CUX1) are drastically reduced. Instead, the SCPN identity marker CTIP2 and others are upregulated in layer 2-4. Additionally, callosal projections through the CC are severely reduced, and axons project alternatively to subcerebral targets in pathways, normally innervated by CTIP2-positive SCPNs (Britanova *et al.*, 2008). High expression of *Satb2* promotes expression of upper layer genes, including *Brn2*, *Cux2* and *Svet1* (Tarabykin *et al.*, 2001; McEvelly *et al.*, 2002; Sugitani *et al.*, 2002; Nieto *et al.*, 2004; Zimmer *et al.*, 2004) which are essential for proper CPN development and represses lower layer genes such as *Ctip2* (Britanova *et al.*, 2008). In contrast, a study of the B. Chen group showed that low expression of *Satb2* activates *Fezf2* expression in SCPNs, while strong *Fezf2* expression inhibits *Satb2* (McKenna *et al.*, 2015). Also, CFuPNs showed low- or medium *Satb2* expression, in accordance with the studies of Arlotta *et al.* (2015) and Leone *et al.* (2014). This possible combinatorial effect of determination genes is further supported by research from our lab. A subpopulation of lower layer neurons co-expresses *Ctip2* and *Satb2* during cortical neurogenesis and this subpopulation is present until late postnatal stages in somatosensory and motor area (Harb *et al.*, 2016).

To conclude, CPN and CFuPN fate determination is highly regulated by fate determination genes. These TFs mainly repress each other and thereby delineate CThPN, SCPN or CPN

identity. In this process, SCPN-determinant *Fezf2* is likely to have a key role, as it interacts with both, CPN and CThPN fate specification TFs.

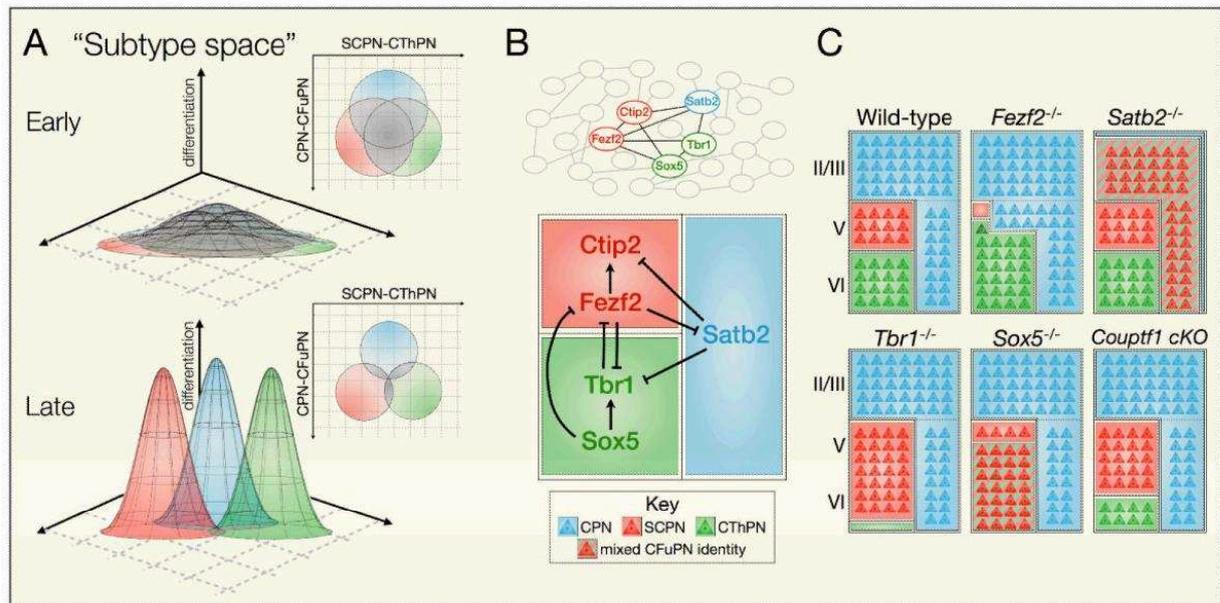


Figure 8: Competing molecular programs direct differentiation of newly-postmitotic projection neurons into one of three broad subtype identities

A. The subtype identities of postmitotic PNs are depicted within a theoretical *n*-dimensional ‘subtype space’ in which individual subtype identities (as defined by gene expression, morphology, dendritic structure, projection patterns, physiology, and other characteristics) occupy distinct coordinates. Boundaries between these identities, preventing neurons of one sub-type from taking on characteristics of another sub-type, are established by the action of cross-repressive molecular controls. One boundary exists between neurons specified as SCPN and those specified as CThPN, and another exists between CFuPN and CPN. Early in corticogenesis, undifferentiated neurons have largely overlapping sub-type identities (top). As development proceeds, neurons differentiate, and sub-types become more distinct from each other (bottom).

B. Known molecular controls represent key nodes of an elaborate transcriptional network, only beginning to be elucidated (top). Arrows indicate known cases of genetic or transcriptional activation or repression, and further interactions and molecular controls remain to be identified (bottom).

C. Changes in expression of these key regulators can cause boundaries between sub-types to shift, with neurons partially or completely acquiring features characteristic of other sub-types. In some mutants, neurons acquire CFuPN identity generally, rather than a well-defined CThPN or SCPN identity. The boundaries between CFuPN and lower layer or upper layer CPN may shift independently of one another., represented by the dashed line between lower layer and upper layer CPN.

CFuPN, corticofugal projection neuron; CPN, callosal projection neuron; CThPN, corticothalamic projection neuron; SCPN, subcerebral projection neuron. Taken from Greig et al. (2013).

3. Layer-specific molecular markers in the neocortex

This section will give an overview of prominent molecular markers in the neocortex, which are expressed in distinct layers throughout development. The presentation of these markers is limited and intended to help the reader for the result section.

a. Upper layer CPN marker CUX1

CUX1 belongs to the family of homeodomain TFs and is characterized by four DNA-binding domains and a homeodomain that contains a nuclear localization sequence (Aufiero, Neufeld and Orkin, 1994). CUX1 can act as repressor or activator, depending on the target gene (Weiss and Nieto, 2018). In the cortex, CUX1 is highly coupled to Cux-ortholog CUX2, with whom it shares 48% amino acid identity (Quaggin *et al.*, 1996). Both have similar expression profiles, they can act in a complementary manner but differ in some functional roles.

Cux1 mRNA in the cerebral cortex can first be observed in VZ and SVZ where it is expressed in cortical precursors (Nieto *et al.*, 2004; Zimmer *et al.*, 2004). Then, CUX1 is exclusively expressed in upper-layer CPNs (Weiss and Nieto, 2018). This expression profile is maintained during the subsequent postnatal cortical development and remains until adulthood. The level of expression differs in an area- and layer-specific manner. CUX1 is drastically decreased in upper layers of motor area compared to upper layers of S1. In the postnatal and adult S1 cortex, CUX1 levels are higher in layer 4 than in layer 2-3 (Nieto *et al.*, 2004; Ferrere *et al.*, 2006). The functional role of *Cux1* seems to be merely restricted to late stages of differentiation of CPNs. *Cux1*-mutant mice show no migration defects or aberrant specification of upper layer neurons (Cubelos, Sebastián-Serrano, Kim, Moreno-Ortiz, *et al.*, 2008; Cubelos, Sebastián-Serrano, Kim, Redondo, *et al.*, 2008). This suggests, that *Cux1* action is not crucial for fate choices or determination of cortical precursor cells (Weiss and Nieto, 2018). Instead, *Cux1* mutant mice show reduction in dendritic length and branching, as well as a reduction in spine density and aberrant spine morphology (Cubelos *et al.*, 2010). On the other hand, only CUX1 seems to be important for axonal projection of CPNs, but not CUX2. Upon *Cux1* deletion, the characteristic topographic projection of CPNs through the CC is drastically reduced (Rodríguez-Tornos *et al.*, 2016). Taken together, CUX1 is a very suitable

marker for upper layer CPNs at early postnatal stages throughout adulthood. Moreover, CUX1 labels upper layers, thereby delineating the cortex from the lower layers.

b. SCPN marker CTIP2

CTIP2 is a zinc finger TF which acts as transcriptional repressor (Avram *et al.*, 2000; Senawong *et al.*, 2003). CTIP2 starts to be present in the cortical plate during the peak of SCPN production between E12 – E14 (**Fig. 3**). However, *Ctip2* does not seem to be critical for precursor specification of SCPNs, as it is not expressed in the VZ and SVZ (Chen *et al.*, 2008). *Ctip2* is a major downstream effector of *Fezf2* and is highly expressed in all post-mitotic SCPNs, regulating the extension of axons towards subcortical targets (Arlotta *et al.*, 2005; Chen *et al.*, 2008). The expression pattern of CTIP2 in L5 SCPNs is maintained from early postnatal to adulthood (Arlotta *et al.*, 2005). The transcription of *Ctip2* is repressed by TFs SATB2 or SOX5, which promote CPN or CThPN fate determination in post-mitotic neurons, suggesting that *Ctip2* is a critical target of transcriptional regulation in developing PNs (**Fig. 8**)

Genetic inactivation of *Ctip2* revealed its function as regulator of axonal outgrowth and collateral refinement. In *Ctip2*^{-/-} mice, SCPNs are still born and migrate to L5, but CSMNs have defects in axonal fasciculation, outgrowth and pathfinding, resulting in failed spinal cord innervation. Axonal projections aberrantly stop at the pons (Arlotta *et al.*, 2005) and instead some axons project to ectopic targets on the dorsal side. Other axonal projections, such as contralateral connections by CPNs are not affected, revealing a specific SCPNs function of *Ctip2* (Arlotta *et al.*, 2005). Consistently, ectopic CTIP2 expression in E15.5 upper layer neurons is sufficient to redirect axonal projections to subcerebral targets: internal capsule, thalamus and spinal cord (Chen *et al.*, 2008). Moreover, *Ctip2* is also highly expressed in medium spiny neurons (MSNs) in the striatum. Here, *Ctip2* regulates proper differentiation and their organization as patch matrix, which is traversed by axonal projection of CSMNs. Furthermore, *Ctip2* is implicated in regulative expression of guidance molecules in MSNs, indicating a non-cell autonomous function (Arlotta *et al.*, 2008). Overall, *Ctip2* is involved in the development of immature post-mitotic SCPNs and its expression pattern makes it a reliable marker for L5 SCPN identity.

c. CThPN marker FOG2 (Friends of GATA2)

FOG2 is a non-DNA binding co-repressor that regulates transcription by interaction with other proteins, for example with family members of COUP-TF (Huggins 2001). Its function has been investigated in few studies, such as heart development (Tevosian *et al.*, 2000) or gonadal differentiation (Tevosian *et al.*, 2002), where its function is implicated in cell differentiation.

In the cortex, FOG2 expression starts in post-mitotic cells at E12.5 and expression continues until E15.5 in the CP. At early postnatal stages (P6), FOG2 is specifically maintained in L6 and the only other expression can be found in the cingulate cortex, while after P7 its specific L6 expression levels drastically decreases (Galazo, Emsley and Macklis, 2016). Even though it has been used as a L6 marker in some studies (McKenna *et al.*, 2011; Rouaux and Arlotta, 2013), a functional role of FOG2 has only recently been described in the cerebral cortex (Galazo, Emsley and Macklis, 2016). Interestingly, the specificity of FOG2 is remarkably limited to CThPNs only and is not expressed in any other cortical subclasses, such as in SATB2- (for CPN identity) or CTIP2- (for SCPN identity) expressing neurons in lower layers. Furthermore, FOG2 is strongly expressed in all thalamocortical retrograde labelled CThPNs in L6, at late embryonic and early postnatal stages. Loss-of-function experiments revealed no difference in arealization or lamination in the developing cortex or any mis-expression of molecular identities (Galazo, Emsley and Macklis, 2016). These data indicate that FOG2 is crucial neither for precursor fate determination nor for proper molecular identification of post-mitotic CThPNs during cortical neurogenesis. However, *Fog2-cko* mice show reduction of CThPNs projections to their natural targets in the motor cortex but not S1 cortex, indicating that FOG2 might have a guidance role in axonal projections of CThPNs. Additionally, a subpopulation of L6 neurons in the motor cortex aberrantly expresses high levels of CTIP2. Consistently, gain-of-function experiments by mis-expressing FOG2 in SCPNs decreases the CTIP2-expression levels (Galazo, Emsley and Macklis, 2016). Thus, FOG2 and CTIP2 seem to inhibit each other in a reciprocal manner, most likely at early postnatal stages when refinement of post-mitotic neurons takes place. Taken together, even though used in earlier studies as L6 marker, the study by Galazo *et al.* (2016) broadens the knowledge of FOG2 as a very specific CThPNs marker.

d. CFuPN marker PCP4

The purkinje-cell protein 4 (PCP4) is a 7.6-kDa IQ motif-containing protein acting as a calmodulin regulator (Johanson *et al.*, 2000). Calmodulin is an important protein kinase involved in synaptic plasticity (Xia and Storm, 2005), mainly expressed in the granular cells of the cerebellum and in the cortex (Cabin, Gardiner and Reeves, 1996). It is known that PCP4 positively regulates neurite outgrowth and neurotransmitter release (Harashima *et al.*, 2011). In addition, deregulation of PCP4 has been observed in various human disorders, including Alzheimer (Slemmon *et al.*, 1994) and Huntington's disease (Utal *et al.*, 1998). This suggests, that precise regulation of PCP4 level expression is associated with normal brain function.

In the cortex, PCP4 is expressed in neurons of nearly all cortical areas, including frontal, temporal, parietal and occipital regions of the brain. Additionally, PCP4 expression is found in subcortical structures such as hippocampus, caudate putamen and thalamus (Renelt, von Bohlen und Halbach and von Bohlen und Halbach, 2014). Radially, *PCP4 RNA* has been found in lower layers 5 and 6 of the whole cerebral cortex, with high expression levels in primary sensory areas (Watakabe *et al.*, 2012). Furthermore, tracing studies on thalamocortical afferents of the lateral geniculate nucleus showed *PCP4 RNA* expression in corticothalamic neurons residing in layer 6 of V1 in the rat. Besides the indication of PCP4 being expressed by CThPNs, another study indicated PCP4 expression in SCPNs (Arlotta *et al.*, 2005). Upon purification of CSMNs, CPNs and CThPNs at different developmental stages, Arlotta *et al.* (2005), were able to identify gene expression of PCP4 in CSMNs, with high level expression at late developmental stages (P6 – P14). In addition, it has been shown that *FEZF2* binds to the proximal promoter of PCP4 in cultured cortical neural progenitors, suggesting a direct transcriptional regulation of PCP4 by *Fezf2* (Lodato *et al.*, 2014). Taken together, PCP4 expression is indicated for late developmental CSMNs and CThPNs, projecting to the lateral geniculate nucleus, therefore representing a specific molecular marker of CFuPNs.

e. CThPN marker DARPP32

Dopamine and CAMP-Regulated neuronal Phosphoprotein 32 (DARPP32) is a protein that plays a crucial role in the physiological activity of dopaminergic neurons. Dopamine and other neurotransmitters act on dopaminergic neurons by changing the phosphorylated

state of DARPP32 (W. W. Wang *et al.*, 2004). Major cell recipients of dopaminergic transmission from various regions of the brain are MSNs in the striatum. Here, DARPP32 is important for cortical excitatory projection of MSNs, which are GABAergic projection neurons and represent the vast majority of cells in the striatum (Gerfen, 1992). Around 95% of MSNs express DARPP32 whereas it is absent in all other cells in the striatum (Ouimet *et al.*, 1984; Anderson and Reiner, 1991; Ouimet, Langley-Gullion and Greengard, 1998). Interestingly, also CTIP2 is strongly expressed in MSNs during development and loss-of-function of *Ctip2* results in failed MSN differentiation and decreased expression of distinct striatal molecular identifiers (Arlotta *et al.*, 2008). *Darpp32* is one among those molecular identifiers that exhibits low expression when *Ctip2* is lost, indicating a possible role of *Ctip2* in regulating *Darpp32* expression in MSN. Beside other brain structures, also the neocortex exhibits *Darpp32* expression. In the cortices of WT mice at postnatal stages, only lower layers show DARPP32 protein expression (W. W. Wang *et al.*, 2004). This is in accordance with ISH data from the Allen brain atlas, where strong *RNA*-expression can be detected in lower layers during postnatal development of the neocortex. More precisely, *Darpp32* expression has been linked to layer 6 CThPNs in the rat cingulate cortex, thus, indicating a specific expression pattern for CThPNs (Ouimet, 1991). Taken together, *Darpp32* is highly expressed in striatal MSNs and in lower layer 6 of the cortex, where it has been suggested to be expressed by CThPN.

4. Areal specialization of post-mitotic projection neurons

Although TFs expressed in the VZ are involved in impinging a pre-identity to cortical progenitors during development, recent studies have shown that expression in early post-mitotic neurons seem to be key in establishing and maintaining areal specification. Among some of these TFs is Basic Helix-Loop-Helix Family Member E22 (BHLHB5), the LIM Domain Only 4 (LMO4), Pre-B-cell leukemia homeodomain 1 (PBX1), the nuclear receptor COUP-TFI, LIM Homeobox 2 (LHX2) and B Cell CLL/Lymphoma 11A (CTIP1, Kashani *et al.*, 2006; Joshi *et al.*, 2008; Huang *et al.*, 2009; Alfano *et al.*, 2014; Perez-Garcia *et al.*, 2015; Greig *et al.*, 2016).

a. BHLHB5

Bhlhb5 is expressed in a high-caudomedial to low-rostralateral gradient in the CP in layer 2-5 neurons of primary sensory areas (Joshi *et al.*, 2008). *Bhlhb5*-deficient mice show normal cortical lamination but the molecular identity in the caudal motor and somatosensory regions is affected. Regionalized markers, such as *Cdh8* that are normally expressed in layers of rostral motor area, are ectopically expressed in S1 of *Bhlhb5* mutant mice. In addition, the sensory area promoting *Coup-TFI* is abnormally expressed in S1 cortex. The barrels of S1 appear to be disorganized in *Bhlhb5*^{-/-} cortices. In L4, barrel fields are organized of walls and of a cell-free hollow, which is innervated by axons from the thalamic VPM nucleus. In *Bhlhb5*-mutant mice, these clear separated barrel walls appear blurry and reveal indistinct borders. Another effect in *Bhlhb5*-deficient mice can be observed in the number of CSMNs. Fewer CSMNs are located in rostral motor cortex and more CSMNs are located in the caudal motor cortex. Lastly, *Lmo4* expression is lost in the somatosensory area, which is expressed in L4 of S1 cortex (Joshi *et al.*, 2008). Thus, BHLHB5 acts as post-mitotic regulator of sensory identity by controlling barrel formation in L4 and CSMNs in L5.

b. LMO4

Developmentally regulated genes that constitute a program of combinatorial molecular genetic controls, mediate the specification and differentiation of subtypes of cortical PNs. *Lmo4* has been shown to establish rostral motor cortex PN subtype diversity (Cederquist *et al.*, 2013). In this study, the motor cortex has been subdivided into rostral (MC_r) and caudal motor cortex (MC_c) by complementary expression patterns of *Lmo4* and *Bhlhb5*. In MC_r, *Lmo4* is highly expressed while *Bhlhb5* expression is absent, *vice versa* for MC_c. *Lmo4* is not required for this defined boundary, as cortex specific KO of *Lmo4* does not alter the expression pattern of *Bhlhb5* in the medial boundary. But, in the absence of *Lmo4* the molecular identity of neurons in the MC_r is disrupted and more homogenous. However, *Lmo4* is expressed in CPNs and mostly absent of CSMNs in MC_r. In contrast, *Lmo4* is expressed in MC_r SCPNs projecting to the brainstem. Upon deletion of *Lmo4*, these neurons aberrantly project to the spinal cord, indicating a role for *Lmo4* in proper development of SCPNs projecting to the brainstem.

The total knockout of *Lmo4* in mice leads to embryonic lethality (Lee *et al.*, 2005) and the few pups that survive, die within two hours after birth (Huang *et al.*, 2009). To overcome this severe phenotype, studies on loss-of-function of *Lmo4* used conditional knock out mice (Kashani, Qiu, *et al.*, 2006; Huang *et al.*, 2009). Upon restricted deletion of *Lmo4* in the CNS, expression of cortical regional markers CDH8 and ID2 (Bishop, Goudreau and O’Leary, 2000) were altered. The expression of *Cdh8* in upper layers of the anterior cortex was reduced in *Lmo4 cKO* cortices and L5 *Id2* expression was shifted rostrally. Deletion of *Lmo4* in post-mitotic neurons leads to perturbations in the development of the barrel fields, as *Lmo4 cKO* cortices showed poorly differentiated and blurred barrels (Kashani, Qiu, *et al.*, 2006). Furthermore, TCAs in *Lmo4 cKO* mice do not segregate into distinct terminal patches. These results show that conditional deletion of *Lmo4* does not prevent thalamocortical projections from reaching the cortex, but it does affect their patterning in the target tissue (Kashani, Qiu, *et al.*, 2006). Interestingly, upon deletion of *Lmo4* in the cortex, mice showed impaired sensory perception and motor control, revealing a role in sensory motor control. Taken together, *Lmo4* is important for the regulation of the shape of cortical functional areas, development of the barrel subfield and is implicated in sensory and motor functions. Moreover, *Lmo4* shows to be important for post-mitotic SCPNs identity and projections as well as barrel field maintenance in S1 cortex.

c. PBX1

Another TF that has been shown to control areal specification in both, progenitors and post-mitotic cells, is Pre-B-cell leukemia homeodomain 1 (PBX1, Golonzhka *et al.*, 2015). In adult mouse brains, *Pbx1* is implicated in the homeostasis of adult neurogenesis in the SVZ (Grebbin *et al.*, 2016). In the developing cortex, *Pbx1* has been shown to be expressed throughout the telencephalic VZ and SVZ at E12.5 with a caudorostral gradient (Toresson, Parmar and Campbell, 2000; Golonzhka *et al.*, 2015). At E18.5, its expression follows a rostral high to caudal low expression in upper layers (Golonzhka *et al.*, 2015). At later stages, *Pbx1* seems not to be expressed in the cortex anymore (P14 – P56), revealed by ISH data (Allen Brain Atlas), suggesting a primary developmental role. However, *Pbx1 cKO* in progenitors and post-mitotic cells show an aberrant areal specification, as revealed by downregulation of *Lmo4* expression in the rostral region of the brain with more severe affection when deleted in progenitors than

in post-mitotic cells (Golonzhka *et al.*, 2015). Interestingly, Reelin is mis-expressed in the deep cortical plate (E15.5) and an inversion of cortical layers in the rostral cortex can be observed at P8, shown by ectopic expression of lower layer markers in upper layers (Golonzhka *et al.*, 2015).

d. Other post-mitotic area regulators

Among being an area patterning gene in progenitors, *Coup-TFI* is also expressed in post-mitotic neurons, where it promotes sensory area identity (Alfano *et al.*, 2014). Also, *Lhx2* or *Ctip1* have been shown to regulate sensory identity in post-mitotic neurons (Perez-Garcia *et al.*, 2015; Greig *et al.*, 2016).

5. The motor area as “output” region

The frontal motor area (M1) is the major center of output signals in the neocortex and is supposed to be the most recent area in placental mammals (Kaas, 2011). The motor area is different from other primary areas, as it does not receive direct input from peripheral signals. Interestingly, it also has some cytoarchitectural unique features compared to primary sensory areas. The adult primary motor cortex contains a large number of CSMNs and has a thick layer 5, whereas S1 is characterized by a thick layer 4 (Tomassy *et al.*, 2010). The prominent staining of L4 stellate PNs in primary sensory areas is lacking in the motor area (Brodmann, 1909). Stellate cells are the main recipients of thalamocortical inputs, have extensive and largely unidirectional projection to the superficial cortical layer (Yamawaki *et al.*, 2014). Stellate cells can easily be detected as they exhibit strong *Rorb*-expression in S1, but only faint expression in M1, in accordance with a very thin L4 in this area. Interestingly, the *Rorb* expression is complementary to *Ctip2* expression: *Ctip2* is more broadly expressed in L5 in the motor cortex, while its expression is narrower in the somatosensory area (see **Fig. 9**, Dehay and Kennedy, 2007; Tomassy *et al.*, 2010; Harb *et al.*, 2016). These observations suggest that the motor area has a considerably less amount of associative projection neurons but instead an increased number of SCPNs, showing broad expression of *Ctip2*. Given its functional role in the neocortex as center of planning voluntary movements, its cytoarchitecture resembles its function and the motor area can be seen as the center of neocortical “output”.

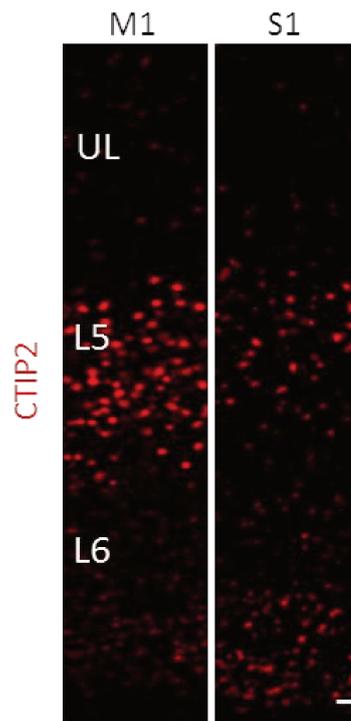


Figure 9: IHC of CTIP2 protein expression in M1 and S1 of WT mice at P7

CTIP2 is more broadly expressed in the L5 of the motor area, compared to its expression in L5 of S1. Scale bar 20 μ m.

Taken together, the laminar identity of PNs is highly regulated by a reciprocal repressing machinery of transcription factors, delineating CPNs from CFuPNs, as well as SCPNs from CThPNs. The area identity in progenitors and post-mitotic neurons is regulated by gradual expression of transcription factors, thereby shaping primary areas.

Given the relevance in this work, a few more details about *Lmo4* and *Fezf2* structures and functions are presented in the following chapters:

D. Fezf2

Fezf2 (or *FEZ1*) belongs to the Forebrain Embryonic Zinc Finger (*Fez*) family, a highly conserved family of transcription factors that play roles in neurogenesis, developmental patterning, cell-fate specification and axon guidance (Shimizu and Hibi, 2009; Eckler and Chen, 2014). The *Drosophila* genome contains only one *Fez* family homologue, *Earmuff* (*erm*), while in vertebrates there are two family members: *Fezf1*, which is also known as *Fez*, and *Fezf2* (also

known as *Fez1* and *Zfp312*, Shimizu *et al.*, 2010; Weng, Golden and Lee, 2010). They have been first discovered in the anterior neuroepithelium of *Xenopus* and zebrafish embryos (Hashimoto, Itoh, *et al.*, 2000; Hashimoto, Yabe, *et al.*, 2000; Matsuo-Takasaki *et al.*, 2000). Both members share an N-terminal Engrailed homology 1 (Eh1) repressor domain, allowing protein-protein interaction, which has also been shown to interact with the Groucho/TLE family of transcriptional co-repressors (Hashimoto, Yabe, *et al.*, 2000; Eckler and Chen, 2014). Furthermore, both Fez members contain six C2H2 zinc-finger DNA binding motifs at the C-terminal. *Fezf1* and *Fezf2* exhibit high levels of homology, particularly within their DNA binding and protein-protein interaction domains (see **Fig. 10**).

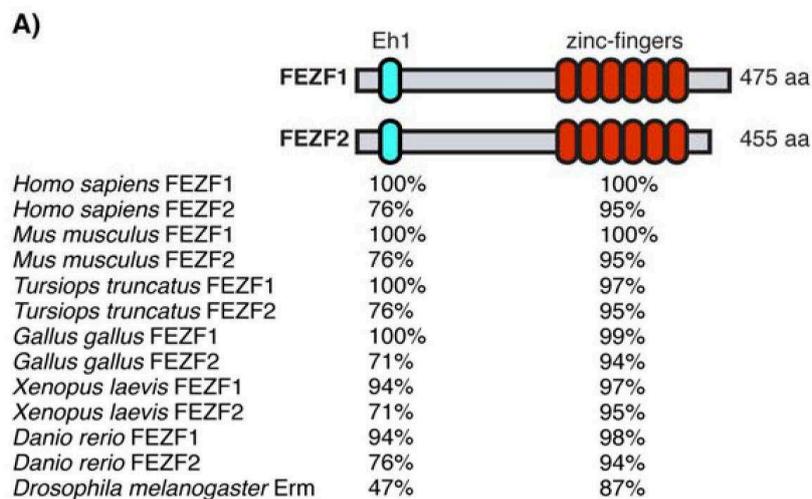


Figure 10: The Fez family of transcription factors.

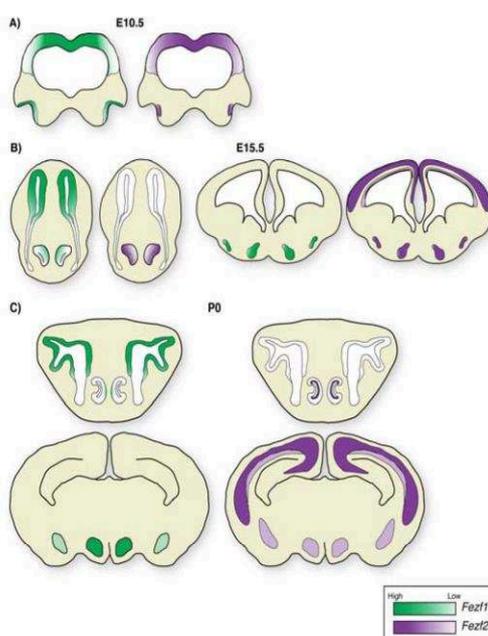
A Schematic of FEZF1 and FEZF2 proteins highlighting the strong evolutionary conservation of the Engrailed homology 1 (Eh1) and zinc-finger DNA binding domains. Conservations is reported as percent homology to *Homo sapiens* FEZF1. Taken from Eckler and Chen (2014).

a. Fez expression during embryogenesis and early postnatal stages

At E8.5, *Fezf2* and *Fezf1* exhibit similar and overlapping expression patterns in the developing forebrain, notably, also in the olfactory system. At E10.5, both Fez members are still expressed in an overlapping pattern in the forebrain and the olfactory system. *Fezf1* is expressed in neural progenitors throughout the forebrain and olfactory pit, *Fezf2* is also expressed in progenitors of the forebrain, but within the olfactory pit it is restricted to progenitors that will give rise to the vomeronasal organ (VNO) (Hirata, 2006a; Eckler *et al.*, 2011; Eckler and Chen,

2014). As development proceeds, both factors start to demarcate distinct neuronal structures. At E15.5, *Fezf1* is broadly expressed in the main olfactory epithelium (MOE) and its expression vanishes from the cerebral cortex, but remains in the developing amygdala, ventral thalamus and hypothalamus (Hirata, 2006b, 2006a; Kurrasch *et al.*, 2007; Watanabe *et al.*, 2009; Eckler *et al.*, 2011). In contrast, *Fezf2* is expressed within the VNO, neocortical progenitor cells and newly born deep-layer projection neurons, with high expression levels in L5 and L6 neurons. In addition, *Fezf2* is expressed in subcortical structures, such as thalamic eminence, prethalamus, developing amygdala and hypothalamus (Inoue *et al.*, 2004; Hirata, 2006b, 2006a; Watanabe *et al.*, 2009; Eckler *et al.*, 2011). At birth, expression of these factors mostly mimics their adult patterns (see **Fig. 11**). Expression of *Fezf1* is confined to progenitor cells and sensory neurons of the MOE as well as neurons of the hypothalamus and amygdala (Eckler and Chen, 2014). *Fezf2* on the other hand is expressed at high levels within L5 neurons of the neocortex and at lower levels within L6 neurons. In subcortical structures, *Fezf2* is expressed in the VNO and neurons in the hypothalamus and amygdala (Kurrasch *et al.*, 2007; Eckler *et al.*, 2011). The dynamic and overlapping expression patterns of both genes underscore their essential function during development of multiple structures within the nervous system.

Figure 11: *Fezf1* and *Fezf2* expression during embryogenesis.



A. At E10.5 *Fezf1* is expressed in progenitor cells in the forebrain and olfactory pit. *Fezf2* is also expressed in forebrain progenitor cells; however, within the olfactory pit its expression is restricted to the future VNO.

B. By late gestation (E15.5), *Fezf1* expression remains within the olfactory epithelium, but is absent from the cerebral cortex, and is decreasing within the VNO. In addition, it is expressed within the developing amygdala and hypothalamus. In contrast, *Fezf2* expression remains high within the developing VNO and cerebral cortex. Within the cerebral cortex, *Fezf2* is expressed at high levels in lower layer post-mitotic neurons and at lower level in progenitor cells. *Fezf2* is also expressed in the hypothalamus.

C. At birth, expression of *Fezf1* and *Fezf2* largely mimic that at E15.5. Within the VNO, expression of *Fezf2* has become restricted to the sustentacular cell layer. Within the cerebral cortex it maintains a high expression level in layer 5 SCPNs and lower levels in layer CThPNs. Green represents *Fezf1* expression, while purple represents *Fezf2* expression

VNO, vomeronasal organ. Taken from Eckler and Chen (2014).

b. Fez genes during development of the olfactory system and diencephalon

As indicated by their expression patterns during embryogenesis, *Fezf1* and *Fezf2* play important roles in the development of the olfactory system. Genetic loss-of-function studies show that *Fezf1* is required for maturation of neurons in the olfactory system and their axonal projection to the olfactory bulb (Eckler *et al.*, 2011). Absence of *Fezf2* during embryogenesis leads to decreased proliferation and apoptosis of sensory neurons in the olfactory system (Eckler *et al.*, 2011). Single knockout of either *Fezf1* or *Fezf2* in the developing diencephalon (composed of prethalamus, thalamus, hypothalamus and pretectum), has no effect on diencephalic patterning. However, double mutant mice reveal redundant roles in patterning of these structures: the prethalamus is completely lost, accompanied by a rostral expansion of the pretectum and marked reduction in thalamic size (Hirata, 2006b). In addition, regional markers such as GBX2 and LHX1 revealed that the rostral diencephalon fail to be specified and thus allowing rostral expansion of the caudal diencephalon. These observations indicate a redundant function of *Fezf1* and *Fezf2* during early diencephalon patterning.

c. Fez genes play unique and redundant roles in forebrain neurogenesis

Expression patterns of *Fezf1* and *Fezf2* transcription factors in the developing cerebral cortex suggest a functional role at early developmental stages. Indeed, both *Fez* genes are implicated in controlling the neurogenic program of neocortical progenitors (Shimizu *et al.*, 2010). In double mutant mice, *Hes5* expression was increased across the forebrain. *Hes5* belongs to the *Hes* genes, vertebrate homologs of *Drosophila hairy* and *enhancer of split*, which encode bHLH transcriptional repressors. HES proteins repress the expression of *bHLH* proneural genes, which promote neurogenesis (Ross, Rao and Kadesch, 2004; Kageyama, Ohtsuka and Kobayashi, 2007, 2008). Hence, depletion of *Fezf1* and *Fezf2* leads to decreased neurogenesis and subsequent decreasing numbers of PNs born during early corticogenesis (see **Fig. 12**). Interestingly, single knock-out of *Fezf1* or *Fezf2* does not lead to any defects in cortical neurogenesis, suggesting a redundant regulation of *Hes5* transcription. *Fezf1* is only expressed during early stages of cortical development. In contrast, *Fezf2* is expressed in neocortical progenitors throughout cortical neurogenesis. *Fezf2* is expressed in RGCs, which give rise to all major PN subtypes as well as glial cells (Guo *et al.*, 2013). This indicates that *Fezf2*

expression in early neocortical progenitors does not direct these cells towards a specific cell fate. Nevertheless, as addressed before, *Fezf2* is essential for the specification of SCPNs during cortical neurogenesis via inhibition of alternative fate genes.

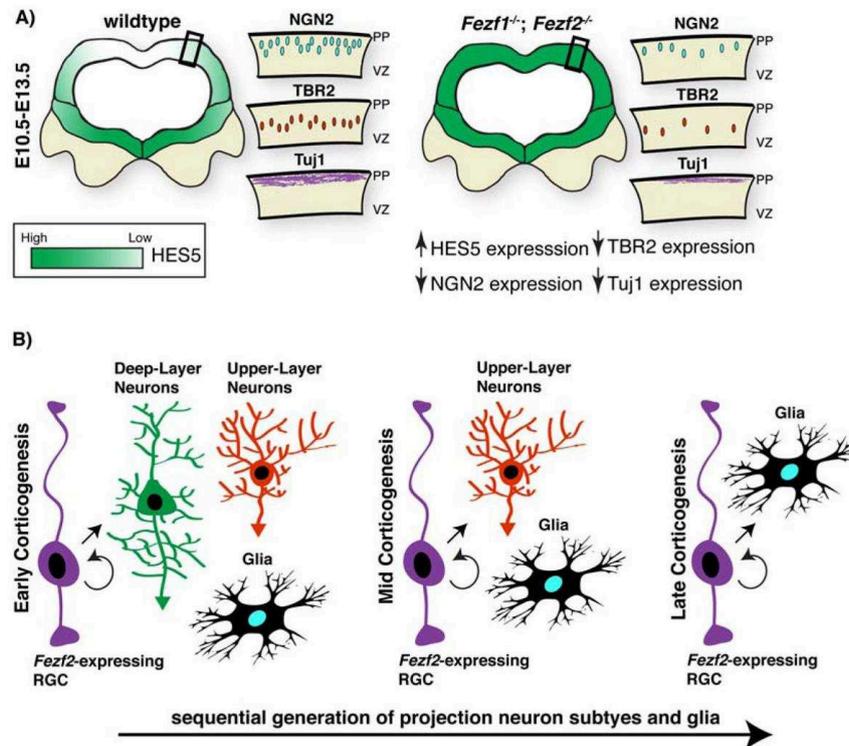


Figure 12: *Fezf1* and *Fezf2* functions during forebrain neurogenesis.

A. Loss of both *Fezf1* and *Fezf2* during early forebrain neurogenesis results in an increase in *Hes5* expression. This leads to decreased neurogenesis and intermediate progenitor generation and decreases in the number of lower layer projection neurons at birth.

B. *Fezf2* is expressed in multipotent RGCs that sequentially generate all major cortical projection neuron subtypes and glia. Taken from Eckler and Chen (2014).

E. *Lmo4*

Lmo4 is a member of the subclass LIM domain only (LMO), a family of nuclear transcription co-regulators (Sang *et al.*, 2014) and is characterized by the presence of two tandem LIM domains and no other functional domain (Deane *et al.*, 2004, see **Fig. 13**). The LIM domain is a highly conserved cysteine-rich zinc finger-like motif, found in a variety of nuclear and cytoplasmic proteins (Sang *et al.*, 2014). The LIM domain does not directly bind to DNA but instead mediates multiple protein-protein interactions (Kenny *et al.*, 1998; Matthews *et al.*, 2008) and can serve as docking site for the assembly of multiprotein complexes (Sánchez-

García and Rabbits, 1994; Dawid, Breen and Toyama, 1998). Therefore, LMO proteins regulate gene transcription by functioning as 'linker' or 'scaffolding' proteins due to their LIM domains and are involved in the formation of multiprotein complexes of DNA-binding factors and transcriptional regulatory proteins (Dawid, Breen and Toyama, 1998; Kenny *et al.*, 1998; Bach, 2000; Sang *et al.*, 2014, see **Fig. 13**).

LMO proteins are a conserved subclass, found in several species such as *C. elegans*, *Drosophila*, Zebrafish and humans (Rétaux and Bachy, 2002; McCollum *et al.*, 2007; Patterson *et al.*, 2007; Matthews *et al.*, 2008). Four members of the LMO subclass have been identified so far: *Lmo1* – *Lmo4*. The latter one, *Lmo4*, was the most recent described family member and was isolated as an interacting protein of LDB1 (also known as NLI or CLIM, Grutz, Forster and Rabbits, 1998; Kenny *et al.*, 1998; Sugihara, 1998; Racevskis *et al.*, 1999). It is the most divergent member of the LMO subclass and is widely expressed throughout embryonic development and adult tissue. In contrast, the expression patterns of *Lmo1*, *Lmo2* and *Lmo3* are more tissue restricted (Sang *et al.*, 2014). The functional role of LMO proteins has been extensively studied in cancerous tissue (Boehm *et al.*, 1990) and observations revealed the main role of LMO proteins as nuclear transcriptional co-regulators, mediating protein-protein interactions of various transcription factors or chromatin modelling proteins which may have either positive or negative effects on gene transcription (Sang *et al.*, 2014).

LMO4 is composed of 165 amino acid residues and has a broad expression spectrum in human tissue and in mice, where its expression can be found in T lymphocyte lineage, cranial neural crest cells, somite, dorsal limb bud mesenchyme, motor neurons and Schwann cell progenitors (Kenny *et al.*, 1998). Furthermore, *Lmo4* plays important roles in several developmental systems, such as epithelial, mammary, ear and neural development (Sugihara, 1998; Hahm *et al.*, 2004; Lee *et al.*, 2005). Also, *Lmo4* is implicated as an oncogene, as it is overexpressed in more than 50% of primary breast tumours and its overexpression markedly reduces differentiation of mammary epithelial cells (Visvader *et al.*, 2001; W. W. Wang *et al.*, 2004). So far, *Lmo4* has been shown to interact with various proteins or protein complexes. For instance, it interacts with the ovarian tumour suppressor protein BRCA1, thereby inhibiting the transcriptional activity of *Brca1* in yeast and mammalian cells (Sum *et al.*, 2002).

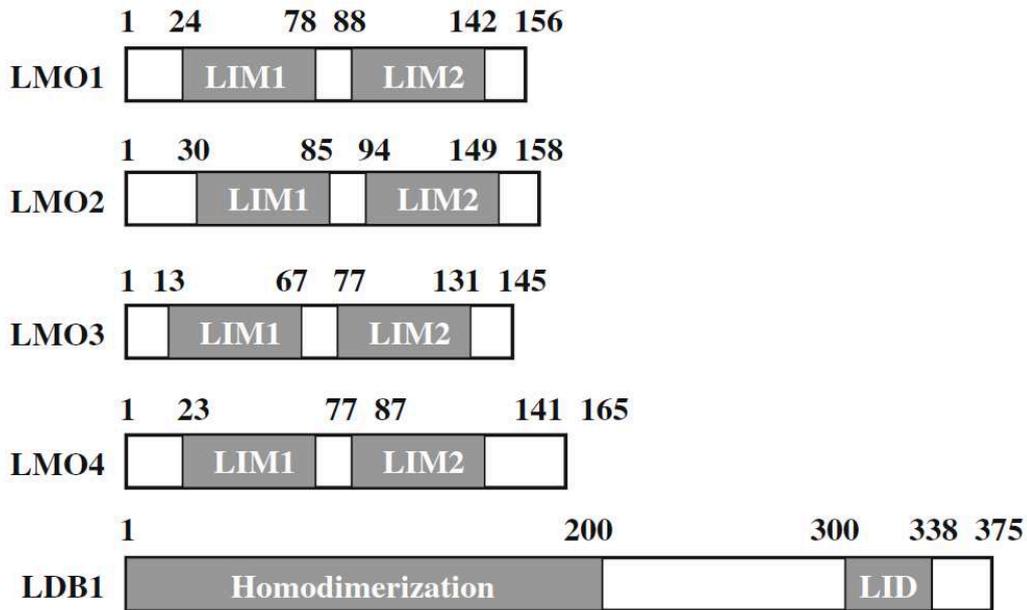


Figure 13: Schematic representation of the human LMOs and LDB1.

The first LIM domain (Lim1) and the second LIM domain (Lim2) of four LMO proteins are indicated. The homodimerization domain and the LIM interaction domain (LID) of LDB1 are also indicated. Taken from Sang et al. (2014).

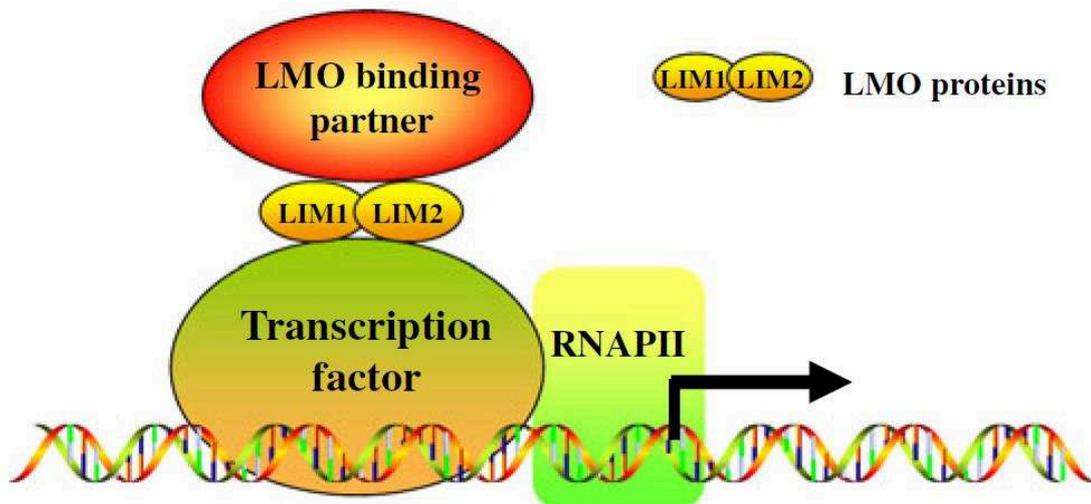


Figure 14: Schematic representation of LMOs in gene transcriptional regulation.

LMOs regulate gene transcription by functioning as 'linker' or 'scaffolding' proteins through their LIM domains and are involved in the formation of multiprotein complexes of DNA-binding factors and transcriptional regulator proteins. 'RNAPII' means RNA polymerase II. Taken from Sang et al. (2014).

In the developing cortex, LMO4 and its binding partner BLI/LDB1/CLIM2 interact with NEUROGENIN2, thereby forming a multi-protein transcription complex. Subsequently, this complex is recruited to enhancers of *neurogenin2* sequence and activates its transcription (Asprer *et al.*, 2011). Therefore, *Lmo4* can act as activator or repressor depending on the proteins it interacts with and depending on the cellular context (see **Fig. 14.**)

Interestingly, *Lmo4* has also been shown to bind to estrogen receptor α (ER α) and MTA1, thereby forming a multi-protein complex consistent of LMO4, ER α , MTA1 and histone deacetylases (HDACs) *in vivo* (Singh *et al.*, 2005). MTA1 is a component of the NURD complex, a group of associated proteins with ATP-dependent chromatin remodelling and histone deacetylase activities (Xue *et al.*, 1998). LMO4 here functioned as repressor of ER α in an HDAC-dependent manner, suggesting that LMO4 may play a role in chromatin remodelling complexes. Indeed, in the postnatal mouse cortex LMO4 is sequestering HDAC1, a critical component of the NURD complex, thereby preventing its binding to the *Ctip2* locus. Therefore, LMO4 acts as de-repressor, allowing *Ctip2* expression (Harb *et al.*, 2016, see **Fig. 15.**)

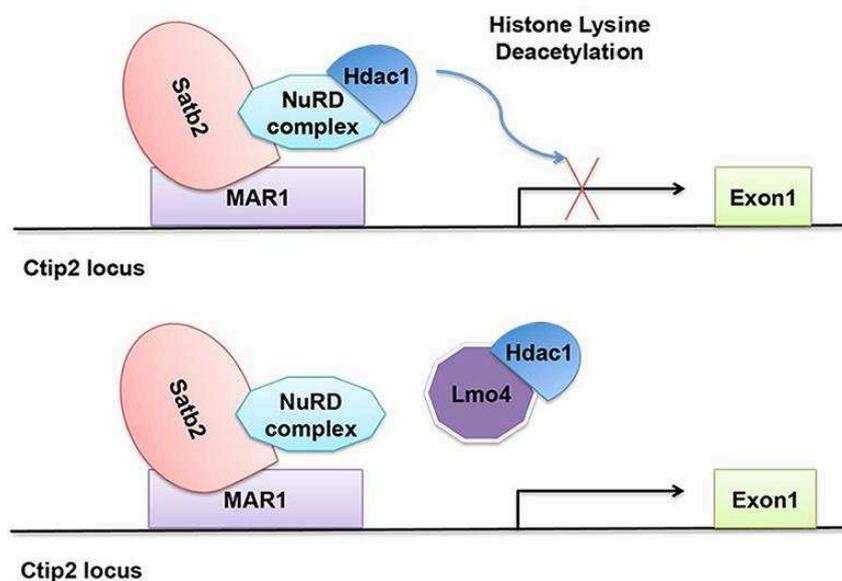


Figure 15: Schematic model of the putative mechanism by which *Lmo4* de-represses *Ctip2* expression

The locus of *Ctip2* is repressed by *Satb2* which is linked to the NuRD complex including HDAC1. *Lmo4* sequesters HDAC1 thereby de-repressing the *Ctip2* locus and allowing *Ctip2* expression. Taken from Harb *et al.* (2016).

a. Expression pattern of *Lmo4*

As mentioned above, *Lmo4* is an important factor for neocortical development and is implicated in arealization and neuronal subtype specification. In the very early stages of cortical development, *Lmo4* is expressed in the VZ and the preplate (E11.5, Asprer *et al.*, 2011). Later on, at E15.5, expression of *Lmo4* is found in the SVZ and the hippocampus. At birth (P0), expression of *Lmo4* is merely expressed in post-mitotic neurons of the cortical plate. Tangentially, *Lmo4* expression is found in the rostral (motor area) and caudal part (visual area) of the cortex, while its expression is almost absent in between, where the presumptive somatosensory area is located. At P5, *Lmo4* is increasingly expressed in upper layers 3 and 4 of the somatosensory area. At P10, *Lmo4* is expressed in all layers of the entire cortex (Huang *et al.*, 2009) and this expression pattern is retained in the adult brain (Allen Brain Atlas). The dynamic expression pattern during cortical development indicates a role for *Lmo4* in defining boundaries between areas and post-mitotic neuron layer-specification.

b. *Lmo4* in cortical subtype specification

The expression of *Lmo4* in the developing neocortex is highly dynamic and region-specific. During early stages of cortical neurogenesis (E11.5 – E18.5), *Lmo4* starts to be expressed in post-mitotic neurons of the PP and the CP (Azim *et al.*, 2009; Harb *et al.*, 2016). Tangentially, *Lmo4* is highly expressed in the rostral/frontal and caudal/occipital part of P0 cortices but at lower levels in the presumptive S1 area (Huang *et al.*, 2009). By the time S1 is fully specified (P10), *Lmo4* is expressed in all layers across the entire cortex (Huang *et al.*, 2009). At the cellular level, *Lmo4* is co-expressed with markers of various cell identity in the CP. At E15.5, *Lmo4* is expressed in SATB2-positive cells, but also in FEZF2/CTIP2-positive cells as well as in SOX5-positive cells, and this co-localization is maintained until P0. At late postnatal stage P6, *Lmo4* is excluded from most *Ctip2*-expressing neurons and nearly all *Lmo4*-expressing neurons have acquired CPN identity (Azim *et al.*, 2009).

Lmo4 together with *Clim1* has been shown to progressively delineate cortical PN subtypes during development (Azim *et al.*, 2009). At E15.5 *Lmo4* and *Clim1* are both expressed post-mitotically in the CP, in an overlapping subset of PNs. Interestingly, a subset of CP neurons expresses both *Fezf2* and *Lmo4*, therefore indicating that a subpopulation of neurons destined to project subcerebrally co-express *Lmo4* in early development stages (Azim *et al.*, 2009).

Additionally, a large number of *Ctip2*-expressing neurons in L5 co-express *Lmo4*, further indicating that many presumptive SCPNs express *Lmo4* during early differentiation. Also, *Clim1* is present in both FEZF2 and CTIP2 positive neurons. SATB2, a transcription factor for CPNs, is co-expressed in a subpopulation with both *Lmo4* and *Clim1*, indicating that *Lmo4* and *Clim1* are expressed in both presumptive CPNs and SCPNs. Lastly, *Sox5*, a TF expressed in the SP and lower layer 5 and 6, is co-expressed with *Lmo4* and *Clim1*, indicating early expression of *Lmo4* in SCPNs. At P0, *Lmo4* is expressed in subsets of neurons broadly throughout the cortical layers, whereas *Clim1* is restricted to neurons in L5. Both markers are still expressed in L5 but start to be segregated into distinct neuronal subpopulations. Despite some overlapping expression of *Fezf2* and *Lmo4*, *Lmo4* becomes partially excluded from SCPN population. At P0, many neurons also co-express *Ctip2* and *Lmo4*, while all *Ctip2*-expressing neurons also express *Clim1*. Also, *Satb2* and *Sox5* are still co-expressed with *Lmo4* and *Clim1* at this stage. At P6, when layer 5 SCPNs have more fully differentiated, *Lmo4* and *Clim1* are expressed in largely distinct L5 neuronal populations with few exceptions. However, *Lmo4* expression is merely excluded from CTIP2-positive SCPNs but instead L5 CPNs co-express *Satb2* and *Lmo4*. *Vice versa*, *Satb2* and *Clim1* expression in CPNs is mostly mutually exclusive and *Clim1* is largely co-expressed in *Ctip2* populations. Moreover, retrograde labelling from contralateral cortex at P3 and examination at P6 revealed that nearly all *Lmo4*-expressing neurons are CPNs, whereas nearly all CPNs do not express *Clim1* (Azim *et al.*, 2009). Overall, these studies indicate a role for *Lmo4* in defining cortical neuron subtypes at embryonic and postnatal stages.

II. Cell reprogramming

A. General Introduction

The ability of a differentiated cell to keep its identity is of eminent importance since each cell plays a unique function within a network of cells that in turn contributes to proper functioning of distinct biological systems. During embryogenesis, differentiating cells become committed to a highly specialized one and due to epigenetic barriers, its ability to convert into a cell type of another lineage is prevented. Epigenetic modulations are modifications of gene expressions, which can lead to their silencing (barrier) or activation. Plasticity of a cell therefore can be described as the ability of a cell to switch to new identities (Filip *et al.*, 2005). This ability of switching to new identities is decreasing as a cell differentiates; therefore, the plasticity is reduced over time.

A problem can arise when one cell or several cells are defective, which can affect the local network and ultimately, the whole system. During evolution various species have found different solutions to either regenerate or repair defective cells. While in *Hydra*, the regeneration potential is so large that it can basically regenerate itself after being fully dissociated (Technau *et al.*, 2000), the regeneration capacity of mammals is very limited. If multipotent or pluripotent stem cells are lacking, terminally differentiated cells cannot simply be replaced. Therefore, biomedical research has great interest in generating specific cell types that can replace defective ones. For example, converting cells into cell-types of defective ones could restore the function of a network. The pioneer study of Gurdon (Gurdon, 1962a) revealed that it is possible to de-differentiate mature cells into an immature state with pluripotent stemness. The feasibility of reprogramming cells led to subsequent observations in different ways and approaches. The study of Davis (1987) has demonstrated for the first time, that converting fibroblasts into myoblasts was feasible by simple overexpression of *MyoD*, revealing single-cell-type-specific transcriptional cell fate change. In 2006, a study of Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) demonstrated that overexpression of a four-component-TF-cocktail can de-differentiate somatic cells into pluripotent stem cells. Besides artificial reprogramming approaches, Zuryn *et al.* (2014), could observe that reprogramming of a mature cell into another cell type is occurring naturally, in this case in *C.*

elegans. In the following section, I would like to decipher differences in reprogramming approaches and their according nomenclature.

B. Nuclear reprogramming

The first attempt to reprogram cells has been conducted by Gurdon *et al.* (1962) even though the intention was different from the reprogramming approaches nowadays. At this time, the aim of Gurdon and colleagues was to understand whether somatic (non-reproductive) cells maintained their genome integrity after differentiation. The experimental approach worked as follows: the nucleus of a somatic cell was isolated, while the chromosomes of an unfertilized egg (*Xenopus laevis*) were destroyed by UV irradiation. Then, the donor nucleus was transferred to a recipient egg via micropipette injection and as a result, a frog containing the genetic origin of the donor could properly develop. This technique paved the way for the first reproductive cloning experiments in mammals, a prominent example is the sheep called Dolly, which represents the first successful cloning of a mammal (Wilmut *et al.*, 1997). These studies indicated that cells retain their genetic makeup and that the plasticity of a cell is strongly dependent on its environment. They also suggested that expression of genes is a dynamic process during development and that silencing of pluripotent genes can be experimentally reversed. Besides ethical issues, reprogramming *via* somatic nuclear transfer has limitations and disadvantages. The reprogramming efficiency using adult somatic cells is very low (1 – 2% normal adult animals), some genes are not properly transcribed after reprogramming (approx. 5%, Humphreys *et al.*, 2002; Vassena *et al.*, 2007) and the normality of nuclear transplant embryonic development decreases with nuclei harbouring increased differentiation states (Gurdon, 1962b). Additionally, as not all genes are properly transcribed, clones usually show abnormalities (Yang *et al.*, 2007) and cross-species nuclear reprogramming in mammals leads to death after few rounds of meiosis (Gurdon and Wilmut, 2011). However, nuclear transfer reprogramming has enlightened our understanding of genetic integrity of differentiated cells and paved the way of cell reprogramming.

C. Cell fusion reprogramming

Another approach of nuclear reprogramming is represented by the fusion between somatic and pluripotent cells (Yamanaka and Blau, 2010). Upon fusion of the membranes, the nuclei of both cells can stay separated in the “newly-hybrid” cell and without cell division form a *heterokaryon*. Another outcome is a *synkaryotic* cell with fused nuclei (hybrid genome) of both cell types, which undergoes cell division (Yamanaka and Blau, 2010). Interestingly, a pluripotent cell (e.g. embryonic stem cell) functions in a dominant way, thereby activating silent genes including pluripotent factors such as *Oct4* (Tada *et al.*, 2001). This approach is useful for elucidating molecular programs that are responsible for the reset of pluripotent genes, for example DNA methylation (Yamanaka and Blau, 2010). Furthermore, *heterokaryons* of mixed species allow deep comprehensive examination of nuclear reprogramming, as gene expression changes can be monitored on the bases of species-specific differences (Yamanaka and Blau, 2010). One major limitation of this approach lies in the fusion of nuclei, as tetraploid cells are unsuitable for therapeutic approaches (Ficz and Reik, 2013). However, cell fusion reprogramming is useful for examining early reprogramming mechanisms, such as rearrangement of methylation sites or chromatin alterations.

D. Cell reprogramming by cell extract treatment

Cells can also be reprogrammed by exposition to protein cocktails from pluripotent stem cells (Taranger *et al.*, 2005). To this purpose, the membrane of differentiated cells (of interest) are permeabilized and incubated with protein extracts of pluripotent stem cells. The membranes can be re-sealed and plated on cell dishes. These cells then start to express previously silent stem cell genes (Taranger *et al.*, 2005). This supports the notion, that changing the environment of the somatic DNA can reprogram somatic cells. The technique allows biochemical modification of the environment and, therefore, careful monitoring of the outcome of introduction or depletion of reprogramming factors from the extract. Additionally, extract-treated cells may also be used as starting material for induced pluripotency or nuclear transfer experiments (Halley-Stott, Pasque and Gurdon, 2013). Limitations arise from poor efficiency of successfully reprogrammed cells and experiments show a low rate of reproducibility (Liu *et al.*, 2011).

E. Natural occurring de-differentiation and trans-differentiation

De-differentiation of cells is a common regenerative mechanism reported for many species including humans (Jopling, Boue and Belmonte, 2011). For example, liver or blood cells are continuously generated from somatic cells. More specifically, some differentiated liver cells are reverted to a less-differentiated state, proliferate and then re-differentiate again into the original cell-type. Blood cells are generated by somatic stem cells which, after activation, proliferate and give rise to differentiated blood cells (Jopling, Boue and Belmonte, 2011). This natural de-differentiation allows local regeneration and is crucial for maintenance of tissues. Another phenomenon occurring naturally is trans-differentiation: conversion of a somatic cell into a cell from another lineage has been reported to occur naturally in certain species (Jopling, Boue and Belmonte, 2011; Beyret and Belmonte, 2014). The term **trans-differentiation** will be used in this work to name the natural phenomenon, whereas **reprogramming** will be used as term for experimental conversion of cells. Trans-differentiation in newts has been reported long time ago, at the end of the 19th century (Call, Grogg and Tsonis, 2005). Upon removal of the lens, pigmented epithelial cells of the iris de-differentiate and proliferate. Subsequently, these cells then create a new lens vesicle and differentiate into mature lens cells, thereby recovering the lost tissue (Jopling, Boue and Belmonte, 2011). A more recent study in *C. elegans* demonstrated trans-differentiation occurring during normal development, without any type of injury. A post-mitotic hindgut cell, named Y cell, detaches from the rectal tube and then converts into a motor neuron, named PDA (Beyret and Belmonte, 2014). This behaviour is occurring with invariant precision and therefore allowed the researches to examine the mechanisms responsible for the efficiency of this event. Briefly, H3K4 methyltransferase Set1 associates with subunits of a complex, which normally ensures embryonic stem cell fate and thereby initiates de-differentiation of the Y cell. Subsequently, this complex cooperates with H3K27me2/3 demethylase and a transcriptional regulator of terminal motor neuron identity Unc-3, to re-differentiate the Y-cell into a motor neuron. These sequential histone-modifications demonstrate how important epigenetic mechanisms are for trans-lineage conversion of cells and reveal useful information for experimental reprogramming approaches.

F. Reprogramming to induced Pluripotent Stem Cells (iPSCs)

The field of reprogramming became more popular thanks to the milestone-publication of Takahashi and Yamanaka in 2006 (Takahashi and Yamanaka, 2006). Virally induced overexpression of four transcription factors - Oct4, Sox2, Klf4 and Myc (OSKM) – converted mouse fibroblasts into pluripotent stem cells, which re-acquired ES cell properties. The pluripotency of these iPSCs were examined by teratoma formation, a benign tumour characterized by rapid growth *in vivo* carrying remnants of all three germ layers. These tumours developed in mice after injection of reprogrammed cells and differentiated into cells of all three germ layers, including neural tissue, cartilage and columnar epithelium (Takahashi and Yamanaka, 2006). This study demonstrated for the first time that somatic cells can be reprogrammed to a pluripotent state by overexpression of ES-type TFs able to silence somatic maintenance factors. Upon reprogramming to iPSCs, these cells can be re-differentiated into any somatic cell type (Halley-Stott, Pasque and Gurdon, 2013). The discovery of iPSCs boosted the field of reprogramming and a tremendous amount of discoveries has been made ever since, too numerous to be covered in this thesis work (see Heinrich, Spagnoli and Berninger, 2015; Masserdotti, Gascón and Götz, 2016; Takahashi and Yamanaka, 2016; Torper and Gotz, 2017). Nevertheless, some major findings, advances and limitations of iPSCs are described below.

After the publication of murine iPSCs, the subsequent study of reprogramming human fibroblasts into iPSCs followed just one year after (Takahashi *et al.*, 2007; Yu *et al.*, 2007). From that moment on, experiments on human pluripotent cells exploded, since ethical issues had been widely extinguished: a problem that existed before by working on human ES cells, as they had to be derived from human embryos. Somatic cells from patients can be derived and reprogrammed into iPSCs, which then are differentiated into the disease-relevant cell-type. These cells are then examined for disease aetiology and pathological mechanisms can eventually be revealed (Shi *et al.*, 2017). Thanks to new genetic modification tools, such as CRISPR-Cas9, iPSCs of a patient can be modified and used as internal control. Patient-derived iPSCs can further be used as a disease model to identify potential drugs for therapy. Another application has been demonstrated by the repair of genetic mutations in murine iPSC and their subsequent transplantation in the diseased mouse model from which the iPSCs originated.

These “newly regenerated” progenitors were able to counteract the disease (Hanna *et al.*, 2007). This approach is now starting to become reality for treating diseases in humans. In Japan for example, macular degeneration has been treated with iPSC-derived retinal pigment epithelium, even though with moderate success (Mandai *et al.*, 2017). In addition, thanks to the iPSC technology, 3D-organoids have been generated from patients to study genetic diseases, thus adding another way of modelling genetic diseases *via* iPSCs (Lancaster *et al.*, 2013; Di Lullo and Kriegstein, 2017).

Nevertheless, a major concern for iPSC technology has been the delivery of OSKM factors into the somatic cells, as the viruses used by Takahashi integrates into the host genome (Yamanaka and Blau, 2010). Advances have been made to overcome this hurdle by using integration-free delivery methods such as synthetic *mRNA* transfection (Warren *et al.*, 2010), episomal plasmid vectors (Okita *et al.*, 2008) or Sendai-virus infection, a virus which does not integrate into the host genome (Fusaki *et al.*, 2009). To date, iPSCs can be easily derived from different cell sources and thus, somatic cells can be more efficiently reprogrammed than initially reported (Kogut *et al.*, 2018), and large numbers of iPSCs can be produced in fully controlled bioreactors in defined culture media (Martin, 2017). In addition, the process of differentiating iPSCs has been improved by using small molecules and biochemical reagents (Martin, 2017).

The undoubtedly success of iPSCs increased our knowledge of cell plasticity, their underlying mechanisms - such as epigenetic landmarks or chromatin accessibility, the discovery of pluripotent factors and the subsequent application opportunities. Despite the advances in cell reprogramming by iPSCs, some hurdles still remain. A major problem in iPSC technology is that several targeted cells do not fully reprogram and therefore can undergo defective differentiation (Takahashi and Yamanaka, 2016). Partially reprogrammed cells exhibit epigenetic landmarks of somatic cells but depict ES cell characteristics, such as proliferation, onset of pluripotency-associated genes or teratoma-forming activity (Takahashi and Yamanaka, 2016). This partial cell reprogramming could explain the widely reported low full conversion rate of iPSC reprogramming. In fact, the reprogramming mechanism seems to be split in two steps. Initially, OSKM factors bind to genes, which are responsible for somatic identity and thus, silence them. The second step is the induction of pluripotent genes, which seems to be largely inefficient due to the presence of repressive methyl-histone marks (Takahashi and Yamanaka, 2016). As a result, even if many somatic cells will silence their

differentiated genetic repertoire, pluripotency fails to be activated by OSKM factors, leading to a partial reprogramming. The homogeneity of fully reprogrammed cells is important for further clinical usage to ensure aberrant development of iPSCs, which could lead to cancerous formations in the host organism (Takahashi and Yamanaka, 2016). Another problem arises from the variability of individual iPSC lines regarding their potential to differentiate into fully functional cells of a defined lineage (Hockemeyer and Jaenisch, 2016). This cause is derived from epigenetic memories, which could be somatic methylation patterns that remained in reprogrammed iPSCs. In turn, using iPSCs as disease model can be tricky if small phenotypic differences between control and patient iPSCs are not the cause of the disease, but rather come from variations between individual iPSC lines (Soldner and Jaenisch, 2012; Hockemeyer and Jaenisch, 2016). Another question is, to which extent differentiated iPSC-derived cells mimic *in vivo* cell maturity. So far, the vast majority of iPSC-directed differentiations produce populations that are more similar to foetal or neonatal cells than to mature cells (Berry *et al.*, 2018). This implies that the right time for iPSC-differentiated cell transplantation has to be carefully evaluated. For instance, a mature cell would be equipped with full functionality, but might not be plastic enough to integrate into the system and *vice versa*. In conclusion, iPSC technology is an exciting field, which not only increased our understanding of cellular plasticity but also gives us a powerful tool to design and generate almost any kind of cell-type *in vitro*. Nevertheless, it shows limitations that need further investigations to overcome.

G. Direct reprogramming

Generation of desired functional cell types is one major goal of regenerative medicine. For this purpose, differentiation of iPSCs has been extensively studied and applied in certain disease situations (FUSAKI *et al.*, 2009; Warren *et al.*, 2010; Zhou *et al.*, 2010; Shi *et al.*, 2017). However, because of the limitations described above, there has been great interest in developing alternative strategies. One of these alternatives is represented by direct reprogramming, which can be defined as inductive conversion of a cell into another one without passing through an intermediate pluripotent state. This definition delineates direct reprogramming from conversion of iPSCs. The first direct reprogramming study has been reported by Davis (Davis, Weintraub and Lassar, 1987), describing conversion of embryonic mouse fibroblasts into myoblasts by overexpression of *MyoD*. This study demonstrated for the first time, that a single TF is able to change somatic cell fate of a committed but not fully

differentiated cell. Subsequent studies revealed TF-driven cell fate conversion between related lineages, such as glia to neuron, pancreatic to liver cell or B cell to macrophage (Shen, Slack and Tosh, 2000; Heins *et al.*, 2002; Xie *et al.*, 2004; Berninger *et al.*, 2007). It has been assumed that it may be easier to reprogram cells from the same germ layer, but further studies were able to demonstrate that cells can be reprogrammed across germ layers by overexpressing a distinct combinations of TFs (Vierbuchen *et al.*, 2010). Therefore, direct reprogramming is not limited to cell conversion within lineages; it is indeed able to transform cells across unrelated lineages or germ layers.

The direct reprogramming toolbox has been improved by alternative molecules besides TFs and increased our understanding in regard to cell plasticity. For instance, epigenetic regulators, such as chromatin modifiers or DNA methylases, have been shown to have an impact on the reprogramming process (Takeuchi and Bruneau, 2009; Dhawan *et al.*, 2011). Epigenetically repressed genes of the target cell-type have to be accessible for the fate determinant factor to be able to convert the target cell. Thus, chromatin modifiers can play an important factor in the cell conversion process. However, it is still not clear on the outcome an epigenetic regulator can have during reprogramming. While, chromatin remodelling complexes can facilitate the conversion of a cell (Takeuchi and Bruneau, 2009), in some case it is hindering reprogramming. For instance, inhibition of chromatin modifiers, like histone deacetylases, facilitated the conversion of germ cells to neurons, which was driven by neuron-specific factors (Tursun *et al.*, 2011; Patel *et al.*, 2012; Xu, Yi and Chien, 2012). Additionally, the deficiency of DNA methyltransferase *Dnmt1* led to a conversion of pancreatic β cells into α cells (Dhawan *et al.*, 2011). Therefore, it is likely that alteration in the epigenetic state of a cell is become crucial for subsequent reprogramming process.

Another important input in direct reprogramming comes from *miRNA*. Overexpression of *miRNAs* have been shown to successfully convert human fibroblasts into cells expressing neuronal markers (Yoo *et al.*, 2011). Additionally, also cardiac lineage conversion has been demonstrated by using *miRNAs* (Jayawardena *et al.*, 2012). Despite the powerful role of *miRNAs* in direct reprogramming, *miRNA*-mediated conversion is less efficient than TF-mediated direct reprogramming (Xu, Yi and Chien, 2012). As *miRNAs* function mainly as translational repressors, the overexpression of a certain *miRNA* may misbalance the

expressions of cell fate genes leading to cell fate change. Also, *miRNAs* could be implicated in epigenome alterations, thereby altering the epigenetic state of the cell, which as for chromatin modulators, can lead to cell fate changes. Indeed, a recent study demonstrated that upon overexpression, pro-neuronal *miRNA - 9/9*124* induced extensive remodelling of the epigenome, including simultaneous activation of a pan-neuronal program and the reconfiguration of chromatin accessibilities (Abernathy *et al.*, 2017). These observations suggest an effective role for *miRNA* in remodelling the epigenome of a cell, which can then be useful for TFs to convert cells more efficiently.

Recently, small molecules gained more and more interest for inducing somatic cell fate changes. Small molecules are low molecular weight organic compounds. The function of small molecules can range from targeting signalling pathways, epigenetic remodelling or influencing metabolic processes during direct reprogramming (Xu *et al.*, 2008; Li and Ding, 2010; Federation, Bradner and Meissner, 2014). They have been used merely to improve transcription factor-induced reprogramming but are also able to change cell fate on their own, even across germ layers (Li *et al.*, 2015). For instance, generation of induced neurons (iNs) from mouse fibroblasts has been reported by using a cocktail of small molecules, such as Forskolin, ISX, CHIR99021 and I-BET 151. Apparently, these small molecules suppressed a fibroblast-specific program, while endogenous neurogenic TF were activated (Li *et al.*, 2015). The advantages of small molecules over genetic manipulations are numerous. They are cost-effective, easily synthesized and standardized and, their biological effects are mostly reversible (Qin, Zhao and Fu, 2017). In clinical studies, small molecules could be used as drugs promoting endogenous regeneration in patients. Altogether, small molecules hold great promises as a replacement for genetic modifications in direct reprogramming. Nevertheless, their efficiency has to be improved and further studies are important to reveal more powerful types of small molecules.

Another notable factor in direct lineage reprogramming is the metabolic state of a cell. It is known that iPSCs and embryonic stem cells (ESCs) differ from differentiated cells regarding their metabolic state (Zhang *et al.*, 2012). ESCs and iPSCs have an elevated dependence on glycolysis under aerobic conditions compared to highly or lowly respiring (metabolic reactions and processes that take in cells to convert biochemical energy) differentiated cell types (Zhang

et al., 2012). During reprogramming to iPSCs, the cell undergoes an active process of metabolic resetting which can influence the reprogramming efficiency. For example, experimental inhibition of glycolysis lowers the reprogramming efficiency whereas increasing glycolysis enhances iPSC reprogramming efficiency (Zhou *et al.*, 2010; Folmes *et al.*, 2012; Panopoulos *et al.*, 2012; Zhang *et al.*, 2012). These observations reveal an important role for the metabolic state of a cell during reprogramming. This might become even more important, when a cell with a certain metabolic state is converted into a cell with a different metabolic state. This was elegantly described during reprogramming of astrocytes and fibroblasts (anaerobic glycolysis and β -oxidation) into neurons (oxidative, McKay *et al.*, 1983; Tsacopoulos and Magistretti, 1996). Gascón *et al.* (2016) discovered that inhibiting ferroptosis, an iron-dependent form of non-apoptotic cell death (Dixon *et al.*, 2012), promoted survival and neuronal conversion of astrocytes. Thus, lipid peroxidation, a hallmark of ferroptosis, was investigated and revealed increasing presence during neuronal reprogramming. In addition, factors inhibiting oxidative stress led to improvements in cell survival, maturation and efficiency (Gascón *et al.*, 2016). This milestone publication demonstrated nicely, that metabolic changes during reprogramming occur and that upon inhibiting oxidative stress, lineage reprogramming (in this case astrocyte-to-neuron) will become more efficient and stable over time. Taken together, the experimental intervention with factors influencing the metabolic state of a cell in favour of the target cell can improve the reprogramming process.

Notably, whether direct reprogramming (here in fully differentiated cells) actually reverts the target cells to transient higher potent state during conversion, or whether it leads to a direct switch between differentiated cell states was not deeply investigated so far. Thanks to the study of Karow *et al.* (2018), a better insight regarding this topic was provided. By deciphering transcriptomes at the population of single cell level, the trajectory underlying conversion of adult human pericytes into iNs was reconstructed (Karow *et al.*, 2018). The transcription factors ASCL1 and SOX2 (AS) upregulated various cell signalling genes during the very early process of reprogramming. These cell signalling genes were further investigated and showed to be markedly enriched in germinal zones of the developing CNS containing neural stem cells in mouse embryos. Following the transcriptomics at later stages of reprogramming, Karow *et al.* (2018) could show that the trajectory can then bifurcate to give rise to differentiated neuronal lineages. Thus, AS-mediated reprogramming unfolded developmental programs,

followed by genes instructing neuronal fates during later stages of the conversion process. These results indicate that cells are undergoing a transient stem cell-like state before converting into a differentiated cell.

The advantages of direct reprogramming over the aforementioned alternative approaches are numerous. For example, direct reprogramming bypasses the procedure of resetting the cell to a de-differentiated state before changing cell fate. This is advantageous as it may be less likely to introduce genetic mutations, which can be a general concern in iPSC reprogramming (Liang and Zhang, 2013). Furthermore, direct reprogramming does not need the introduction of oncogenes like *Oct-3/4* used in iPSCs, therefore reducing the risk of possible tumorigenesis for *in vivo* applications. Another advantage for direct reprogramming is that it does not reset the age of the starter cell, which is especially important for disease modelling, e.g. when using patient derived cell biopsies (Mertens *et al.*, 2015; Tang *et al.*, 2017). Notable, direct reprogramming appears to be faster and more cost-effective than iPSC generation, as it also does not necessarily need an *in vitro* culture system using rather expensive reagents.

A big advantage of direct reprogramming is that it can be conducted *in vivo*. Some studies showed OSKM-mediated reprogramming *in vivo* too, which resulted in pluripotent gene expression (Senís *et al.*, 2018). The issue here is the control over the induced pluripotent cells and the cancerous appearance of teratomas. Direct reprogramming allows to overcome these hurdles as it does not generate pluripotent cells as well as it does not necessarily need oncogenic factors. It therefore may be used in the future for cell replacement therapy, bypassing difficulties of transplantation (e.g. rejection of the transplanted tissue)(Xu, Du and Deng, 2015). The advantage of *in vivo* reprogramming is the provision of a niche that supplies factors to support the functional maintenance of target cell types, which might also facilitate functional conversion (Xu, Du and Deng, 2015).

Major limitations and hurdles of direct reprogramming are mostly common with other reprogramming approaches and are described below:

First, the selection of the starter cell-type may ease or impede the conversion process. It is likely to assume that lineage-related cells might be easier to convert into each other as they share a common origin. For example, glia are efficiently converted into functional neurons by single overexpression of one transcription factor (Heins *et al.*, 2002; Berninger *et al.*, 2007;

Heinrich *et al.*, 2010). In contrast, conversion of one neuronal subtype into another one appeared to be rather difficult and has been achieved only in immature cells (Rouaux and Arlotta, 2013; Gascón *et al.*, 2017). Furthermore, the same reprogramming factor can trigger different outcomes when it is induced in different cell-types. For instance, *Neurogenin-2* has been shown to convert astrocytes into glutamatergic neurons (Heinrich *et al.*, 2010; Masserdotti *et al.*, 2015), while in foetal fibroblasts it triggers cell conversion into a cholinergic motor-neuron like cell fate (Liu *et al.*, 2013). These observations highlight the importance of the crosstalk between the reprogramming factors and the cellular context in which they operate.

Another important hurdle in reprogramming is to improve the efficiency of converting cells, as it was the case for the initial protocol of iPSC generation (Takahashi and Yamanaka, 2006). Subsequent studies revealed factors which can facilitate conversion, such as *Glis1* or *Mbd3*, and barriers whose removal drastically increased reprogramming efficiency (Maekawa *et al.*, 2011; Rais *et al.*, 2013; Di Stefano *et al.*, 2014). A prominent factor hindering reprogramming efficiency is *p53*. For example, expression of a dominant-negative form of *p53* or its knockdown improves direct conversion of mouse embryonic fibroblasts into dopaminergic neurons (Liu and Qian, 2014; Jiang *et al.*, 2015). Thus, efficiency of direct reprogramming can be improved by adding factors facilitating conversion or by blocking factors hindering conversion.

Furthermore, generating different types of fully functional mature cells remains a major challenge (Xu, Du and Deng, 2015). A common concern is that the direct converted cells fail to silence the expression programs of the initial population, indicating that it still possess an immature phenotype (Cahan *et al.*, 2014). For instance, mouse hepatocyte-like cells induced by hepatic fate determination TFs remained functionally immature, reflected by incomplete hepatocyte differentiation and expression of hepatoblast markers (Huang *et al.*, 2011; Sekiya and Suzuki, 2011). Thus, fate determination factors alone may not be sufficient to achieve functional maturation of reprogrammed cells. Combination of lineage-specific TFs and factors promoting cell maturation could represent an effective combination to achieve functional maturation by direct reprogramming. Lastly, it is crucial to evaluate how far the reprogrammed cells resemble the endogenous population. Several methods have been used to evaluate converted cells: analysis of marker gene expression, global gene expression and

epigenetic patterns, silencing of exogenous factors and the epigenetic state of master genes (Cohen and Melton, 2011). As these assays are primarily based on whole cell populations and therefore cannot reveal the heterogeneity of converted cells, further deep analysis such as single-cell *RNA* sequencing, could be applied for gaining additional information.

A straightforward test to evaluate converted cells is to assay their function *in vivo*. Several studies demonstrated successful integration and survival of reprogrammed cells after transplantation in mice (Ring *et al.*, 2012; Bredenkamp *et al.*, 2014). The function of converted cells can be further tested in mouse disease models. For example, mouse fibroblasts reprogrammed into thymic epithelial cells formed a complete and functional thymus *in vivo*, which supported T cell development in an athymic mouse model (Bredenkamp *et al.*, 2014).

Overall, from the field of reprogramming, cells can be seen as maintaining a stable state (represented as islands in **Fig. 16**) which can be reverted upon changes of the epigenetic environment and induction of factors able to induce a certain cell fate (“boats”). Converting cells into cell-types from close related lineages can be instructed by using few TFs (“orange and yellow sails”), whereas reprogramming of cells from different germ-lines (“difficult routes, storms and reefs”) might need a more complex combination of several TFs (“blue and red sails”), or combination with *miRNA* or small molecules.

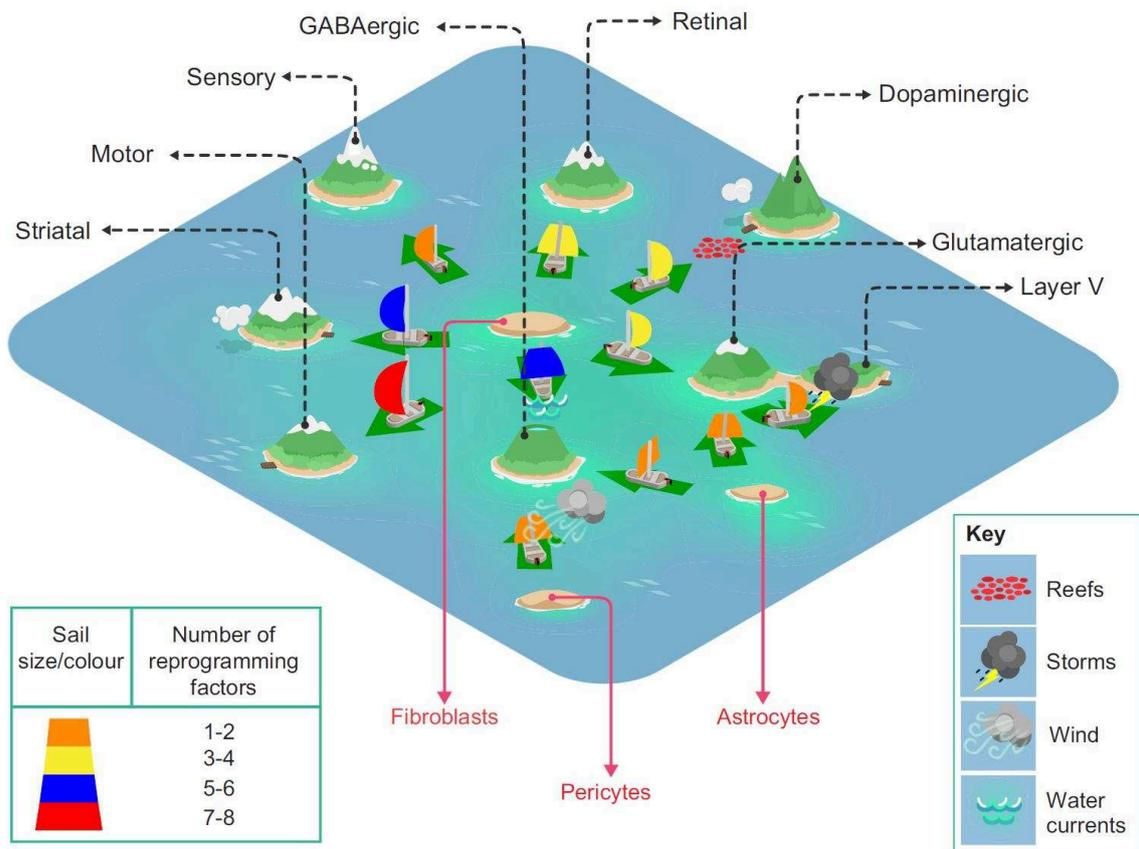


Figure 16: The Cook Islands model applied to neuronal reprogramming

In the Cook Islands model, each island represents a different stable state. In essence, a boat, namely a cell, can move from island to island freely and in reprogramming such transitions are instructed by the combinatorial expression of specific transcription factors (depicted in the model as different colours in the sails of the boats). More complicated routes need bigger sails with several transcription factors, for example the reprogramming of fibroblasts to striatal or dopaminergic neurons. By contrast, small sails, representing fewer transcription factors, are sufficient to push boats to more closely related neuronal sub-types, for example from astrocytes to glutamatergic or GABAergic neurons. Certain routes are easier than others due to various factors such as distance and/or the relative water level, representing the various barriers to reprogramming as they occur both in vitro and in vivo. Other routes are particularly difficult due to the presence of strong opposing sea currents (see fibroblasts to GABAergic neurons), sudden storms (see glutamatergic to Layer V) or reefs (fibroblasts to dopaminergic). The presence of these hurdles may require novel routes to be taken, or more potent and equipped boats; that is, new strategies for reprogramming may be required to overcome cell type-specific barriers to reprogramming. Taken from Masserdotti, Gascón and Götz (2016).

1. Direct neuronal reprogramming *in vivo*

The great advantage of direct reprogramming over other reprogramming approaches is the straightforward conversion *in vivo*. This strategy may represent an interesting alternative for regenerative medicine as it aims to replace damaged or lost cell to restore organ function. Especially organs with low intrinsic capacity of self-renewal in the adult organism, such as the pancreas, the heart or the CNS, are in need of strategies to replace cells that haven been damaged by disease or injury. Here, the focus will be on the brain, as it is the main interest of my thesis work.

The adult mammalian brain lacks a significant regenerative capacity and is therefore especially vulnerable to damaging events resulting in the loss of neural cells. Notably, there are few regions in the brain, which retain a neural stem cell niche even during adulthood. Neural stem cells (NSCs) give rise to newborn neurons throughout life, a process called adult neurogenesis. Two main locations in the adult brain possess NSCs: the SVZ of the lateral ventricles and the dentate gyrus of the hippocampus. NSCs in the SVZ give merely rise to interneurons which migrate to the olfactory bulb, whereas NSCs in the SGZ (subgranular zone) of the dentate gyrus generate granule neurons (Braun and Jessberger, 2014). Both processes are contributing to olfactory-and hippocampus-dependent learning and memory processes (Deng, Aimone and Gage, 2010). Although these stem cell niches do not seem to be designed for neuronal renewal after injury, they could be eventually exploited for brain repair. The idea is to boost NSCs proliferation and guided chemokine-directed migration towards lesioned tissue (Li *et al.*, 2012).

An alternative strategy for self-repair aims at fate converting resident cells into the desired cell type (Heinrich, Spagnoli and Berninger, 2015), named direct reprogramming *in vivo*. The field of developmental biology here serves as a fundamental source of knowledge for the identification of potential reprogramming candidates. Again, TFs are mostly used for reprogramming, as their fate specification in development is known and thus, can possibly predict the outcome of converting a cell. For an experimental design of *in vivo* reprogramming, defining the careful sequential steps that are essential for setting up a direct reprogramming experiment is crucial (Heinrich, Spagnoli and Berninger, 2015): first, the cell type source for

reprogramming has to be characterized; second, the factors required to obtain a certain cell-type after conversion must be identified, as well as a safe and efficient delivery method; third, reprogrammed cells of the same molecular identity and phenotype as the desired cell type, while erasing its original molecular identity, must be obtained. So far, these considerations are shared by *in vitro* approaches for direct reprogramming, as mentioned above. However, two other considerations are more specific to *in vivo* reprogramming approaches. Host environments can differ in their permissiveness to accommodate new cells and may sometimes create barriers against their functional integration. Cell networks with limited self-regenerative potential may have constraints for new cells and identifying these barriers can help overcoming them. Lastly, the full functional integration of reprogrammed cells is the final step to be accomplished (Heinrich, Spagnoli and Berninger, 2015). Therefore, factors promoting the functional integration have to be identified and then, integration into the system has to be carefully examined. So far, several studies reported successful direct reprogramming *in vivo*. Below, the presented studies are divided into reprogramming in an injured environment and reprogramming under physiological conditions, as both conditions differ in the plasticity of targeted cells.

a. Direct neuronal reprogramming in vivo in the injured brain

Despite their fatal impact, brain injuries also provide an opportunity for reprogramming, as they produce glial cells, known to be cells with a certain plasticity (Gascón *et al.*, 2017). For instance, during the acute phase of injury, astrocytes become hypertrophic, resume proliferation and upregulate expression of the intermediate filament proteins glial fibrillary acidic protein (GFAP) and vimentin in a process called reactive gliosis (Torper *et al.*, 2013; Z. Guo *et al.*, 2013; Aravantinou-Fatorou *et al.*, 2015; Brulet *et al.*, 2017). After injury, astrocytes become proliferative, and in some cases begin to express markers of neural stem/progenitor cells and neurogenic differentiation (Anderson, Ao and Sofroniew, 2014; Bayraktar *et al.*, 2015; Duan *et al.*, 2015; Nato *et al.*, 2015; Brulet *et al.*, 2017). In addition, other glial cells, such as NG2 cells and microglia undergo reactive responses to injury and form a defence system against the invasion of micro-organisms and cytotoxins from the surrounding tissues (Pekny and Nilsson, 2005; Sofroniew and Vinters, 2010; Robel *et al.*, 2011). Once activated, many reactive glial cells stay in the injury site and secrete neuroinhibitory factors and eventually

form a glial scar inside the brain (Sofroniew and Vinters, 2010). Nevertheless, many studies took advantage of the environmental change that occurs upon brain injury.

Guo *et al.* (2014) demonstrated, upon brain injury, reprogramming of reactive glial cells, including astrocytes and NG2 cells, into functional neurons in the adult mouse cortex (Guo *et al.*, 2014). After stab injury, reactive proliferative glial cells were infected by a retrovirus encoding for *NeuroD1* (a proneural TF) and then analysed for neuron-like properties. Infected astrocytes were converted into glutamatergic neurons, while NG2 cells were converted into both glutamatergic and GABAergic neurons, demonstrating again the importance of the starting cell-type for the reprogramming outcome. Interestingly, *NeuroD1*-converted neurons expressed *Ctip2* and *Tbr1*, lower layer markers of the cortex. Additionally, *NeuroD1*-driven reprogramming of glial cells was successfully employed in an Alzheimer's disease mouse model (Guo *et al.*, 2014). These results show that reprogramming in the injured brain, mechanically or diseased, can be potentially achieved and reactive glial cells can be eventually converted into functional neurons.

Another study from Heinrich *et al.* (2014) demonstrated successful conversion of NG2-glia into induced neurons (Heinrich *et al.*, 2014). By performing stab wound injury and subsequent injection of retrovirus - coding for *Sox2* alone or in combination with *Ascl1* - forced reactive NG2 cells could be converted into DCX or NeuN-positive neuronal cells (immature neuronal marker and mature neuronal marker, respectively). Interestingly, *Sox2* was able to convert NG2 cells into iNs alone but did not succeed to do so without stab wound injury (Heinrich *et al.*, 2014). Thus, *Sox2*-mediated reprogramming of NG2 cells needs a more plastic environment, similarly to what happens after brain injury.

Other studies demonstrated reprogramming in the injured brain of

- 1) parenchymal astrocytes into NeuN-positive neurons in the striatum, by using a transgenic mouse model, (Torper *et al.*, 2013);
- 2) glial cells into neurons by *Neurog2/Bcl-2* coding retroviruses and additional anti-oxidants in the cortex (Gascón *et al.*, 2016);
- 3) dorsal midbrain astrocytes into functional neurons by overexpression of *Ascl1* delivered *via* a GFAP-adenoviral-associated virus vector (Liu *et al.*, 2015);

- 4) functional dopamine neurons from mouse astrocytes in a Parkinson's model (Rivetti Di Val Cervo *et al.*, 2017).

Taken together, *in vivo* reprogramming of reactive glial cells has been successfully employed and might represent a powerful tool for cell replacement in the injured brain. A more detailed overview can be found in Torper and Götz (2017).

b. Direct neuronal reprogramming in vivo under physiological conditions

So far, most studies have made use of an altered, more plastic environment after brain injury to reprogram glial cells into neurons. Thus, it was not clear whether glia-to-neuron conversion was feasible under physiological conditions, which could potentially facilitate future clinical approaches long after the acute injury phase. Recent publications demonstrated that it is indeed possible to reprogram glia cells into neurons in a healthy brain. For example, Brulet *et al.* (2017) showed neuronal conversion of non-reactive astrocytes in the adult mouse brain. By injecting an AAV9 virus with an expression cassette for *NeuroD1* and a transgenic mouse labelling GFP in astrocytes (GFAP promoter), they specifically targeted astrocytes in the cortex and the striatum. Only astrocytes of the striatum showed NeuN-expression after transfection (around 2%, Brulet *et al.*, 2017). However, there was a possibility that SVZ-stem cells (persisting in the adult brain) could have migrated into the striatum and converted into neurons upon *NeuroD1* overexpression, resulting in false-positive results.

Pereira *et al.* (2017) showed reprogramming of NG2 cells in the intact mouse brain. NG2 cells are an interesting target cell-type, as they retain some ability of self-renewal (Simon, Götz and Dimou, 2011), which avoids depletion of an endogenous population upon reprogramming. In their study, they have overexpressed *Ascl1*, *Lmx1a* and *Nurr1* using AAV vectors and a transgenic mouse line, which expresses GFP upon neuronal identity (genes under neuron-specific synapsin promoter) after transfection (Torper *et al.*, 2015). NG2 glia in the striatum revealed successful conversion into neurons showing parvalbumin-containing interneurons (Pereira *et al.*, 2017). Other approaches sought out to directly reprogram neurons into a distinct cellular type. The advantage is that neuron-to-neuron reprogramming does not need to switch the metabolic state, as it is instead the case for astrocyte-to-neuron conversion (Gascón *et al.*, 2016). On the other hand, reprogramming of neurons will result in a depletion

of the targeted cells. In case of conversion of a small population it might be less dramatic, but these alterations have to be further investigated. In the brain, successful direct reprogramming has been reported in the striatum and in the neocortex. Upon overexpression of *Sox2* together with dopaminergic-specific TFs, Niu *et al.* (2018) were able to convert striatal neurons into dopaminergic neuron-like cells in the adult brain. Moreover, electrophysiological investigations of reprogrammed cells revealed functional connections with other neurons (spontaneous postsynaptic currents, Niu *et al.*, (2018)). This revealed an important property, since it not always clear whether reprogrammed neurons can properly integrate into the host system.

Instructing a reprogrammed cell into a precise and reproducible neuronal sub-type in the brain is still in its infancy. Only few studies have been able to show reprogramming to a specific neuronal sub-type (Gascón *et al.*, 2017). One good example comes from the *in vivo Fezf2*-driven reprogramming shown to convert one neuronal sub-type into another one.

In 2010, Rouaux and Arlotta (Rouaux and Arlotta, 2010) demonstrated in the developing striatum conversion of striatal progenitors, normally fated to become medium spiny neurons, into CFuPNs upon *Fezf2* overexpression. Via *in utero* electroporation, a *Fezf2* expression vector was delivered into neural progenitors of the lateral ganglionic eminence. Molecular profiles of the electroporated cells revealed expression of *Sox5*, *Tbr1* and *Fog2* – markers of lower layer projection neurons in the cortex. Furthermore, converted neurons showed morphology characteristic of pyramidal neurons and projections to expected corticofugal targets, such as the thalamus and cerebral peduncle. As the striatal niche normally does not host cortical projection neurons, these results demonstrated that cell-autonomous signals that drive neuron type-specific development in the cortex can override extracellular niche restrictions and instructions over progenitor cell fate (Rouaux and Arlotta, 2010).

In 2013, two groups independently demonstrated *Fezf2*-driven reprogramming of upper layer cortical post-mitotic neurons into corticofugal neurons *in vivo*. De la Rossa *et al.* (De La Rossa *et al.*, 2013) used a special technique, named iontoporation, to deliver a *Fezf2*-expressing plasmid in L4 stellate neurons. Iontoporation worked as follows: plasmids are injected into the lateral ventricles of P1 pups followed by an electric pulse allowing transfection of cells. After ectopic expression of *Fezf2* in L4 stellate cells, transfected cells showed up-regulation of the

lower layer marker *Er81* and down-regulation of upper layer markers, such as *Satb2* and *Cux1*. In addition, reprogrammed cells revealed aberrant projection to striatum. L4 stellate cells normally form local neurocircuits and do not project outside the cortex. Electrophysiological examinations further revealed characteristic physiological properties of lower layer projection neurons of reprogrammed cells. Moreover, intracortical inputs were altered upon *Fezf2*-driven conversion of L4 neurons. Normally, these neurons do not receive input from L2-3 neurons, but, in contrast, reprogrammed cells received L2-3 neuronal input. Thus, reprogramming of post-mitotic neurons into corticofugal-like projection neurons in the cortex *in vivo* had been successfully demonstrated.

In parallel, Rouaux and Arlotta (Rouaux and Arlotta, 2013) showed *Fezf2*-driven direct reprogramming of callosal projection neurons into CFuPNs *in vivo*. In their study, they made use of a plasmid, which expressed *Fezf2* (with GFP reporter) under a *cdk5r*-promoter, which is specifically turned on in post-mitotic neurons. By *in utero* electroporation at E14.5, they were able to target post-mitotic migrating upper layer neurons. Investigation of their molecular identity (P7) revealed that *Fezf2*-electroporated cells expressed lower layer markers such as ER81, CRYM, TLE4, CTIP2 and FOG2. The upper layer marker CUX1 was also reduced. Molecular identity of converted cells was maintained at later stages, even if with a lower percentage. No significant cell death was found when GFP-positive neurons were stained for CASPASE-3. Moreover, by using an inducible expression plasmid for *Fezf2*, they could show that reprogramming at P3 still led to molecular identity changes, even though with very reduced efficiency (Rouaux and Arlotta, 2013). Interestingly, *Ctip2*, *Tle4* and *Fog2* expressions could not be observed anymore when *Fezf2* was induced at P3. Induction of *Fezf2* at later stages than P3 failed to convert upper layer neurons into lower layer ones. In addition, *Fezf2*-electroporated cells projected aberrantly to corticofugal targets, such as thalamus, internal capsule, cerebral peduncle and some axons were also found in the spinal cord. The number of axons crossing the CC, which CPN normally project through, was reduced, but not absent. When *Fezf2* was induced at E17.5, few neurons projected into the internal capsule and the thalamus. Induction of *Fezf2* at later stages did not show any aberrant projections anymore.

Taken together, these data demonstrate that post-mitotic upper layer neurons at early developmental stages retain a certain degree of plasticity, being able to be reprogrammed

into corticofugal neurons. However, this window of reprogramming capacity vanished after P3, suggesting that with increasing maturity, neurons lose the ability to change cell identity.

c. Data leading to the thesis work

A recent study from our lab demonstrated a de-repressing function of *Lmo4* in the SATB2-mediated *Ctip2* repression (Harb *et al.*, 2016). Furthermore, this study also revealed an interplay between *Lmo4* and *Ctip2* expression in lower layers of S1. Upon cortical deletion of *Lmo4* (cKO), the total number of *Ctip2*-expressing neurons in lower layers L5 and L6 was reduced in P7 brains. On the other hand, overexpression of *Lmo4* at E13.5 showed an increase of CTIP2-positive neurons in lower layers in P7 brains (Harb *et al.*, 2016). These results indicated a regulative role of *Lmo4* for *Ctip2*-expression in lower layer neurons of the developing cortex, and therefore implicating *Lmo4* in SCPN specification.

Thus, we wondered whether *Lmo4*-ectopic expression in upper layers could instruct SCPN fate conversion of CPNs as it has been demonstrated for *Fezf2*. Furthermore, the de-repressing mediation of the *Ctip2*-locus by LMO4 motivated us to investigate whether *Fezf2* could possibly take advantage of the opened access of *Ctip2* locus and thus, more efficiently convert CPNs into SCPNs in comparison to single *Fezf2*-reprogramming as shown in Rouaux and Arlotta (2013) and De la Rossa *et al.* (2013).

III Aims of the thesis

As aforementioned, the brain lacks a significant regeneration capacity and thus, has no or very little capacity for self-repair upon injury. Direct reprogramming has been shown to be a potential tool for restoring lost neurons by converting neighboring cells into the lost cell-type. Transcription factors, which are known for their role in cell fate specification during development, are good candidates for directly converting one cell-type into another one. The transcription factor FEZF2 is necessary and sufficient to specify L5 subcerebral projection neuron identity. Its ectopic expression in cortical upper layers and in the striatum can specifically convert cells into corticofugal neurons *in vivo*, revealed by molecular identity and axonal projections. However, these studies lacked efficiency in reprogramming, and conversion in the cortex was restricted to immature neurons.

Our lab has demonstrated that the nuclear co-adaptor *Lmo4* can act as a de-repressor of the *Ctip2*-locus. *Ctip2* is a downstream target of *Fezf2* and is highly expressed by L5 subcerebral projections neurons. Hence, the aims of the thesis have been the following:

- 1) Investigating whether *Lmo4* can as a potential reprogramming factor in the conversion of upper layer neurons into corticofugal neurons.
- 2) Examining the combinatory effect of *Lmo4* and *Fezf2* as a reprogramming cocktail for a more efficient way of converting upper layer neurons into corticofugal neurons.
- 3) Carefully following axonal projections of reprogrammed neurons *in vivo* by identifying an improved reporter strategy.
- 4) Exploring until which postnatal stage upper layer neurons can still be converted into a corticofugal sub-type.
- 5) Searching for downstream and induced targets of reprogrammed neurons as a strategy for identifying novel factors and/or pathways useful in direct *in vivo* reprogramming.

MATERIAL AND METHODS

I. Animals

All *in utero* electroporations were conducted on RjHan:NMRI mice obtained from Janvier. Pregnant mothers from this outbred strain have a drastically higher number of embryos, when compared with mothers from other strains, they are good breeders and they exhibit strong fitness. Due to these features, this strain has been chosen as the experimental model. Female and male RjHan:NMRI mice were put in matings in the evening, a positive plug on the next day has been considered as pregnant stage of 0.5 days.

II. Plasmids

A. *pCdk5r-Lmo4-IRES-GFP*, *pCdk5r-Fezf2-IRES-GFP* and *pCdk5r-IRES-GFP*

For reprogramming at embryonic stage E14.5, *pCdk5r-Lmo4-IRES-GFP*, *pCdk5r-Fezf2-IRES-GFP* and *pCdk5r-IRES-GFP* plasmids were used. *pCdk5r-Fezf2-IRES-GFP* and *pCdk5r-IRES-GFP* were donated by the lab of Paola Arlotta (Rouaux and Arlotta, 2013), while *pCdk5r-Lmo4-IRES-GFP* has been generated by Kawssar Harb (Harb *et al.*, 2016).

B. Inducible *pCAG-Lmo4-IRES-GFP*

To induce the expression of *Lmo4* at postnatal stages after *in utero* electroporation, *Lmo4* has been subcloned into a vector with a floxed stop codon sequence just before the gene of interest, a subsequent *IRES* sequence and a *Gfp* reporter (gift of V. Tarabykin lab).

1. Insert

For cloning, *Lmo4* has been amplified by PCR from the *pCdk5r-Lmo4-IRES-GFP* plasmid while adding restriction sites on both ends of the strands (**EcoRV**). The used primers had the following sequence:

- 1) Forward: 5' GTCATG**GATATC**CATGGTGAATCCGGGCAGCAGC 3'
- 2) Reverse: 5' GTCATG**GATATC**TCAGCAGACCTTCTGGTCTGG 3'

And the respective PCR program:

95°C – 5 min
95°C – 30 s
60°C – 30 s
72°C – 30 s
72°C – 8 min

} 35 cycles

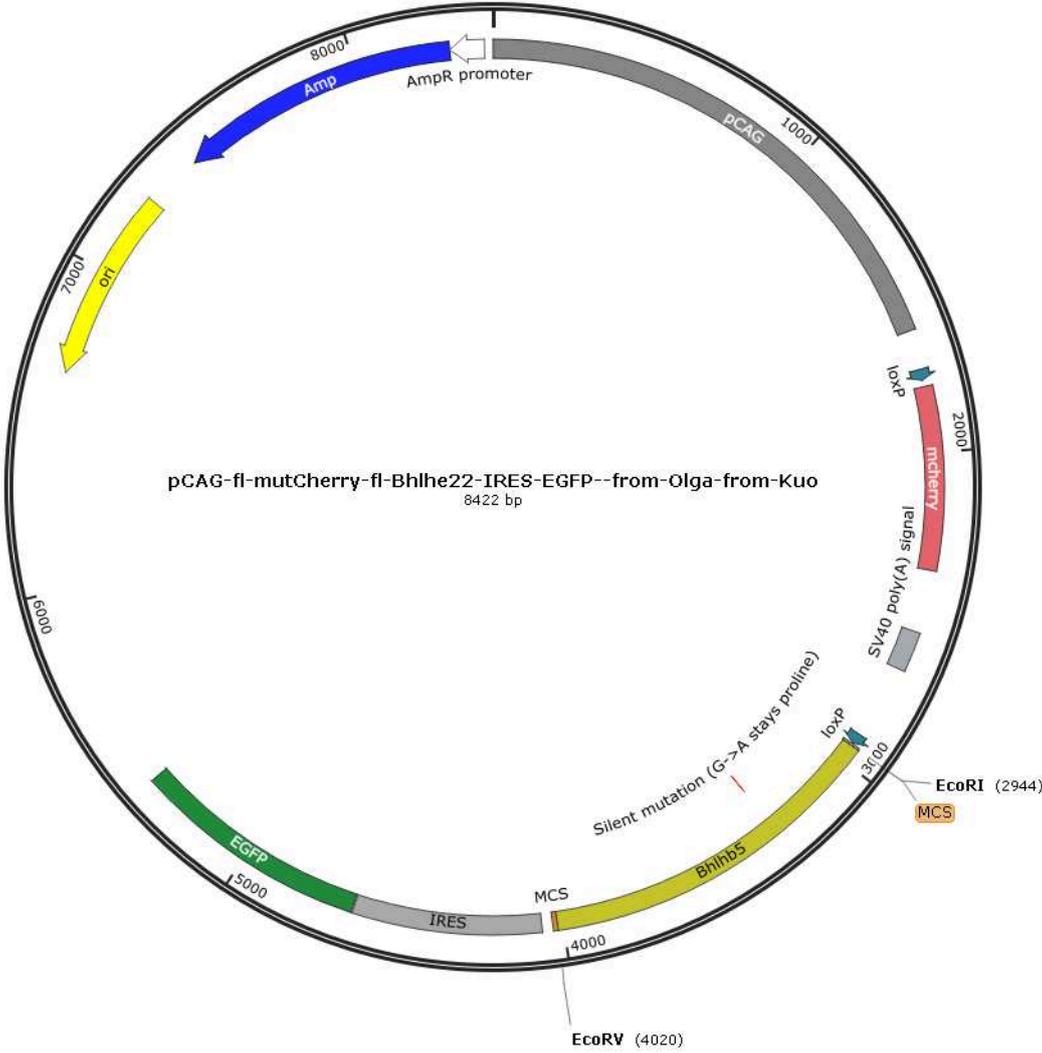
Subsequently, the PCR product has been digested with restriction enzyme EcoRV to create blunt ends:

30 µl Digestion mix
1 µg DNA
3 µl Buffer
1 µl Restriction enzyme
Filled up to 30 µl with ddH ₂ O

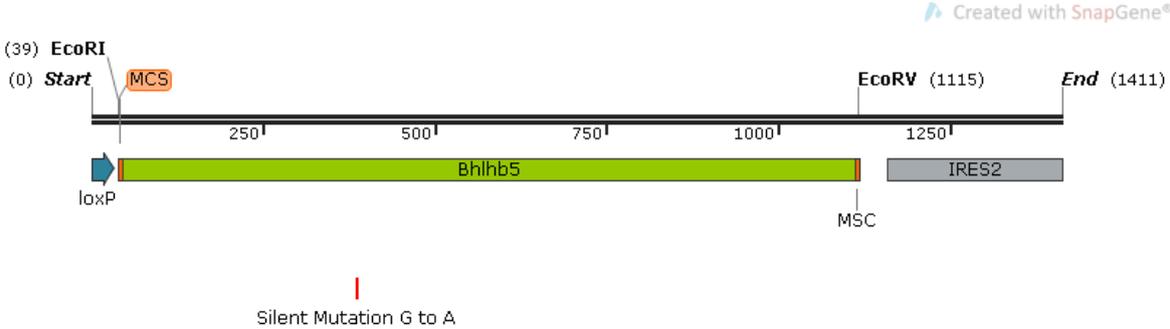
Incubated for 1h at 37°C

At the end, the digested PCR product has been purified by gel-electrophoresis (1%) and purified using DNA Gel Extraction Kit (Qiagen, #28705).

2. Vector



Thanks to the gift of the Tarabykin lab, a plasmid with conditional expression could be used as vector to generate an inducible *Lmo4* plasmid.



The plasmid has been digested with EcoRI-HF and EcoRV-HF to remove the *Bhlhb5* sequence. The resulting sticky end of EcoRI has been filled-in using Klenow-Fragment (NEB (M0210):

Fill-in protocol (30µl)
1 µg DNA
1 µl Klenow
3 µl T4 DNA Ligase buffer
1 mM dNTPs
Filled up to 30 µl with ddH2O

Reaction stop by heating up to 75°C for 20min

Then, the plasmid has been dephosphorylated to prepare it for the final ligation step:

Dephosphorylation
1 µg DNA
4µl Buffer 2
1 µl Alkaline Phosphatase, Calf Intestinal (CIP)
Filled up to 40 µl with ddH2O

As a final step, the vector has been purified on a gel. For the ligation process, 200 ng of the vector have been used, while the quantity of the insert has been calculated according to the

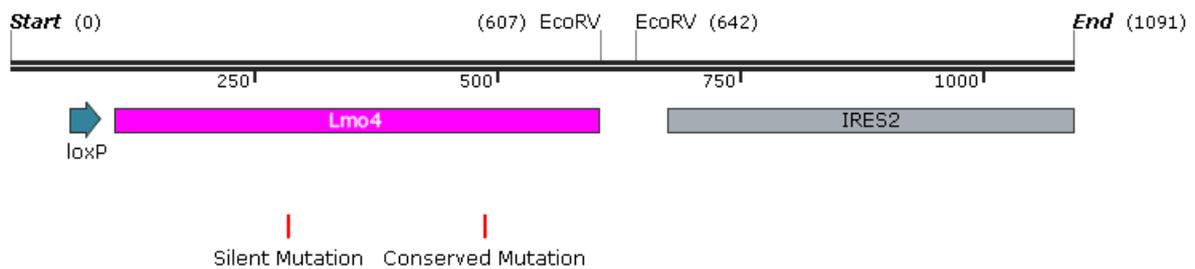
following calculation:

$$x = \left(\frac{200 \text{ ng}}{\frac{x \text{ bp vector}}{x \text{ bp insert}}} \right) * 4$$

Ligation occurred according to the following protocol:

Ligation
200 ng vector
X ng insert
1 µl T4 DNA Ligase
1 µl T4 DNA Ligase Buffer (10X)
Filled up to 10 µl with ddH2O

The ligation was incubated at 16°C ON. Note that between each incubation step, the DNA has been purified using QIAquick PCR Purification Kit (Qiagen, #28104). The ligation product has been mechanically transfected in bacteria and plate on LB agar (Amp+) plates. Grown colonies were picked and amplified in 4 ml LB buffer + 1 mg/ml Ampicillin. The plasmid of the resulting was purified using EndoFree Plasmid Maxi Kit (Qiagen, #12362).

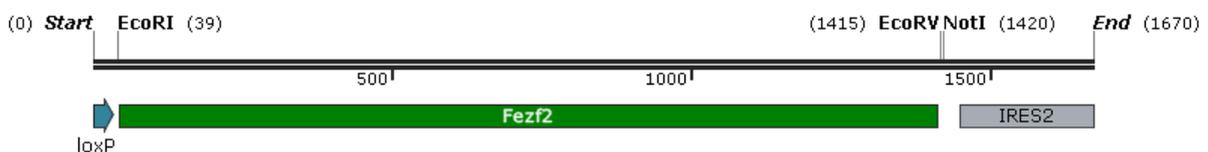


3. Inducible pCAG-Fezf2-IRES-GFP

The same target vector has been used as for inducible *Lmo4* described above. The *Fezf2* sequence has been amplified from the *cdk5r-Fezf2-IRES-GFP* plasmid by PCR and flanked with EcoRI and EcoRV restriction cassettes using the following primers:

- 1) Forward: 5' CCGAATTCCATGGCCAGCTCAGCTTC 3'
- 2) Reverse: 5' CCGATATCTCAGCTCTGAACTGTCC 3'

Subsequently, the PCR-generated Fezf2-insert and the target vector have been digested with EcoRI and EcoRV and then ligated to finally generate the inducible *Fezf2* plasmid:



4. Inducible pCAG-IRES-GFP

To generate a control plasmid which only expresses GFP upon action of CRE recombinase, the Inducible *pCAG-Fezf2-IRES-GFP* has been digested with EcoRI and NotI in order to remove the

Fezf2 sequence and, sticky ends were filled up and the open plasmid then has been ligated again.



5. pCAG-ERT2-CRE-ERT2

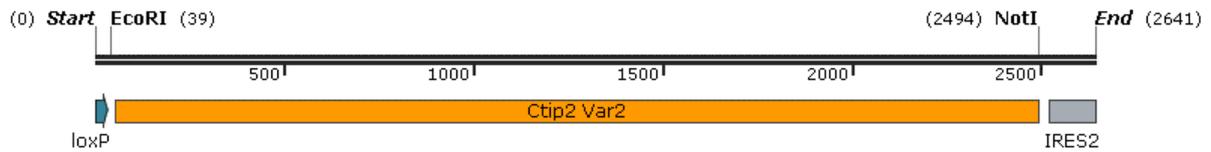
In order to remove the floxed stop codons from *inducible Gfp*, *Lmo4* or *Fezf2* plasmids, we made use of a conditionally regulated plasmid which expresses CRE upon tamoxifen application. The plasmid is commercially available from addgene under the reference number #13777.

6. pCAG_smFP FLAG

As the GFP reporters of inducible *Lmo4* and *Fezf2* plasmids were not sufficient to express signal in fine axons and collaterals of electroporated cells, *pCAG_smFP FLAG* has been used to overcome this hurdle. Adding this plasmid to the reprogramming factors at postnatal stages, we were able to improve the signals in fine axons and collaterals of transfected cells. The plasmid is commercially available from addgene under the reference number #59756.

7. Inducible pCAG-Ctip2-IRES-GFP

This plasmid has been cloned by removing the *Fezf2* sequence from inducible *pCAG-Fezf2-IRES-GFP* via digestion with EcoRI and NotI and ligation of a *Ctip2*-insert with EcoRI and NotI-restriction sites. The insert has been cut out from another plasmid containing the *Ctip2* sequence which fortunately was flanked by EcoRI and NotI too (kind gift from V. Tarabykin).



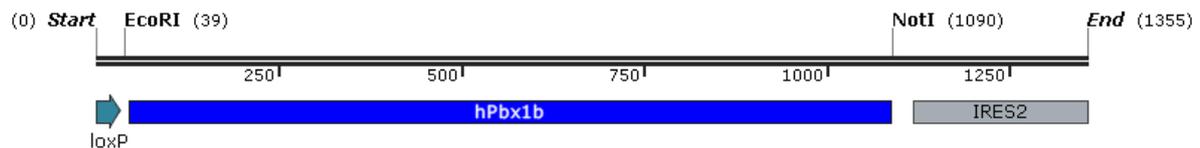
8. Inducible pCAG-hPbx1-IRES-GFP

Also, *hPBX1* has been subcloned using inducible *Fezf2* as backbone. Briefly, *Fezf2* has been cut out from the vector via digestion of **EcoRI-HF** and **NotI-HF**. The insert of *Pbx1* has been amplified from a plasmid (kind gift of Licia Selleri) via PCR using the following primers:

Forward: 5' GACAG**AATTC**ATGGACGAGCAGCCCAGGGC 3'

Reverse: 5' CAG**GCGCCGCT**ACTGTATCCTCCTGTC 3'

Then, after digestion of the PCR product, both vector and insert were ligated.

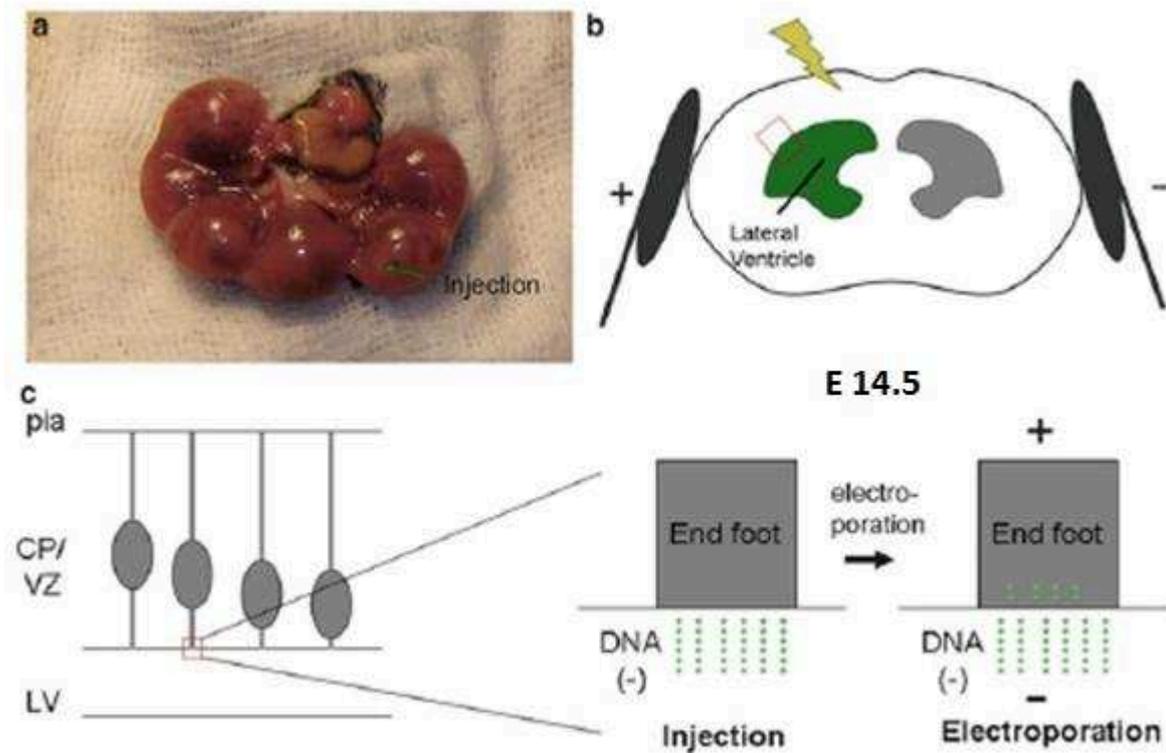


III. *In utero* electroporation

The experimental procedure for *in utero* electroporations were performed according to the protocol in Saito and Nakatsuji (2001) with several modifications as stated in the following:

Endofree-toxin plasmids were diluted in TE-Buffer (Qiagen, #1018499) until it reached a total concentration of 1 µg/µl in a final volume of 20 µl, including 1x Fast Green FCF (Sigma-Aldrich, #F7252). For co-electroporations of multiple plasmids, the concentration of each

single plasmid has been lowered until they together reached a final concentration of 1 $\mu\text{g}/\mu\text{l}$. For instance, co-electroporation of four plasmids together would mean a concentration of 0.25 $\mu\text{g}/\mu\text{l}$ for each plasmid. After a pregnancy duration of exact 14.5 days (E14.5), mothers were deeply anesthetized via Intraperitoneal (IP) injection of 10 μl Ketamin/Xylazine (10mg/ml) per gram body weight. Then, the mice were shaved on the upper abdomen and cleaned with disinfectant (Pierre Fabre, #716431). Afterwards, a lateral laparotomy has been performed and the uterus was exposed by carefully pulling it out with ring forceps. Via microinjection of the DNA mix with capillaries (Harvard-Apparatus, #30-0016), which have been generated by pulling with a micropipette puller (Sutter Instrument, Model P-1000; Parameters: Heat 459, Pull 60, Vel 75, Time/del 100, Pressure 200) and cut at approximately 60 μm away from the tip. The capillary has been inserted into a holder of a microinjector (Eppendorf FemtoJet 5274 V2.02; Parameters: Pi[hPA] 100 – 300, ti[s] 0.7, Pc[hPA] 7) and approximately 1 μl have been injected into one of the two lateral ventricles (see **Fig. 17**). Then, by placing tweezer electrodes (3 mm, Nepagene, #CUIY650P3) onto the uterine wall with the plus node on the premature M1 or S1 area underneath, square electric pulses were applied (Without poring pulse; Transfer pulse with Voltage: 37, Pulse length: 50 ms, Pulse interval: 999 ms, Number of pulses: 4, Decay rate: 5%) via an electroporator device (Nepagene Superelectroporator, Nepa21 Typell). To finish, uterus with embryos was repositioned into the original location and the incision was sewed up with sutures (Péters Surgical; 6/0 #87002F for peritoneum and 5/0 #87001F for skin). 120 μl Meloxicam (Metacam) and 200 μl Gentamicin (1 mg/ml, Sigma #G1272) were subcutaneously injected to prevent inflammation. Electroporated mice were housed in solitary and in a ventilated black box for 24h after the surgery to recover.



Procedure of in utero electroporation. **A.** Embryos were exposed by lateral laparotomy. **B.** DNA was injected in the lateral ventricles of mouse brains. **C.** Schema showing how DNA is transfected in cortical progenitors. Taken and modified from (Wang and Mei, 2013).

IV. Tamoxifen injection

To induce the expression of CRE recombinase from *pCAG-ERT2-CRE-ERT2* plasmid, tamoxifen has been applied at the desired developmental stage of the mice. Briefly, 10ml of 10 mg/ml tamoxifen has been prepared as followed:

100 mg tamoxifen (Sigma, #T5648-1G) has been taken up in 500 μ l Ethanol absolute and transferred in an eppendorf tube. Next, the tube has been put on a rotator for 20 min at RT and then taken up with 9.5 ml of corn oil (Sigma, #C8267). Then, the tube has been thoroughly vortexed and put back on a rotator until the tamoxifen completely dissolved. 1 ml aliquots have been stored at -20°C until usage.

Mice received tamoxifen subcutaneous under the neck depending on their age:

Age	μ l tamoxifen
P3	50
P7	50
P10	100
P14	100
P21	150

V. Cell Dissociation

All reagents were prepared according to the manufactures protocol (Worthington Papain Dissociation System, Cat. No. LK003150)

48h after *in utero* electroporation at stage E14.5, pregnant mice were sacrificed via cervical dislocation. The embryos have been removed from the uterus in icecold HBSS+Glucose (6g/l) with dissection tools and the brains have been extracted under a fluorescent microscope. Only GFP-positive areas were dissected, and 2-3 of them were dissociated together in one Eppendorf tube with 1 ml of papain-mixture for 10 min at 37°C. To mechanically digest the tissue, the suspension has been pipetted up and down 20 times with a P1000 pipette. The suspension has been transferred on a 70 μ m cell strainer and filtrated and then centrifuged at 400 g, 4°C for 5 min. Subsequently, the liquid has been removed and the remaining pellet was resuspended in 0.6 ml of Ovo-mixture by pipetting up and down ten times. Next, the suspension was filtrated again with a 70 μ m cell strainer and centrifuged at 400 g, 4°C for 5min. The liquid has been discarded and the pellet was resuspended in 1 ml of ice-cold DPBS (1X) by pipetting up and down 5 times and centrifuged again at 400 g, 4°C for 5 min. Finally, liquid has been removed and the pellet was resuspended in 300 μ l PBS-FBS (3%) in a tube.

VI. Cell sorting

Sorted cells were resuspended in 100 μ l RA1 + 2 μ l TCEP (see RNA extraction for further references) and stored for short term at -20°C.

VII. RNA Extraction

For RNA extraction of the GFP-positive sorted cells, the NucleoSpin RNA XS kit from MACHEREY-NAGEL (Ref 740902) has been used. The stored sorted cells were thawed up on ice for 20 min and then vortexed thoroughly two times for 5 s. Afterwards, the RNA extraction was done according to the manufacturer's manual. The quality and quantity of extracted RNA has been analyzed using a Bioanalyzer2100 (Agilent). At the end, the extracted RNA has been stored at -80°C.

VIII. Intracardiac Perfusion

At the planned developmental stage, mice underwent intracardiac perfusion for fixation of the brain tissue. For anesthesia without reawakening, dolethal has been IP injected and the body opened from the sternum to the neck. A butterfly needle was inserted into the left valve of the heart and varying amount of 4% PFA (Sigma-Aldrich, #P6148; were used to perfuse. Afterwards, the head has been cut and dissected. After checking for successfully electroporated brains (GFP-positive) under a fluorescence-microscope, brains were post-fixed in 4% PFA for 2h at RT. Finally, brains were washed one time in PBS and stored in PBS – Azide (0.05%; Sigma-Aldrich, #S8032) at 4°C for long-term storage.

IX. Embedding and Slicing

Successfully electroporated brains were embedded in 4% Select Agar (Sigma-Aldrich, #A5054) in PBS. Therefore, 4 g of Select Agar were dissolved in PBS and heated up with a microwave. As soon as the agar cooled down to 37°C, the brain has been embedded. and then sliced on a vibratome (Leica VT 1000S; Speed: 7, Frequency 7). All brains were cut coronally in 100 µm thick sections and preserved in PBS – Azide (0.05%) until further processing.

X. Immunofluorescence

Floating slices were incubated in 1 ml blocking buffer per well ON at 4°C, gently rocking. Then primary antibodies were diluted in 500 µl antibody buffer per well for two days at 4°C. Subsequently, slices were washed three times for 10 min at RT and two times for one hour. Secondary antibodies were diluted 1:400 in antibody buffer and 500 µl have been added to

each well and slices incubated ON at 4°C. Again, slices were washed as described before and 500 µl of PBS with DAPI (Invitrogen, #H3570) was added and incubated at RT for 5 min. Finally, slices were mounted on glass microscope slides with 200 µl of mounting medium and enclosed with a cover slip.

50 ml Blocking buffer	50 ml Antibody buffer
10% Goat serum	3% Goat serum
3% BSA	3% BSA
0.03% Triton-X100	0.03% Triton-X100
Filled up with PBS (1X)	Filled up with PBS (1X)

Mounting Medium
6g Glycerol
2.4g Mowiol
6 ml ddH2O
12 ml 0.2M Tris (pH 8.5)

Antibody	Host	Dilution	Reference
GFP	Chicken	1:500	ab13970
Flag	Rat	1:200	NBP1-0671255
Cux1	Rabbit	1:200	ABE217
Ctip2	Rat	1:200	ab18465
Fog2	Rabbit	1:200	sc-10755
PCP4	Rabbit	1:200	PA5-52209
Darp32	Rabbit	1:200	ab40801
Lmo4	Rat	1:500	Gift from Jane Valsvader
Fezf2	Rabbit	1:200	JP18997
RFP	Rabbit	1:200	ab124754

Antibody	Host	Dilution	Reference
Chicken488	Goat	1:400	A-11039
Rabbit488	Goat	1:400	A-11034
Rat488	Goat	1:400	A-11006
Rabbit594	Goat	1:400	A-11012
Rabbit647	Goat	1:400	A-21245
Rat647	Goat	1:400	A-21247

XI. Microscopy

Overview images (mosaics, 10X) of IHC floating slices were taken using Axio Imager 2 (Zeiss) while images for counting cells were taken using a Zeiss 710 confocal or Zeiss 780 NLO confocal. The confocal images have been taken and processed as mosaic and z-stack (2 μ m between layers).

XII. Analysis

To process the images taken from microscopes, Fiji2 has been used. Each immunofluorescent slice has been carefully checked for the right neocortical area (M1 or S1) and their respective anatomy using Allen Brain Atlas or Atlas of the Developing Mouse Brain (Paxinos *et al.*, 2007, ISBN 0-12-547622-1). Experiments were given random numbers to ensure bias was avoided. Four layers of confocal images were stacked (2 μ m x 4) and the total number of GFP-positive cells were counted. Then, markers were screened for co-localization with GFP-positive cells using orthogonal view for 3D presentation. Each marker that has been identified as co-localized with GFP-positive has been counted and divided by the number of total GFP-positive cells. The result was represented as a percentage and error bars were calculated as standard error of the mean (SEM). For comparisons between plasmids, 1-way ANOVA has been applied together with a tukey post-hoc test. For grouped comparisons between areas together with plasmids, a 2-way ANOVA has been applied together with a tukey post-hoc test. Image analysis has been performed with Fiji (ImageJ 1.51n), graphs and statistical analysis have been performed with Graphpad Prism 7. Scale bars are indicated on the images or in the figure legends.

RESULTS

Section I.: Reprogramming upper layer neurons in the embryonic mouse brain

A. Ectopic expression of *Lmo4* induces lower layer molecular markers in post-mitotic upper layer neurons at low efficiency

Direct neuronal reprogramming aims at changing cell fate of differentiating or differentiated cells into neurons. In the mammalian brain, significant neuronal generation is limited to early post-mitotic and postnatal stages. Once a neuron is born, it does not change its cell identity until the end of its lifespan. Seminal work has demonstrated that post-mitotic neurons retain a certain plasticity and new-born upper layer CPNs can be converted into lower layer corticofugal projection neurons (CFuPNs) by ectopic expression of one transcription factor *in vivo*. These reprogrammed cells expressed lower layer markers, including SCPN identity marker CTIP2.

Lmo4 is expressed in an area- and subtype specific fashion and controls post-mitotic projection neurons diversity in the rostral motor cortex (Cederquist *et al.*, 2013). Furthermore, previous observations found out that *Lmo4* over-expression led to an increase of CTIP2 in L5 neurons (Harb *et al.*, 2016). Thus, we sought out whether the nuclear co-adaptor LMO4 has the capacity of reprogramming upper layer neurons, into CFuPNs.

Therefore, we used the *Cdk5r-Lmo4-IRES-GFP* plasmid or control *Cdk5r-GFP* to deliver *Lmo4* or control *GFP* into target cells. The plasmid drives gene expression only in migratory, post-mitotic neurons thanks to the *Cdk5r* gene promoter (Tsai *et al.*, 1994; Delalle *et al.*, 1997). Furthermore, the expression of *Lmo4* was revealed by a reporting GFP signal. Mouse embryos at E14.5 were electroporated *in utero*, to deliver *Cdk5r-Lmo4-IRES-GFP* (*cLmo4*) or control *Cdk5r-GFP* (*cGFP*) in cortical progenitors at the position of the presumptive somatosensory area (S1, see Fig. 17A). The developmental stage of E14.5 marks peak generation of upper layer (UL) neurons, while the generation of lower layer neurons is almost at its end (Greig *et al.*, 2013). As a next step, we perfused and extracted GFP-positive brains of pups at P7, when laminarization of the cortex has almost finished. Then, brains were cut with a slicing vibratome into coronal sections. As a last experimental step, we applied immunohistochemistry (IHC) for visualizing proteins, which are specific molecular markers of UL or LL neurons and we analyzed

expression of these markers in GFP-positive neurons located in upper layers of S1 (**see Fig. 17B and B'**). Here, we applied IHC to reveal the expression of CUX1, a protein that is selectively expressed in upper layer CPNs (Molyneaux *et al.*, 2007), CTIP2, a protein specifically expressed in lower layer SCPNs and CThPNs (Molyneaux *et al.*, 2005), FOG2, a protein restricted to lower layer CThPNs (Galazo, Emsley and Macklis, 2016), PCP4, a protein found in SCPNs and CThPNs (Arlotta *et al.*, 2005; Watakabe *et al.*, 2012) and DARPP32, a protein restricted to lower layer 6 CThPNs (Ouimet, 1991).

The data were then generated by counting the UL GFP-positive neurons expressing molecular markers (**see Fig. 17C**). Results were then presented as a percentage of electroporated GFP-positive UL neurons expressing molecular markers (**see Fig. 17D**).

The examination of *cLmo4*-electroporated cortices revealed, that the percentage of targeted UL neurons expressing CUX1 was very similar to *cGFP*-control electroporated cortices ($94.13 \pm 1.45\%$ vs. $92.57 \pm 2.51\%$ in *cGfp*). Furthermore, *cLmo4* did not induce expression of CTIP2 (2.8 ± 0.84 vs. $0.53 \pm 0.53\%$ in *cGfp*) or PCP4 ($0.33\% \pm 0.3\%$ vs. 0% in *cGFP*). Interestingly, some targeted UL neurons expressed FOG2 ($7.86 \pm 1.43\%$ vs. $0.37 \pm 0.31\%$ in *cGFP*) and, a significant proportion showed DARPP32 expression ($12.43 \pm 2.4\%$ vs. $0.97 \pm 0.27\%$ in *cGfp*).

Taken together, the ectopic expression of *Lmo4* did not lead to a downregulation of *Cux1*, nor an upregulation of *Ctip2* or *Pcp4* gene expression in targeted UL neurons, with a subtle induction of lower layer markers FOG2 and DARPP32 in UL neurons was observed. These results indicate that *Lmo4* does not lead to an efficient conversion of molecular identity in UL neurons, especially in regard that UL neurons maintained CUX1 expression. Furthermore, a similar effect of *Ctip2* by *Lmo4* as reported upon overexpression in post-mitotic LL neurons could not be observed.

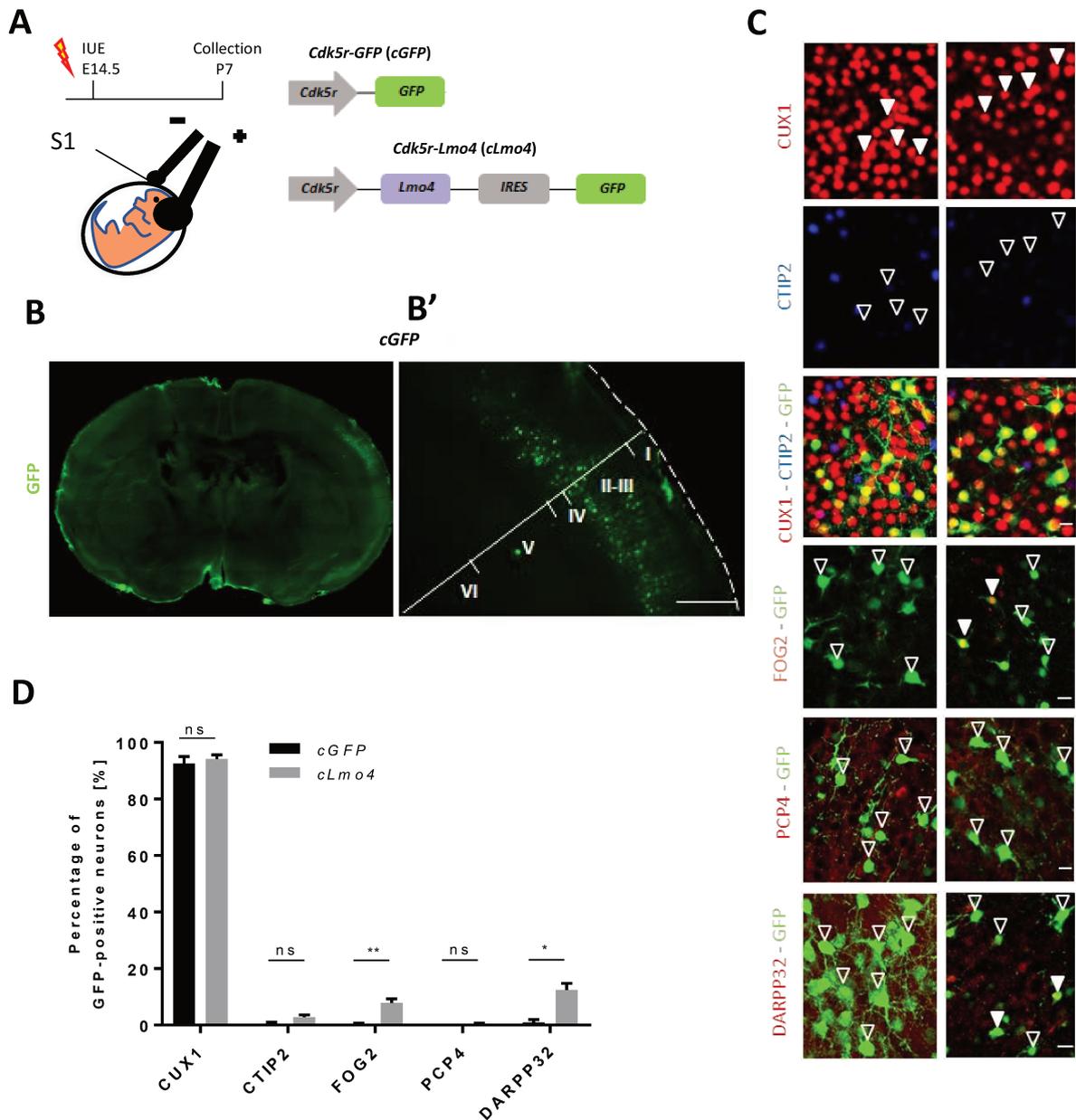


Figure 17: Ectopic expression of Lmo4 induces lower layer markers at low efficiency in post-mitotic upper layer neurons

A. Schematic representation of the experimental procedure and vectors used in the experiment. *Cdk5r-Lmo4 (cLmo4)* or control *Cdk5r-GFP (cGFP)* vector were electroporated at E14.5 and brains were collected at P7. **B.** Immunohistochemistry (IHC) for GFP on a P7 brain slice showing successful electroporation of cGFP in upper layer (UL) neurons of the somatosensory area. White box indicates magnification shown on the right side. **C.** IHC for upper layer marker CUX1 and lower layer markers CTIP2, FOG2, PCP4, DARPP32. Full arrowheads indicate expression of protein, empty arrowheads indicate absence of expression. **D.** Quantification of percentages of UL neurons expressing the markers described on the left. Results are expressed as mean \pm s.e.m. The unpaired two tailed t-test was used for statistical analysis. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$, ns = not significant. CUX1, $p = 0.62$; CTIP2 = 0.083; PCP4 = 0.37. $n = 3$ brains for each plasmid. Scale bars, 1000 μm (B), 200 μm (B'), 20 μm (C) in confocal images, M2, secondary motor cortex, M1 primary motor cortex, S1 primary somatosensory cortex

B. Ectopic expression of *Lmo4* in UL neurons does not alter their axonal projection

The previous results indicated that upon *Lmo4* induction in UL neurons, a strong change of molecular identity did not occur. Nevertheless, LL marker FOG2 and DARPP32 were found in a significant higher number upon *Lmo4* ectopic expression.

Neuronal identities are defined by a combination of morphology, electrophysiological properties or connectivity features. Under physiological conditions, many upper layer neurons project through the corpus callosum (CC) towards the contralateral hemisphere, some UL neurons in addition project ipsilaterally within the cortex and to the striatum (Str, Fame, MacDonald and Macklis, 2011). In contrast, CFuPNs project outside the cortex, to the striatum, the thalamus (Th) and within the pyramidal tract which includes the internal capsule (IC), the cerebral peduncle (CP) and the spinal cord (SC).

Thus, to further investigate any possible identity changes in targeted UL neurons, we sought out whether axonal projections are changed upon *Lmo4* ectopic expression. Therefore, we investigated sequential coronal sections, from rostral to caudal, of electroporated brains and followed GFP-positive axonal projections.

Examination of axonal projections revealed numerous GFP-positive axons in the corpus CC and few in the striatum (Str) in control *cGFP* and *cLmo4*-electroporated brains (**see Fig. 18A, B**). Projections of targeted UL neurons were not present in subcerebral trajectories and structures (including IC, Th, CP or spinal cord SC).

These results showed axonal projections that are characteristic of CPNs, the most abundant neuronal cell-type in the upper layers of the cortex. Furthermore, no axonal projections were detected in corticofugal structures. Thus, reprogramming of UL neurons upon ectopic expression of *Lmo4* is not conclusive regarding alterations of axonal projections.

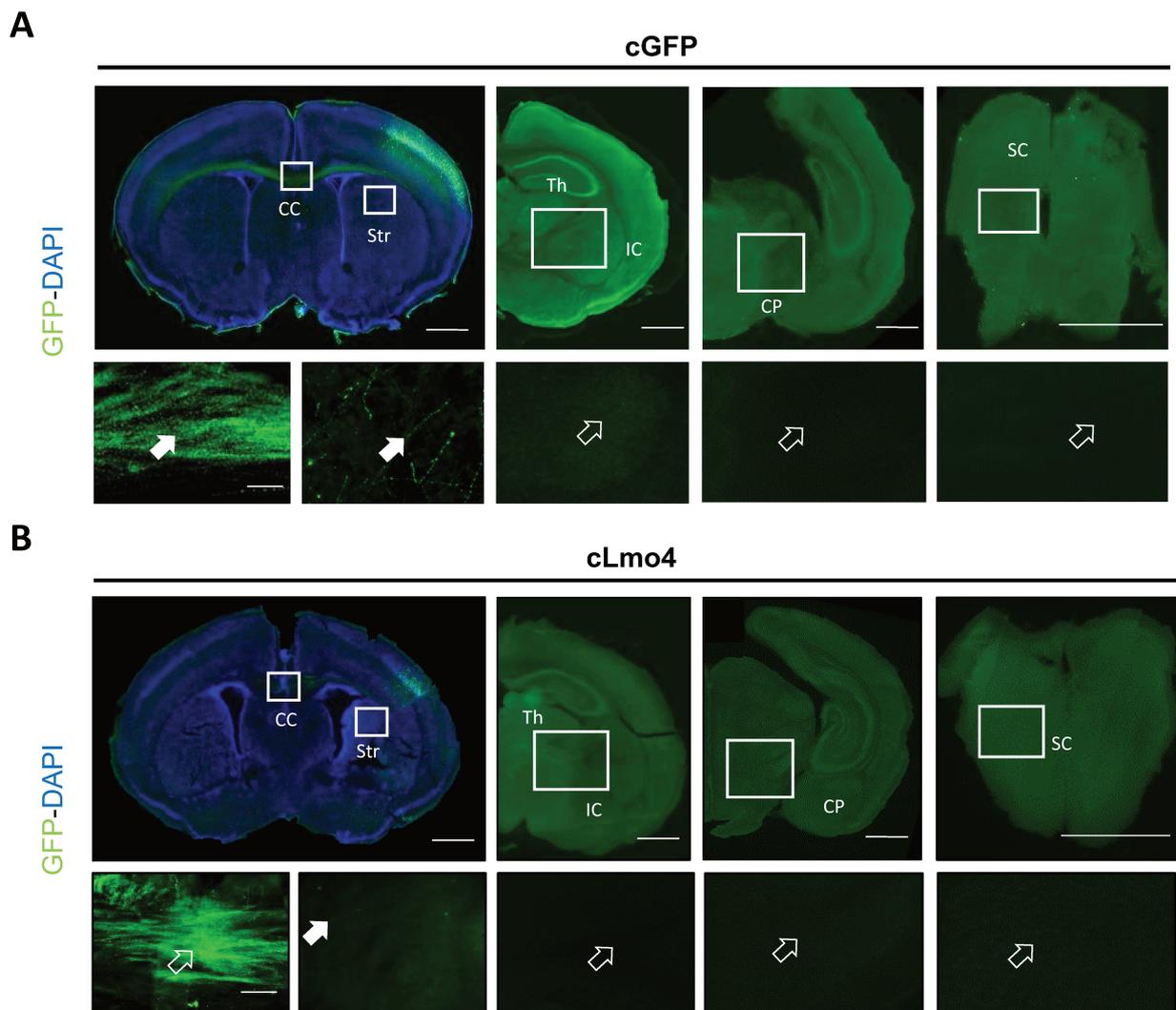


Figure 18: Ectopic expression of *Lmo4* in UL neurons does not alter their axonal projection

A. and **B.** GFP-positive axons of upper layer neurons in brains electroporated with cGFP or cLmo4 vectors are crossing the corpus callosum to the contralateral hemisphere. Few GFP-positive axons are found in the striatum. No GFP-positive axons are present in corticofugal targets. Scale bars, 1000 μm in high magnification images, 200 μm in detailed images. White boxes show axonal targets which are presented on the bottom in higher magnification. Empty arrows indicated absence of axons, full arrows indicate presence of axons. CC, corpus callosum, Str, striatum, IC, internal capsule, CP, cerebral peduncle, SC, spinal cord. $n = 3$ brains for each plasmid.

C. *Lmo4* in combination with *Fezf2* efficiently induces lower layer markers in UL neurons

Concluding that LMO4 is not inducing efficient reprogramming of UL neurons, we wondered whether LMO4 could improve FEZF2-driven reprogramming of UL neurons into CFuPNs. FEZF2 is a transcription factor which promotes SCPN cell fate in post-mitotic LL neurons, and studies demonstrated that ectopic expression of FEZF2 in UL neurons can change their molecular identity (De La Rossa *et al.*, 2013; Rouaux and Arlotta, 2013). *Ctip2* is a direct target of FEZF2 and ectopic expression of FEZF2 in UL neurons showed, among other CFuPN genes, upregulation of *Ctip2* (Rouaux and Arlotta, 2013). Based on previous studies which showed that LMO4 is implicated in de-repressing the *Ctip2* locus, we wondered whether a possibly more accessible *Ctip2* locus could be targeted by FEZF2 to induce CTIP2 expression more efficiently. Furthermore, this could also be the case for other CFuPN identity genes.

Therefore, we co-electroporated *Cdk5r-Fezf2-IRES-GFP (cFezf2)* together with & *cLmo4* in embryo brains at E14.5, targeting UL progenitors (see Fig. 19A). To ensure that both proteins are expressed in GFP-positive cells, we carefully examined co-localization of FEZF2 and LMO4 in electroporated neurons. FEZF2 and LMO4 protein was present in all examined GFP-cells, thus revealing both plasmids are collectively delivered in targeted neurons (see Fig. 19B).

Next, we quantified molecular marker expression of CUX1, CTIP2, FOG2, PCP4 and DARPP32 in *cFezf2* & *cLmo4*-electroporated UL neurons (see Fig. 19C, D). UL neurons electroporated with *cFezf2* & *cLmo4* revealed a significant decrease of cells expressing CUX1 ($3.43 \pm 0.29\%$ vs. $92.57 \pm 2.51\%$ in *cGfp*), CTIP2 was present in a significant higher number of GFP-positive cells ($64.1 \pm 2.28\%$ vs. $0.53 \pm 0.33\%$ in *cGfp*), as well as FOG2 ($29.67 \pm 2.91\%$ vs. $0.37 \pm 0.21\%$ in *cGfp*), or PCP4 ($54.13 \pm 7.16\%$ vs. 0% in *cGfp*) or DARPP32 ($47.37 \pm 2.67\%$ vs. $0.97 \pm 0.42\%$ in *cGfp*, see Fig. 19E).

As it could not be excluded that the driving factor here was solely FEZF2, we electroporated single *Fezf2* in UL neurons and compared quantified marker expression with the data of *cFezf2* & *cLmo4*. Expression of CUX1 ($3.43 \pm 0.29\%$ vs. $9.27 \pm 2.39\%$ in *cFezf2*), FOG2 ($29.67 \pm 2.91\%$ vs. $21.63 \pm 8.57\%$ in *cFezf2*) and DARPP32 ($47.37 \pm 2.67\%$ vs. 57.27 ± 4.55 in *cFezf2*) was not significantly different. Interestingly, CTIP2 ($64.1 \pm 2.28\%$ vs. $35.97 \pm 1.93\%$ in *cFezf2*) or PCP4

($54.13 \pm 7.16\%$ vs. $27.53 \pm 1.4\%$ in *cFezf2*) was expressed by a larger number of *cFezf2* & *cLmo4*-electroporated UL neurons (see Fig. 19E, F)

In conclusion, *cFezf2* & *cLmo4* ectopic expression in UL neurons triggered CFuPN gene identity expression. Furthermore, the efficiency of *cFezf2* & *cLmo4* in inducing CTIP2 or PCP4 was significantly higher compared to single *cFezf2*, while expression of CUX1, FOG2 or DARPP32 markers were not significantly different in electroporated UL neurons.

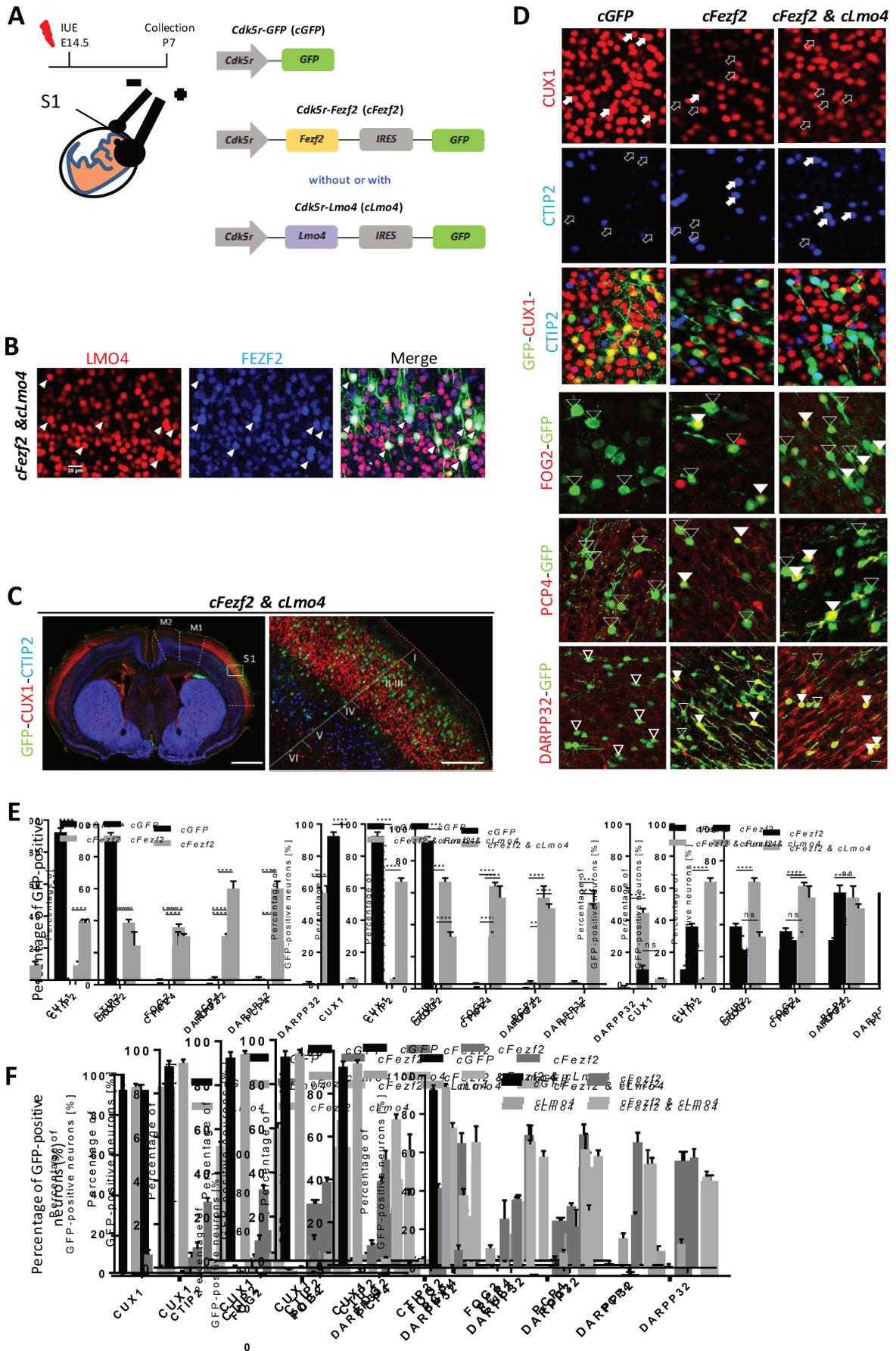


Figure 19: Ectopic Expression of single Fezf2 or Fezf2 in combination with Lmo4 drives post-mitotic UL neurons to acquire molecular features characteristic of CFuPN.

A. Schematic representation of the experimental procedure and used vectors. Mouse embryos were electroporated at E14.5 and their brains were collected at P7. For reprogramming approaches cFezf2 or cFezf2 & cLmo4 were electroporated. cGFP served as control. **B.** IHC for LMO4 and FEZF2 on slices of cFezf2 & cLmo4-electroporated brains. Full arrowheads indicate protein expression. **C.** Immunohistochemistry (IHC) of upper layer CUX1 and lower layer marker CTIP2 on a coronal slice of cFezf2 & cLmo4-electroporated brains. White box indicates magnification image on the right side. **D.** Representative images of IHC of CUX1, CTIP2, FOG2, PCP4 and DARPP32. Full arrows or triangles indicate that the protein is expressed in GFP-positive cells. Empty arrows or triangles indicate absence of protein expression in GFP-positive cells. **E.** Quantification of the percentage of electroporated cells expressing upper layer marker CUX1, or lower layer markers CTIP2, FOG2, PCP4 and DARPP32. Results are expressed as mean \pm s.e.m. One-way ANOVA with multiple comparisons was used for statistical analysis, * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$, ns = not significant. CUX1, $p = 0.58$; FOG2, $p = 0.32$; DARPP32, $p = 0.16$. **F.** Overview of quantification of the percentage of electroporated UL neurons expressing upper layer marker or lower layer markers. $n = 3$ brains for each plasmid.

D. *cFezf2* & *cLmo4* or single *cFezf2* ectopic expression alter axonal projection of post-mitotic UL neurons towards corticofugal targets

Next, we wanted to investigate whether the induction of CFuPN molecular identity in *cFezf2* & *cLmo4*-electroporated UL neurons also established a CFuPN-like connectivity.

Thus, we followed the trajectory of GFP-positive axons in *cFezf2* & *cLmo4*-electroporated brains (see Fig. 20A, B). No GFP⁺-axons in the CC of *cFezf2* & *cLmo4*-electroporated cortices were observed, whereas in the Str, the Th and in the pyramidal tract GFP⁺-axons were present. These results strongly indicate a loss of UL neuron identity at the expense of a CFuPN-like identity. Interestingly, labelled axons project both to the Th and the IC. This observation is raising the question whether Th-targeting projections of electroporated UL neurons are branches of axons *en route* towards subcerebral targets, whether these are independent axonal projections or, if CFuPN-like cells resemble a heterogenous population of SCPNs or CThPNs identity.

A similar change in axonal projections was observed in *cFezf2*-electroporated brains, but GFP⁺-axons were still present in the CC. This might indicate that *cLmo4*-addition to *cFezf2* facilitates *Fezf2*-driven reprogramming of UL neurons into CFuPNs-like cells.

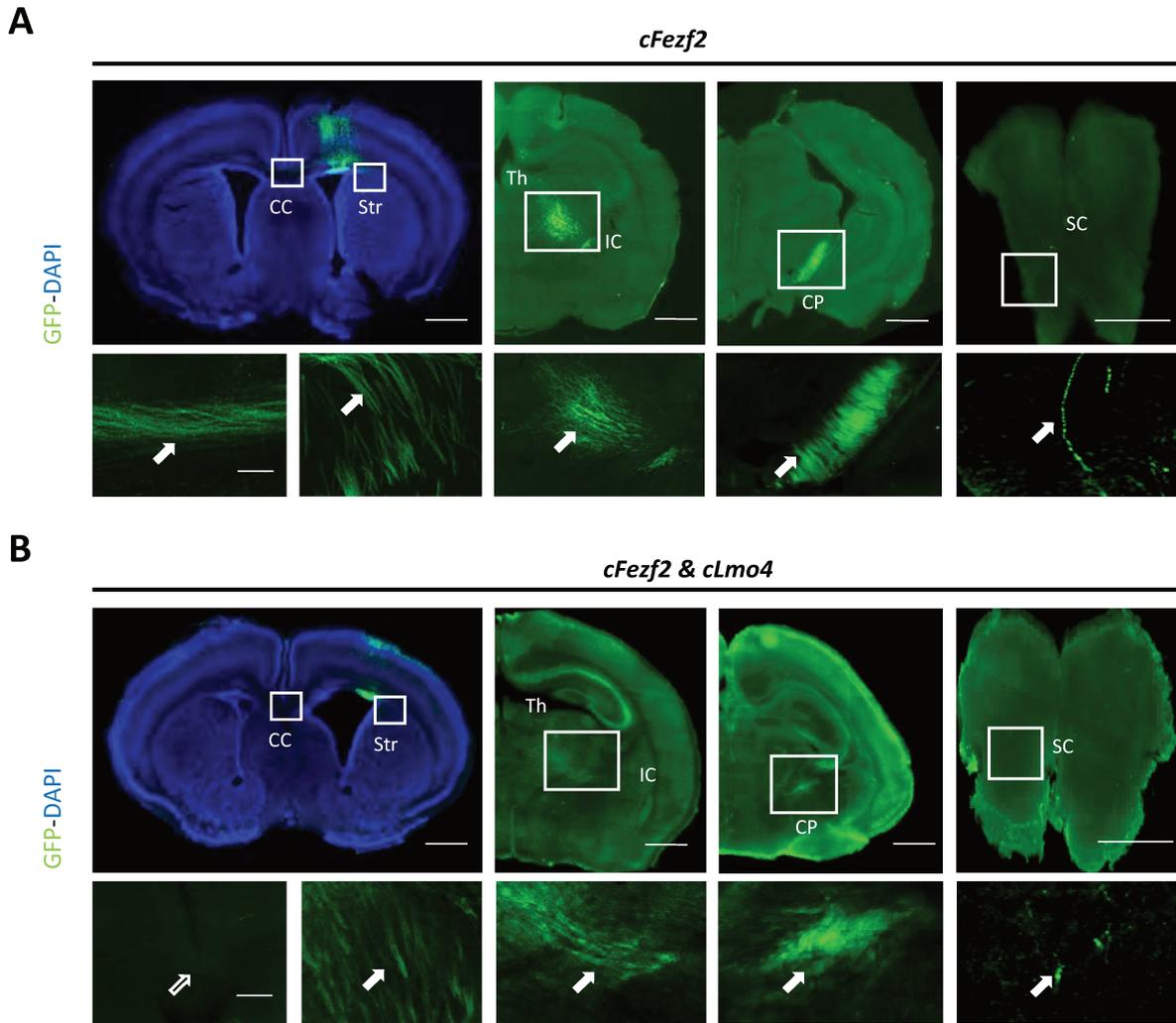


Figure 20: *cFezf2* & *cLmo4* or single *cFezf2* ectopic expression alter axonal projection of post-mitotic UL neurons towards corticofugal targets

A. and **B.** IHC for DAPI and GFP. Few GFP-positive axons crossing the CC are present in brains electroporated with *cFezf2*. No GFP-positive axons in the CC can be found in *cFezf2* & *cLmo4* electroporated brains. GFP-positive axons reaching corticofugal targets such as IC, Th, CP and SC are present in both *cFezf2* and *cFezf2* & *cLmo4*. Scale bars, 1000 μm in high magnification images, 200 μm in detailed images. White boxes show axonal targets which are presented on the bottom in higher magnification. Empty arrows indicated absence of axons, full arrows indicate presence of axons. CC, corpus callosum, Str, striatum, IC, internal capsule, CP, cerebral peduncle, SC, spinal cord. $n = 3$ brains for each plasmid.

E. The motor area is a more instructive environment for CFuPN-reprogramming

As a next step, we wanted to investigate whether the cellular environment may have an impact on reprogramming efficiency. Therefore, we chose to electroporate our plasmids into post-mitotic UL neurons of the presumptive motor area (see Fig. 21A, B). The motor area differs in its cytoarchitecture from the somatosensory cortex. Lower layer 5 and 6 are enlarged in the motor area with a broader and denser population of CSMN, compared to the somatosensory area. Accordingly, the thicker lower layer 5 is resembled by a larger CTIP2 expression in the motor area (Tomassy *et al.*, 2010). Thus, the motor area might be a more instructive environment for CFuPN reprogramming of UL neurons.

As a result, CTIP2 and DARPP32 markers were induced in a larger population of *cFezf2* & *cLmo4*-targeted UL neurons in the motor area, in comparison to reprogramming in S1 (see Fig. 21C). In *cFezf2* & *cLmo4*-electroporated cortices, CTIP2 ($79.47 \pm 1.01\%$ vs. $64.1 \pm 2.28\%$ in S1) and DARPP32 ($61.87 \pm 2.53\%$ vs. $47.37 \pm 2.68\%$ in S1) revealed expression in a significantly higher number of electroporated UL neurons, while CUX1 ($2.6 \pm 1.74\%$ vs. 3.43 ± 0.29 in S1), FOG2 ($23.13 \pm 0.95\%$ vs. $29.67 \pm 2.91\%$ in S1) or PCP4 (45.73 ± 2.33 vs. $43.97 \pm 1.55\%$ in S1) were not differently induced in a significant way, in GFP-positive UL neurons of M1 (see Fig. 21D). Interestingly, the expression of molecular markers in M1 compared to S1 was not significantly different in control, single *cLmo4*, or *cFezf2*-electroporated animals,

To conclude, the cellular environment of M1 had an impact on reprogramming efficiency of *cFezf2* & *cLmo4*, while control *cGfp*, *cLmo4* or *cFezf2* electroporated UL neurons appeared to express molecular markers in a similar number compared to S1. These results suggest, that the cellular environment within an organ can indeed affect the reprogramming efficiency and thus, selecting the cell niche for *in vivo* reprogramming approaches, appear to be an important factor.

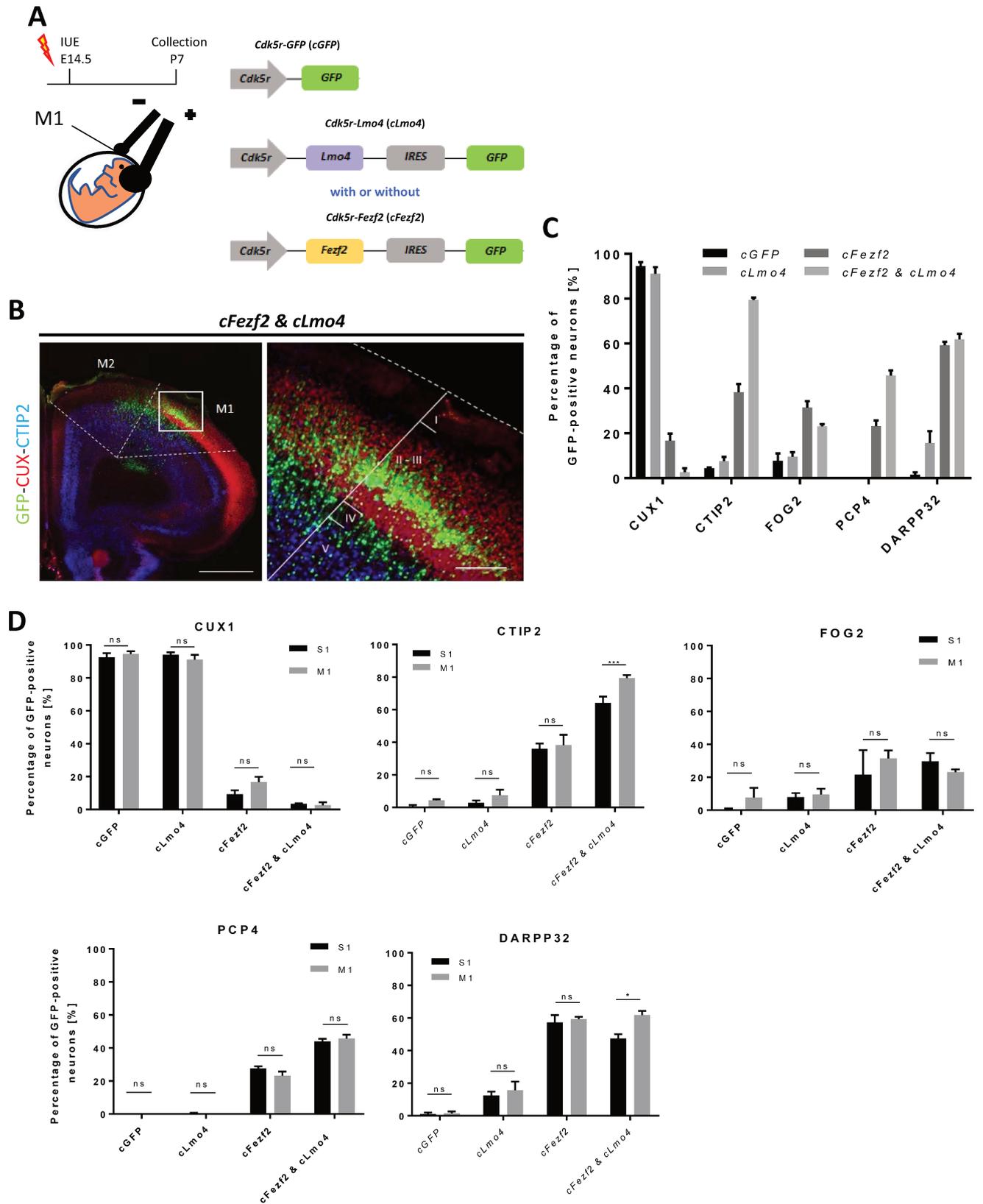


Figure 21: Ectopic expression of cFezf2 & cLmo4 in UL neurons of the motor area induces higher levels of corticofugal identity markers CTIP2 and DARPP2 compared to somatosensory area.

A. Schematic representation of experimental procedure. Embryos were electroporated with cGFP, cLmo4, cFezf2 or cFezf2 & cLmo4 in the motor cortex. **B.** Representative IHC image of GFP, CUX1 and CTIP2 on a brain slice electroporated with cFezf2 & cLmo4. White box indicates the location of the detailed image. **C.** Overview of quantification of the percentage of electroporated cells expressing upper and lower layer markers. Results are expressed as mean \pm s.e.m. **D.** Comparisons of quantified percentage of markers in electroporated cells by area. Two-way ANOVA with Tukey as post-hoc with multiple comparisons was used for statistical analysis, applied to all results together. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$, ns = not significant. Scale bars 1000 μm in overview image (left), 200 μm in detail image (right). $n = 3$ brains for each plasmid.

F. Reprogrammed post-mitotic UL neurons maintain stable regarding their altered molecular identity

As reprogrammed post-mitotic UL neurons might not be able to maintain their altered expression profiles and thus, revert to their original identity, we investigated animal brains electroporated with either control *cGFP*, *cFzf2* or *cFzf2 & cLmo4* at P35 (see Fig. 22A, B). Then, we compared the quantified percentages of GFP-positive UL neurons in P35 with the results of quantified percentage at P7.

Whereas in control *cGfp*-electroporated brains, expression of CUX1 was present in nearly all examined UL neurons ($96.75 \pm 0.35\%$ vs. $94.5 \pm 1.81\%$ at P7, see Fig. 22C), The number of cells expressing CUX1 in single *cFzf2*- ($6.75 \pm 1.85\%$ vs. $9.27 \pm 2.39\%$ at P7) or *cFzf2 & cLmo4* - electroporated ($6.95 \pm 1.75\%$ vs. $3.43 \pm 0.29\%$ at P7) UL neurons at P35 remained low. Furthermore, control *cGfp*-electroporated brains showed CTIP2 expression in a similarly number of UL neurons at P35 ($4.05 \pm 0.45\%$ vs. $0.53 \pm 0.53\%$ at P7), whereas the number of electroporated UL neurons expressing CTIP2 remained almost unaltered in *cFzf2* ($33.45 \pm 13.95\%$ vs. $35.97 \pm 1.93\%$ at P7), with a slight reduction in *cFzf2 & cLmo4*-electroporated UL neurons ($42.5 \pm 8\%$ vs. $64.1 \pm 2.28\%$ at P7). FOG2 expression was almost absent in P35 control *cGFP*-electroporated UL neurons as it was the case when brains were collected at P7 ($1.95 \pm 1.64\%$ vs. $0.37 \pm 0.37\%$ at P7). *cFzf2*- ($24 \pm 5.4\%$ vs. $21.63 \pm 8.57\%$ at P7) or *cFzf2 & cLmo4*- ($19.7 \pm 8.6\%$ vs. $29.67 \pm 2.91\%$ at P7) electroporated brains at P35 show almost unaltered number of cells co-expressing FOG2 compared to examination in P7 brains. The number of UL neurons expressing PCP4 at P35 did not change for *cGfp*-electroporated UL neurons ($2.15 \pm 1.14\%$ vs. 0% at P7), and expression pattern at P35 also resembled the quantified percentages of P7 brains that were electroporated with *cFzf2* ($34.85 \pm 2.55\%$ vs. $27.53 \pm 1.35\%$ at P7) or *cFzf2 & cLmo4* ($65.85 \pm 4.15\%$ vs. $54.13 \pm 7.16\%$ at P7). Interestingly, the percentage of DARPP32-expressing UL neurons in cortices electroporated with *cGfp* remained unaltered low ($3.95 \pm 0.75\%$ vs. $0.97 \pm 0.97\%$ at P7), whereas DARPP32-expressing UL neurons in both *cFzf2* ($15.4 \pm 1.2\%$ vs. $57.27\% \pm 4.55\%$ at P7) and *cFzf2 & cLmo4* ($11.65 \pm 2.65\%$ vs. $47.37 \pm 2.68\%$ at P7) dropped drastically at P35 when compared to P7.

Overall, reprogrammed UL neurons seem to keep their CFuPN molecular identity over time. DARPP32 is not maintained in reprogrammed UL neurons. An explanation could be, that

DARPP32-expression is age-dependent and gets downregulated over time. ISH data from the Allen Brain Atlas however show *RNA*-signals of *Darpp32* in lower layers at adult stages. More insights on the expression profile of DARPP32 in the cortex over time are necessary for a more conclusive explanation.

Here, we showed that the plasticity of early-postmitotic UL neurons can indeed be exploited to reprogram neurons, which remain stable altered molecular marker expression, even in an environment that is not in favour of their newly-acquired identity.

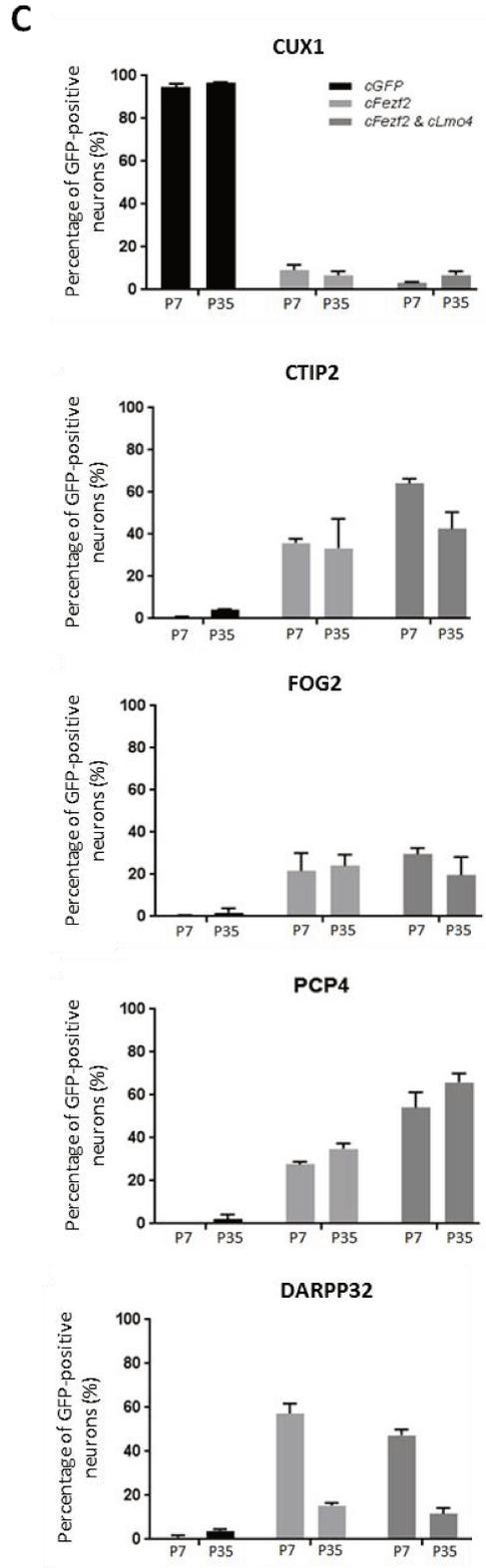
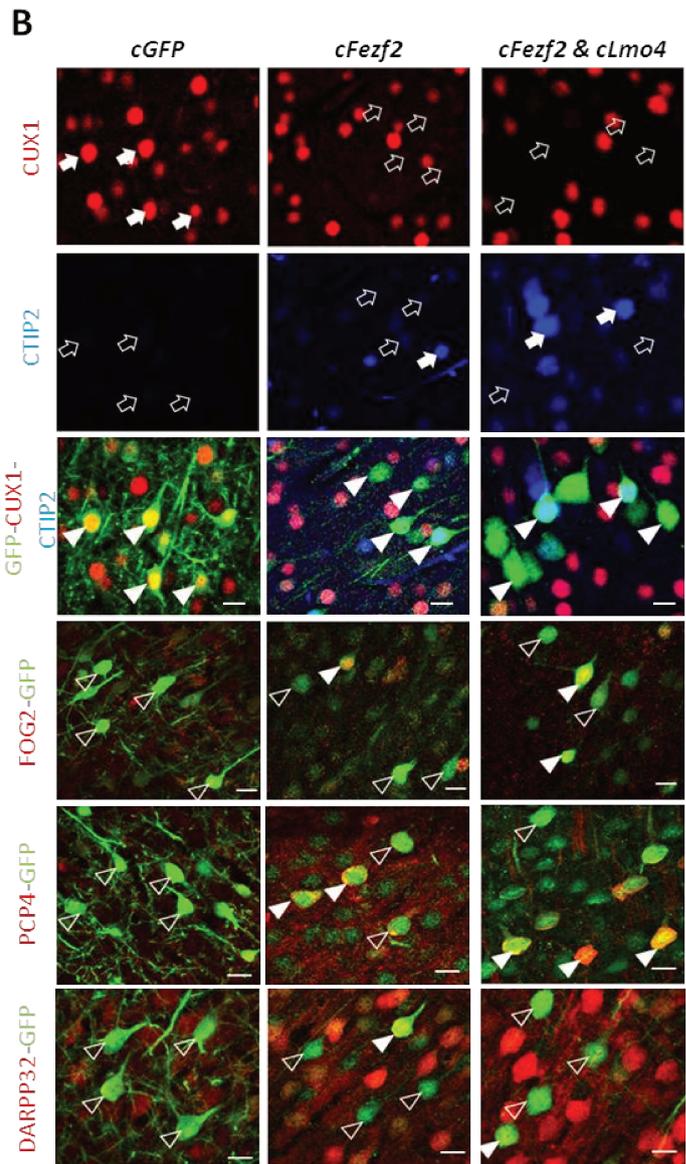
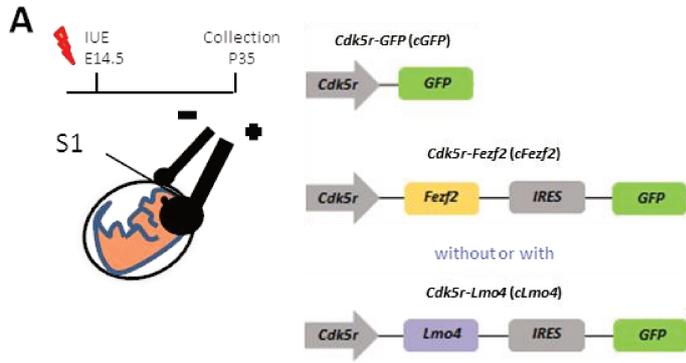


Figure 22: Reprogrammed post-mitotic UL neurons maintain stable regarding their altered molecular identity

A. Schematic representation of the experimental procedure. This time, brains were collected at P35. Brains were electroporated with vectors *cGFP*, *cFezf2* or *cFezf2 & cLmo4*. **B.** Representative images of IHC of upper layer and lower layer markers on brain slices electroporated with the 3 different vectors (left). Filled arrows or triangles indicate expression of marker, empty arrows indicate their absence. **C.** Quantification of upper and lower layer markers expressed in electroporated UL neurons compared by their expression levels at P7 and P35. Results are expressed as mean \pm s.e.m. Scale bars, 20 μ m. *n* = 2 brains for each plasmid.

Section II.: Reprogramming upper layer neurons at postnatal stages

A. Postnatal induction at P3 of single *Fezf2* or *Fezf2 & Lmo4* alters molecular identity of UL neurons

Given the high efficiency of reprogramming UL neurons by induction of *cFezf2 & cLmo4* at embryonic stages, we wondered whether this effective combination could be able to reprogram these neurons at later post-mitotic stages. Single *cFezf2* induction at P1 or P3 showed low efficient reprogramming in previous studies (De La Rossa *et al.*, 2013; Rouaux and Arlotta, 2013). Thus, we investigated if UL neurons can be reprogrammed at P3 by applying *cFezf2 & cLmo4* and, whether this factor cocktail would reveal a higher efficiency compared to single *cFezf2*.

To test this hypothesis, we subcloned *Fezf2* or *Lmo4* into an inducible vector *pCAG-fl-mutCherry-fl-IRES-EGFP* (gift from V. Tarabykin). This plasmid consists of a *stop* sequence flanked by *loxP* sites in same directional orientation before the actual gene, a subsequent *IRES*-sequence and a GFP-reporter (see Fig. 23A). The pCAG promoter drives high levels of gene expression in mammalian expression vectors, but is not limited to specific cell types or the developmental stage of a cell (Niwa, Yamamura and Miyazaki, 1991). All vectors were co-electroporated with *pCAG-ER^{T2}-CRE-ER^{T2}* (Matsuda and Cepko, 2007), a plasmid that expresses CRE-recombinase protein upon administration of 4-hydroxytamoxifen (TAM). The gene expression of inducible vectors is halted until activity of CRE, which can be induced at any given time by administration of TAM. As single *Lmo4* was not sufficient to reprogram UL neurons at earliest post-mitotic stages, it has not been applied for later postnatal stages. Thus,

only control *pCAG-Ind-GFP*, from now on referred to as *iGFP*, *pCAG-Ind-Fezf2*, now *iFezf2* and *pCAG-Ind-Fezf2* together with *pCAG-Ind-Lmo4*, from now on *iFezf2* & *iLmo4* were electroporated in the somatosensory area. We made this choice due to the difficulty of targeting the motor area and the observations of a higher embryonic lethality when plasmids are electroporated in presumptive M1. Here, we injected TAM in electroporated mice at P3, collected the brains at P7 and analyzed GFP-positive UL neurons for expression of upper and lower layer markers.

As a first step, we investigated whether *iFezf2* & *iLmo4* express their respective proteins in GFP-positive cells. Indeed, FEZF2 and LMO4 are co-expressed in *iFezf2* & *iLmo4*-electroporated cells (**see Fig. 23B**). Furthermore, to ensure that inducible vectors are not expressing their respective proteins without TAM administration, we electroporated embryos with *iFezf2* & *iLmo4*, *pCAG-ER^{T2}-CRE-ER^{T2}* and an *mCherry* plasmid (to reveal successful electroporation). Then, at P3, we injected corn oil **without** TAM into electroporated mice and analyzed their brains by application of IHC. The expression of CHERRY revealed electroporated cells. No GFP signal was found in CHERRY-positive cells, demonstrating that inducible vectors do not express their respective proteins **without** TAM administration (**see Fig. 23C**)

Next, we electroporated embryo brains at E14.5 with either *iGfp* or *iFezf2* or *iFezf2* & *iLmo4* and induced expression of these genes at P3 via injection of TAM (**see Fig. 24A, B, C**), Induction of *iFezf2* & *iLmo4* reduced the number of GFP-positive cells expressing CUX1 ($29.89 \pm 14.67\%$ vs. $93.83 \pm 1.33\%$ in control, **see Fig. 24D**) in an efficient way, compared to single *iFezf2* ($71.75 \pm 4.75\%$ vs. $93.83 \pm 1.33\%$ in control). Interestingly, CTIP2-expression was not induced anymore in P3 UL neurons, neither with *iFezf2* ($0.45 \pm 0.45\%$ vs. 0% in control) - confirming observations published by Rouaux and Arlotta (2013) - nor with *iFezf2* & *iLmo4* (0% vs. 0% in control). FOG2 was expressed by few numbers of P3 UL neurons induced with *iFezf2* & *iLmo4* ($10.8 \pm 4.96\%$ vs. $5.07 \pm 2.46\%$ in control), as it was the case for *iFezf2* ($10.4 \pm 2.4\%$ vs. $5.07 \pm 2.46\%$ in control).

Very interestingly, GFP-positive UL neurons were expressing PCP4 in high numbers, after induction of *iFezf2* & *iLmo4* ($66.67 \pm 15.5\%$ vs. 0% in control), whereas *iFezf2* ($24.05 \pm 24.05\%$ vs. 0% in control) revealed lower numbers of PCP4-expressing UL neurons., The quantified percentage of GFP-positive UL neurons expressing DARPP32 was low in *iFezf2* induced cells

($3.2 \pm 1.2\%$ vs. $3.75 \pm 2.81\%$ in control), whereas *iFezf2* & *iLmo4* induced DARPP32-expression in three-times higher numbers of UL neurons ($12.4 \pm 3.02\%$ vs. $3.75 \pm 1.91\%$ in control).

These results demonstrate that UL neurons at P3 retain the ability of changing their molecular identity. Again, *iFezf2* & *iLmo4* showed drastic higher numbers of UL neurons ectopically expressing CFuPN-markers, compared to single *iFezf2*. More specifically, PCP4 was the marker that showed the most drastic increase in numbers of UL neurons targeted with *iFezf2* & *iLmo4*. The percentage of PCP4-expressing UL neurons was even higher than in *cFezf2* & *cLmo4* reprogrammed cells at embryonic stages. This may indicate a later developmental role for PCP4 in CFuPN specification, as it was reported for CSMN (Arlotta *et al.*, 2005). Taken together, the reprogramming cocktail of *iFezf2* & *iLmo4* revealed efficient molecular identity alterations when induced at P3 in UL neurons, revealing a novelty in reprogramming postnatal cortical projection neurons.

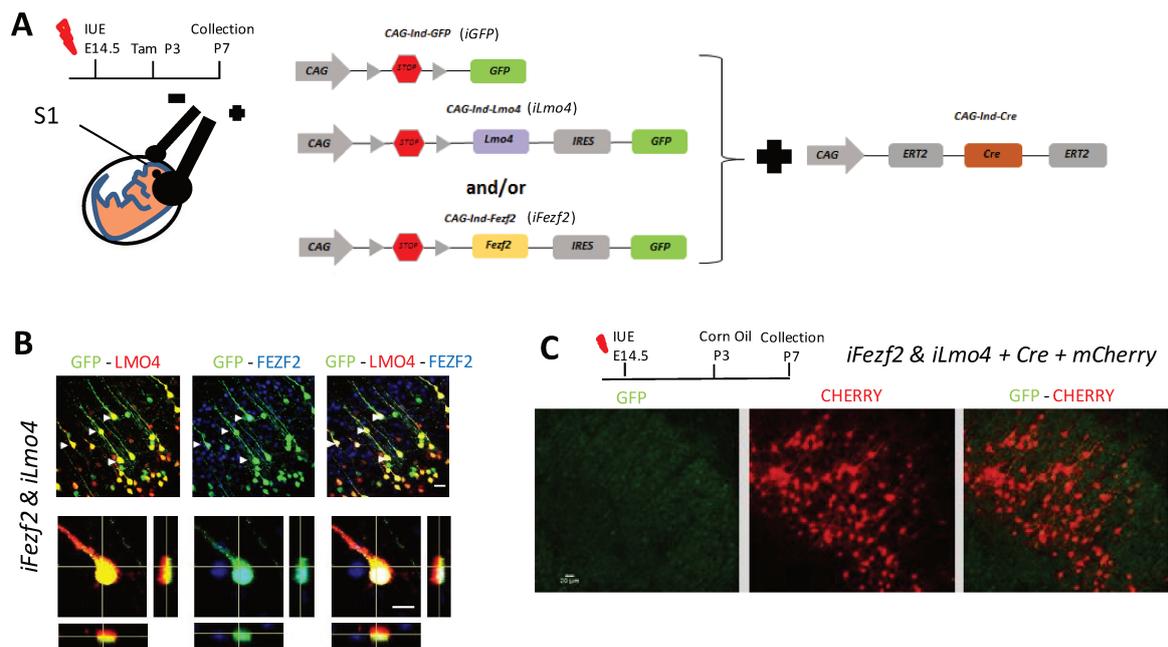


Figure 23: A. Verification of *iLmo4* and *iFezf2* induction

A. Schematic representation of experimental procedure. Inducible vectors of GFP, *Fezf2* or *Fezf2* & *Lmo4* were co-electroporated with a tamoxifen-dependent Cre-recombinase vector at E14.5. Expression of genes was induced at P3 by application of tamoxifen, collection at P7. **B.** Single cell level examination of FEZF2 and LMO4 protein expression in GFP-positive neurons after TAM application. **C.** Verification that inducible vectors only express their respective proteins upon TAM administration. To reveal electroporated cells, mCherry plasmid was co-electroporated. Sale bars 10 μm , 20 μm .

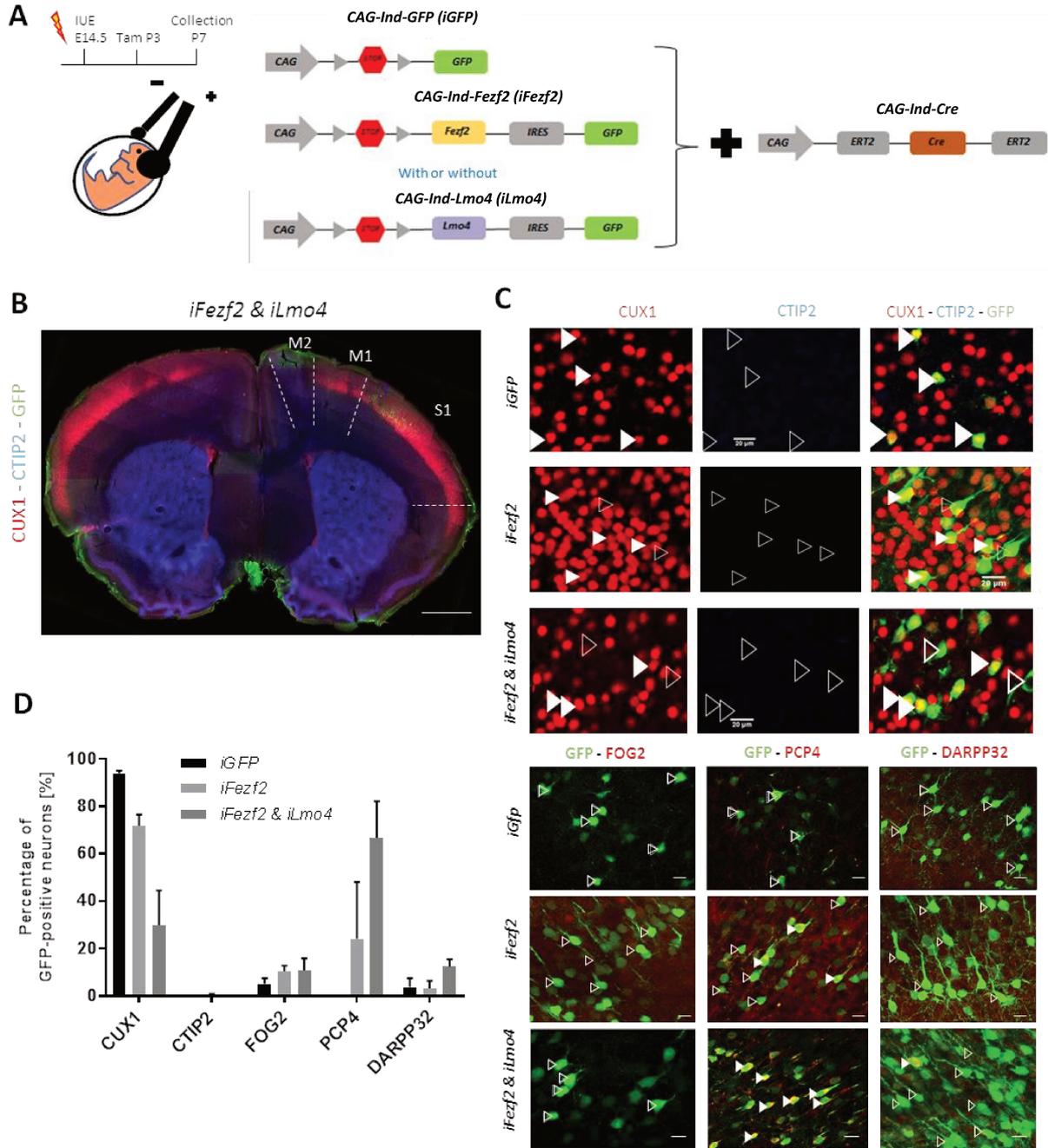


Figure 24: Postnatal induction at P3 of single *Fezf2* or *Fezf2* & *Lmo4* alters molecular identity of UL neurons

A. Schematic representation of experimental procedure. Inducible vectors of GFP, *Fezf2* or *Fezf2* & *Lmo4* were co-electroporated with a tamoxifen-dependent Cre-recombinase vector at E14.5. Expression of genes was induced at P3 by application of tamoxifen, collection at P7. **B.** Representative IHC image of GFP, CUX1 and CTIP2 on a brain slice electroporated with *iFezf2* & *iLmo4*. **C.** Panels of IHC images showing marker expression in GFP positive cells. Filled triangles indicate marker expression, empty triangles indicate absence of marker expression. **D.** Quantification of the percentage of electroporated cells expressing upper and lower layer markers. Results are expressed as mean \pm s.e.m. Scale bars, 1000 μ m, 20 μ m. $n = 3$ brains for *iGFP* and *iFezf2* & *iLmo4*. $n = 2$ for *iFezf2*.

B. Postnatal induction of single *iFezf2* or *iFezf2* & *iLmo4* combined with *iCtip2* or *iPBX1* does not increase reprogramming efficiency

Next, we sought out whether additional factors could improve the reprogramming efficiency of *iFezf2* & *iLmo4* at this postnatal stage. To test this hypothesis, we subcloned *Ctip2* into *pCAG-fl-mutCherry-fl-IRES-EGFP* (*iCtip2*) to obtain an inducible vector carrying this gene. CTIP2 is a TF, known for its specificity of SCPNs and in addition, known for regulating CST formation during development (Arlotta *et al.*, 2005; Chen *et al.*, 2008). Moreover, ectopic expression of *Ctip2* in UL progenitors was demonstrated to re-route their axonal projections towards subcerebral targets (Chen *et al.*, 2008).

Thus, we co-electroporated *iFezf2* & *iLmo4* together with *pCAG-ER^{T2}-CRE-ER^{T2}* and *iCtip2* and applied TAM at P3 (see Fig. 25A). The resulting quantified percentage of UL neurons did not reveal drastic higher numbers, besides obvious CTIP2-expression, compared to *iFezf2* & *iLmo4*: CUX1 (53.3% vs. $29.89 \pm 14.67\%$ in *iFezf2* & *iLmo4*), FOG2 (6.3% vs. $10.8 \pm 4.96\%$ in *iFezf2* & *iLmo4*), PCP4 (69.5% vs. $66.67 \pm 15.5\%$).

This result showed that the addition of *Ctip2* to the reprogramming factors *Fezf2* & *Lmo4* did not increase efficiency of reprogramming UL neurons. Together with the observation that CTIP2 is not expressed in targeted P3 UL neurons, we assume that the role of CTIP2 is limited to early post-mitotic development of CFuPNs.

Another factor we investigated for possible improvement in reprogramming was PBX1. PBX1 is implicated as pioneer factor in adult neurogenesis and as patterning gene for arealization and cortical laminarization during development (Golonzhka *et al.*, 2015; Grebbin *et al.*, 2016). Pioneer factors are especially well-suited to initiate cell fate changes as they can recognize their target sites in barely accessible chromatin and initiate a cascade of events that allows for later transcriptional activation of the respective genes (Grebbin and Schulte, 2017).

To test this whether hPBX1 could improve reprogramming efficiency, we electroporated an inducible vector carrying a *PBX1b* sequence (*iPBX1*) together with *iFezf2* & *iLmo4* and *pCAG-ER^{T2}-CRE-ER^{T2}* and applied TAM at P3 (see Fig. 25B). The effect of adding *PBX1* to the reprogramming cocktail did not increase the number of reprogrammed UL neurons compared

to *iFezf2* & *iLmo4*: CUX1 (60.4% vs. $29.89 \pm 14.67\%$ in *iFezf2* & *iLmo4*), CTIP2 (0% vs. 0% in *iFezf2* & *iLmo4*), FOG2 (4.3% vs. $10.8 \pm 4.96\%$), PCP4 (64.3% vs. $66.67 \pm 15.5\%$).

Thus, the addition of *Pbx1* to the reprogramming factors *Fezf2* & *Lmo4* did not increase efficiency of molecular identity change in targeted UL neurons. It could be, that the target genes of PBX1 are not involved in CFuPN specification, or that FEZF2 and LMO4 do not interact with PBX1 in the cortex.

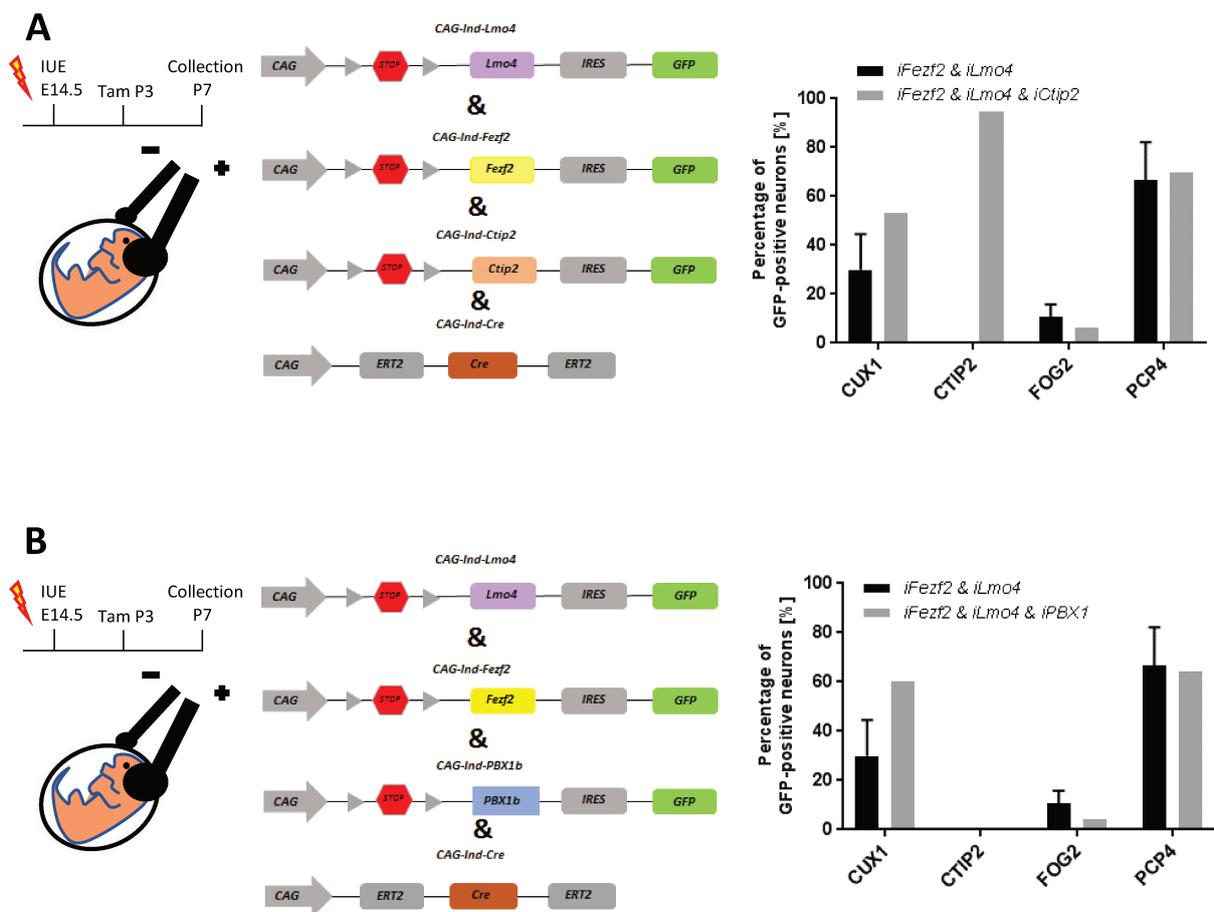


Figure 25: Postnatal induction of single *iFezf2* or *iFezf2* & *iLmo4* combined with *iCtip2* or *iPBX1* does not increase reprogramming efficiency

A. Schematic representation of experimental procedure and used vectors. *iCtip2* has been added to *iFezf2* & *iLmo4* together with the tamoxifen-dependent Cre recombinase vector. On the right side, quantification of percentage of GFP-cells expressing upper layer marker CUX1 or lower layer markers compared to *iFezf2* & *Lmo4* experimental results. **B.** Schematic representation of experimental procedure and used vectors. *iPBX1b* has been added to *iFezf2* & *iLmo4* together with the tamoxifen-dependent Cre recombinase vector. On the right side, quantification of percentage of GFP-cells expressing upper layer marker CUX1 or lower layer markers in comparison with *iFezf2* & *Lmo4* experimental results. $n = 1$ brain for *iFezf2* & *iLmo4* & *iCtip2*, $n = 1$ for *iFezf2* & *iLmo4* & *iPBX1*.

C.1. Postnatal induction of single *iFezf2* or *iFezf2 & iLmo4* drives UL neurons to change their axonal projections towards corticofugal targets

To further investigate reprogrammed UL neurons, we followed their axonal projections. An issue to overcome was the absence of GFP-positive axons when vectors were electroporated at postnatal stages (data not shown). A recent publication demonstrated that an engineered probe was able to reveal signal in fine axons and collaterals upon electroporation in neurons of the cortex and the hippocampus (Viswanathan *et al.*, 2015). This protein, called “spaghetti monster” *pCAG_smFP_FLAG*, is equipped with a *CAG* promoter, the engineered *probe* attached with 3x polypeptide protein tag. The “spaghetti monster” protein itself does not emit any fluorescence but can be detected by anti-FLAG antibodies and subsequent secondary antibody binding.

Therefore, we co-electroporated either *iGfp*, *iFezf2* or *iFezf2 & iLmo4* together with *pCAG-ER^{T2}-CRE-ER^{T2}* and *pCAG_smFP_FLAG* and induced expression of inducible vectors at P3 (see **Fig. 26**). Brains were collected at P10. When vectors were induced at P3, FLAG-positive axons could be observed in the CC, in the Str, the Th and the IC in all conditions. Note, that only very faint signal was observed in control *iGFP* in subcerebral targets, Th and IC. In cortices electroporated with *iFezf2*, FLAG-axons were found in the CP and in the SC, while more FLAG-positive axons could be observed in cortices electroporated with *iFezf2 & iLmo4* in CP and the SC, indicating a stronger reprogramming efficiency specifically into SCPNs.

To conclude, UL neurons can change their axonal projections even at postnatal stages. Nevertheless, axons in the CC of *iFezf2 & iLmo4* induced cortices, reveal that the reprogramming efficiency seems to decline with higher developmental stages of UL neurons. Furthermore, only very few FLAG-axons were found in Th, suggesting that at P3 the majority of reprogrammed UL neurons turn into SCPNs.

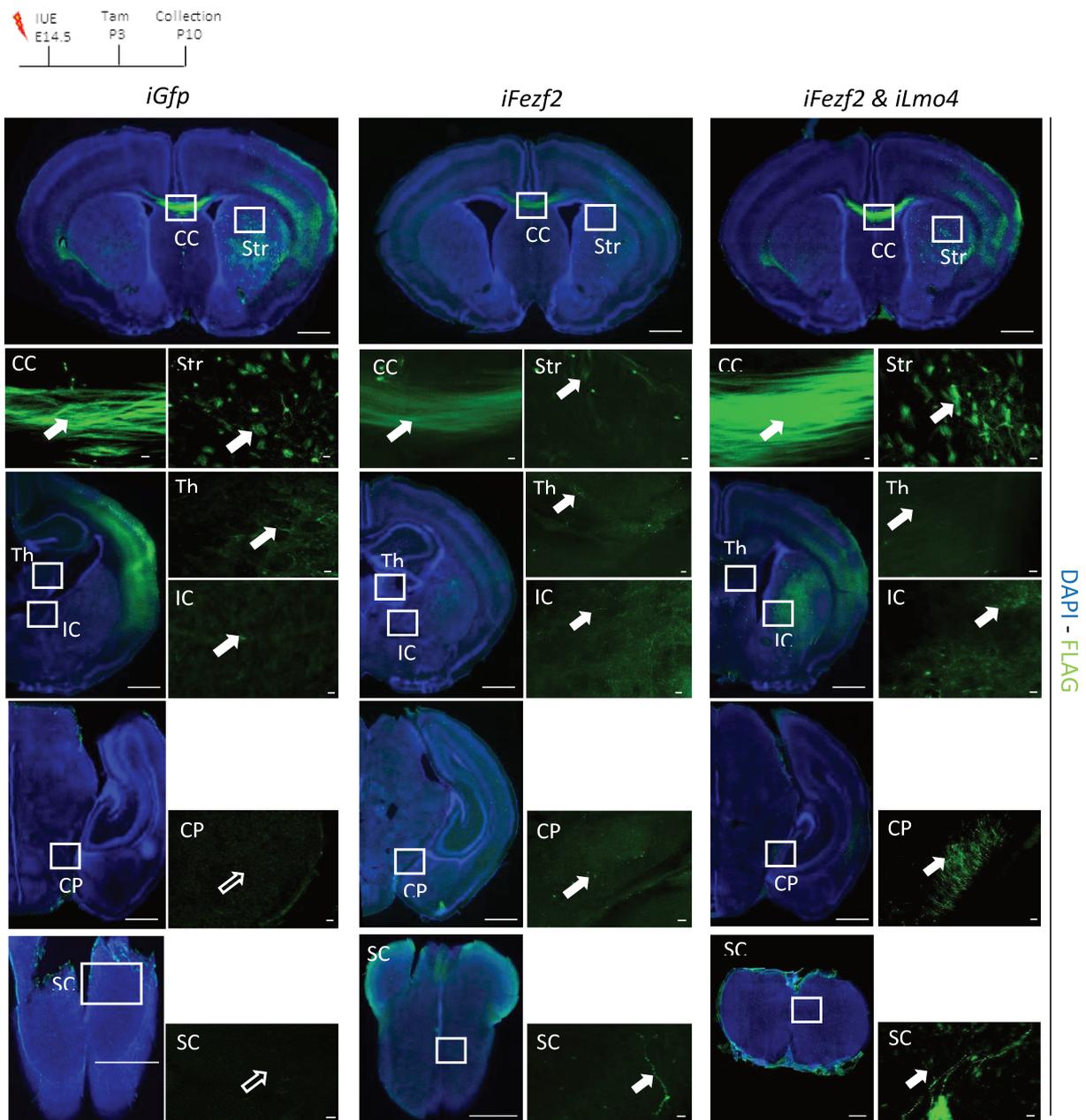


Figure 26: Postnatal induction of single *iFezf2* or *iFezf2 & iLmo4* drives UL neurons to change their axonal projections towards corticofugal targets

Induction of vectors *iGfp*, *iFezf2* or *iFezf2 & iLmo4* at P3, collection at P10. Note, that all plasmids were co-electroporated with *smFP_Flag*, a plasmid carrying 3x FLAG, which allows tracing of fine axons. Signals were then amplified by using a FLAG antibody. FLAG-positive axons of electroporated UL neurons show crossing of the CC and reveal signal in the Str, the Th and IC in all conditions. FLAG-signal of axons is present in CP and SC of brains electroporated with *iFezf2* and *iFezf2 & iLmo4*. White boxes indicate location of examination with their respective magnification on the right side. Filled arrows indicate FLAG-signal while empty arrows indicate the absence. Scale bars 1000 μ m, 20 μ m in high magnification images. $n = 3$ brains for each plasmid.

D. Postnatal induction at P7 of *iFefz2* & *iLmo4* still drives UL neurons to express lower layer marker PCP4

The next step was to investigate whether UL neurons can still be reprogrammed at later developmental stages. Therefore, we induced expression of vectors control *iGFP* or *iFefz2* & *iLmo4* at P7 and analysed electroporated brains at P14 (see Fig. 27A, B, C). Quantification of percentage of UL neurons expressing upper and lower layer markers revealed alteration of molecular identity in cortices electroporated with *iFefz2* & *iLmo4*. Upper layer marker CUX1 was expressed in significant fewer numbers of GFP-positive UL neurons ($62.8 \pm 5.76\%$ vs. $89.97 \pm 2.13\%$ in control), while again lower layer marker CTIP2 was not expressed (0% vs. 0% in control). FOG2 expression was not significantly induced upon *iFefz2* & *iLmo4* electroporation ($0.67 \pm 0.67\%$ vs. $5.9 \pm 2.07\%$ in control), but interestingly PCP4-expression was significantly induced ($49.27 \pm 9.48\%$ vs. $0.67 \pm 0.33\%$ in control).

Thus, UL neurons at P7 be changed regarding their molecular identity towards a CFuPN sub-type revealed by fewer number of cells expressing CUX1 and an increased number of cells expressing PCP4 upon induction of *Fefz2* & *Lmo4*. Furthermore, these results support the assumption that PCP4 is involved in late developmental CSMN specification.

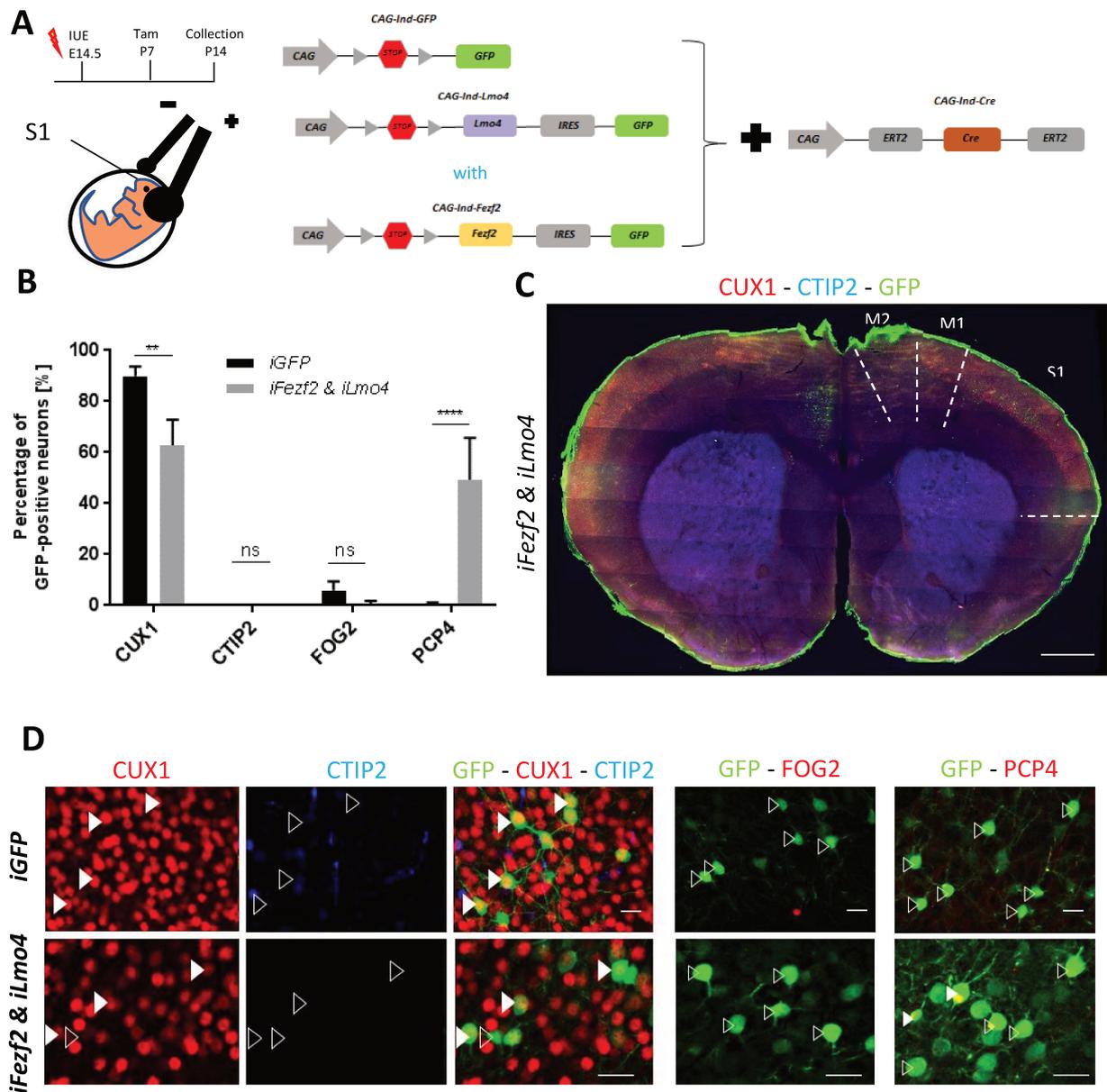


Figure 27: Postnatal induction at P7 of iFezf2 & iLmo4 still drives UL neurons to express lower layer marker PCP4

A. Schematic representation of experimental procedure and used vectors. Note that only control iGFP and iFezf2 & iLmo4 were used this time. **B.** quantification of the percentage of electroporated cells expressing upper and lower layer markers. Results are expressed as mean \pm s.e.m. Two-way ANOVA was applied for statistical analysis. * $p < 0.5$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$, ns = not significant **C.** Representative IHC image of GFP, CUX1 and CTIP2 on a brain slice electroporated with iFezf2 & iLmo4. **D.** Representative images of IHC of CUX1, CTIP2 and GFP as well as FOG2-GFP and PCP4-GFP. Full triangles indicate that the protein is expressed in GFP-positive cells. Empty triangles indicate non-expression in GFP-positive cells. Scale bars, 1000 μ m, 20 μ m. $n = 3$ brains for each plasmid.

D.1. UL neurons receiving expression of single *iFezf2* or *iFezf2 & Lmo4* at P7 show aberrant projections to corticofugal targets

Again, to test whether P7 UL neurons can also change their axonal projections at this stage, we induced vector *iGFP*, *iFezf2* or *iFezf2 & iLmo4* by TAM application and followed their axonal projections.

Induction of vectors at P7 and showed axons of targeted UL neurons in the CC and in the Str in all conditions (**see Fig. 28**). Moreover, no FLAG-signal was detected in the Th in control cortices, while both *iFezf2* or *iFezf2 & iLmo4* induction revealed signal in the Th. In all conditions, axons of FLAG-positive UL neurons showed projection into the IC, while only in cortices electroporated with reprogramming factors *iFezf2* or *iFezf2 & iLmo4* FLAG-positive axons were present in the CP. Interestingly, *iFezf2*-induction in UL neurons did not show axonal projections in the SC, while *iFezf2 & iLmo4* reprogrammed UL neurons reached the SC.

Taken together, UL neurons can still be instructed to change axonal projection towards corticofugal targets. In particular, *iFezf2 & iLmo4* induction revealed innervation of the SC, the most caudal target of SCPNs. These results reveal an unknown plasticity of UL neurons at P7, and adds up a time point, where neurons in the cortex can still be converted.

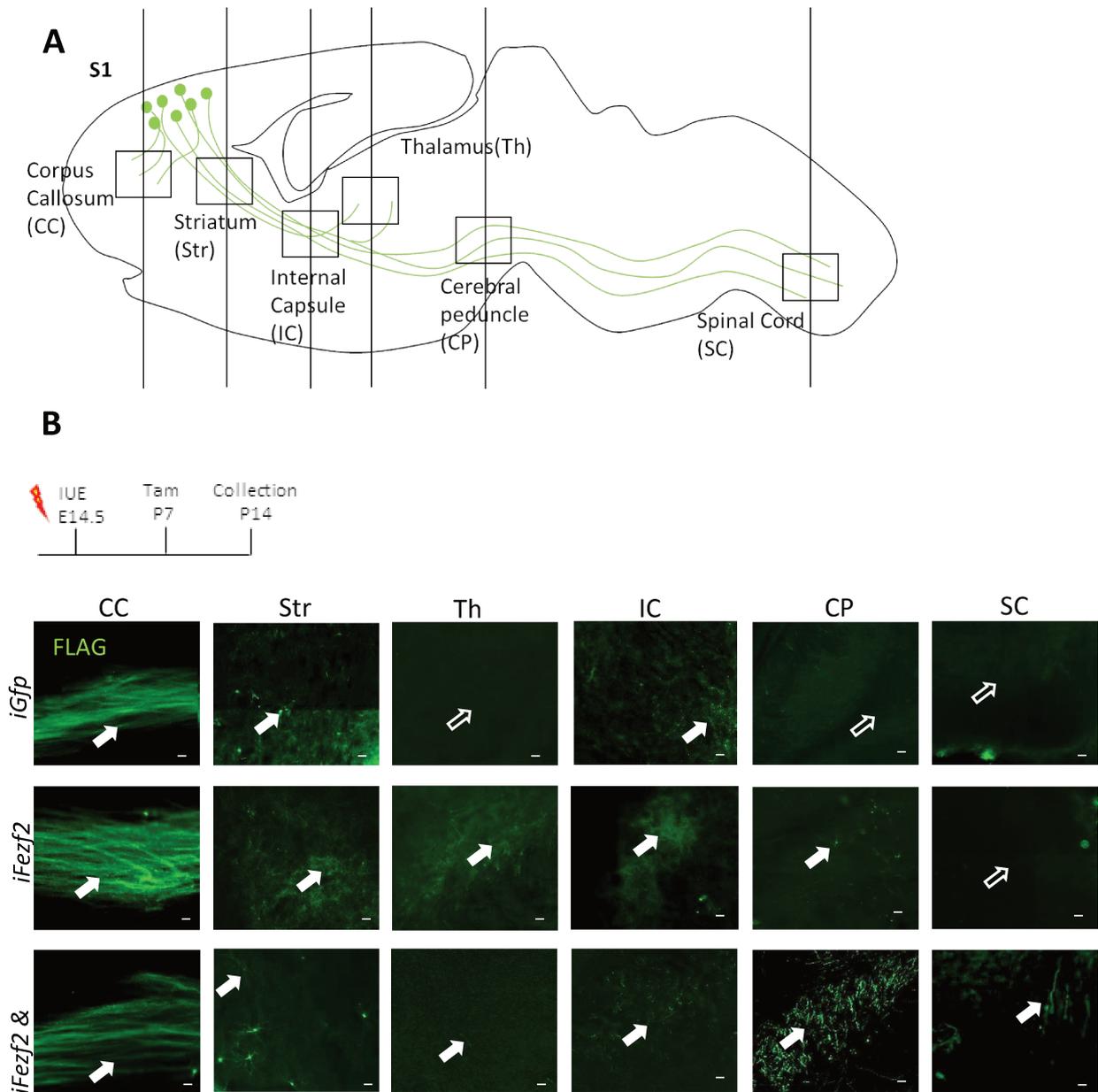


Figure 28: UL neurons receiving expression of single *iFefz2* or *iFefz2 & Lmo4* at P7 show aberrant projections to corticofugal targets

A. Schematic representation of a sagittal view on a brain with indicated coronal sections and investigated structures.

B. Induction of vectors at P7, collection at P14. In all conditions, targeted UL neurons show axonal projections through the CC, to the Str and to the IC. No FLAG-signal in the Th is present in control brains, but present for *iFefz2* or *iFefz2 & iLmo4*. FLAG-signal is present in IC and CP of brains electroporated with *iFefz2* and *iFefz2 & iLmo4* but absent in control brains. Only axons of *iFefz2 & iLmo4* electroporated UL neurons reach the SC. Scale bars, 20 μ m. $n = 3$ brains for each plasmid.

E. Ectopic expression of single *iFezf2* or *iFezf2 & Lmo4* in late postmitotic UL neurons show corticofugal projections

We next explored whether we can reprogram UL neurons at later developmental stages. Induction of *iFezf2 & Lmo4* at P10, did not show expression of CFuPN markers anymore, thus our examination was limited to connectivity features (data not shown).

At P10, induction of vectors mimicked phenotypes of P3 and P7 regarding projections in CC and Str in all conditions (see Fig. 29A and B). *iGfp*-induction in UL neurons did not reveal any signal in subcortical targets, whereas *iFezf2*-induction in UL neurons showed FLAG-positive axons in the CC, Str, Th, IC and CP but not in SC, similar to results obtained when induced at P3. In contrast, *iFezf2 & iLmo4* electroporated cortices revealed FLAG-positive axons of UL neurons in CC and Str, but also subcortical structures Th, IC, CP and signal was even detected in the SC. These results indicate that UL neurons can still be reprogrammed at postnatal day P10, showing axonal projections characteristic of CFuPNs. The addition of *iLmo4* to *iFezf2* seems to facilitate the projections of converted UL neurons to the SC, which could not be observed in single *iFezf2* at P7, nor P10.

These data demonstrate for the first time that reprogramming of postnatal P10 cortical neurons is feasible and reveal an unknown plasticity of differentiating cortical neurons.

Thus, we went one step further and induced plasmid expression at later developmental stages in UL neurons. Induction at P21 showed axonal projections in all examined structures of the brains electroporated and induced with *iGFP*, *iFezf2* or *iFezf2 & iLmo4*, besides absence of FLAG-signal in Th of cortices of control *iGfp* or *iFezf2 & iLmo4* induced vectors (see Fig. 29B). As subcortical targets such as CP and SC were even innervated by FLAG-positive axons of control *iGfp*-induced UL neurons, qualitative conclusion of reprogramming was not possible. Thus, whether UL neurons can be still reprogrammed at P21 is not clear at this stage and further investigations are necessary to gain a better understanding of these results.

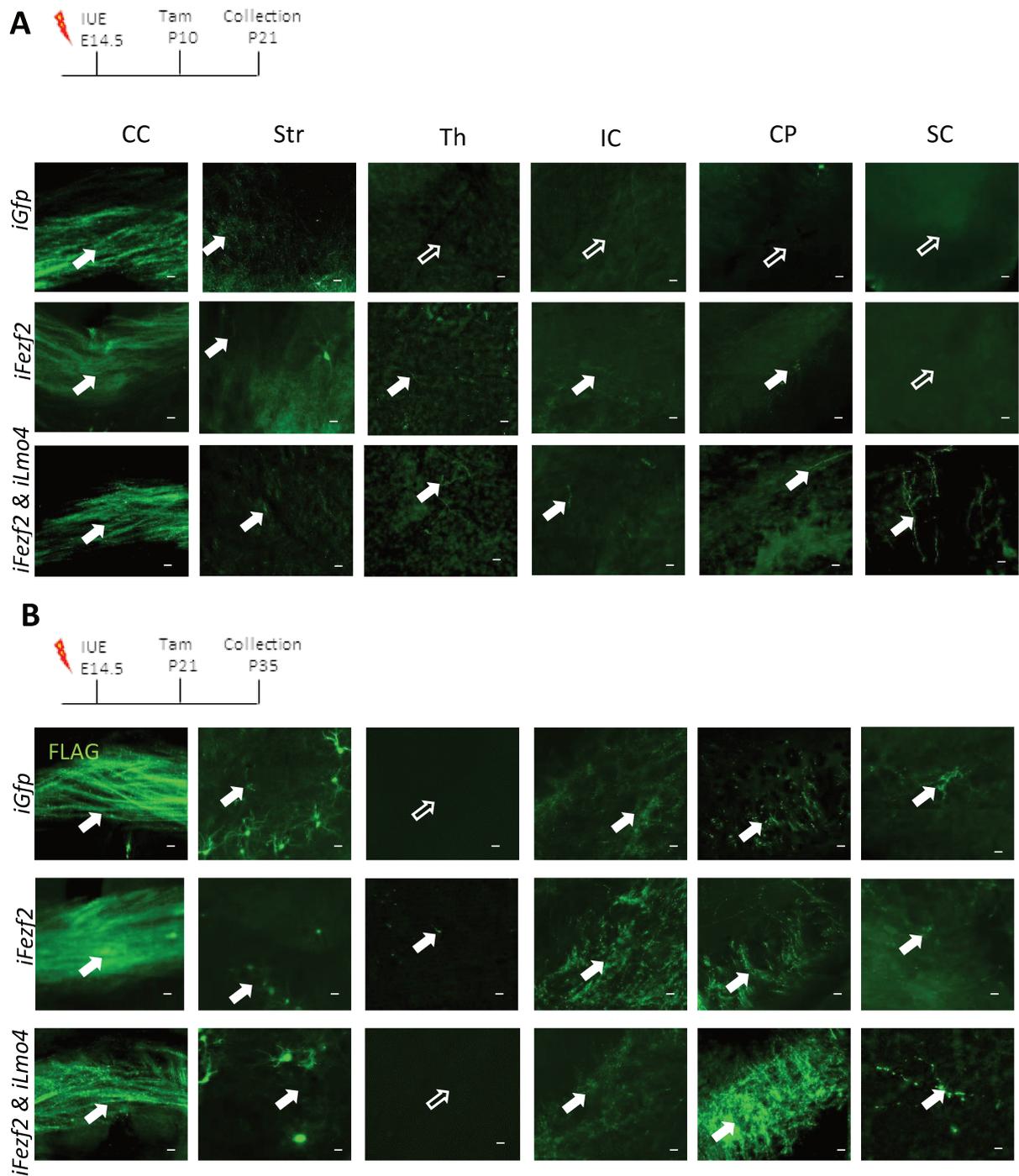


Figure 29: Ectopic expression of single *iFezf2* or *iFezf2 & Lmo4* in increasingly differentiating UL neurons show corticofugal projections

A. Induction of vectors at P10, collection at P21. At this developmental stage, *iFezf2* and *iFezf2 & iLmo4* electroporated UL neurons send axons to corticofugal targets Th, IC and CP, while no FLAG-positive axons are present in these structures in control brains. In the SC, only *iFezf2 & iLmo4* electroporated brains reveal FLAG-positive axons. **B.** Induction of vectors at P21, collection at P35. In all conditions, FLAG-positive axons reach corticofugal targets IC, CP and SC. Only, *iFezf2* electroporated brains show axons in the Th, while they are absent in the control and in *iFezf2 & iLmo4* brains. $n = 3$ brains for each plasmid.

Section III.

RNA extraction from UL neurons electroporated with *cGFP*, *cFezf2* or *cFezf2 and cLmo4* to screen for downstream targets *via* RNA-sequencing

As a last step, we were interested in examining downstream targets upon reprogramming UL neurons at embryonic stage E14.5. RNA-sequencing is a common technique to screen for genes showing altered expression levels upon comparing test and control conditions. Thus, finding genes which show up- or downregulation could help us to better identify reprogrammed cells and reveal possible downstream targets of FEZF2 or LMO4.

Therefore, we electroporated embryo brains at 14.5 with either *cGFP*, *cFezf2* or *cFezf2 & cLmo4* and collected brains two days later at E16.5 (see Fig. 30). Subsequently, GFP-positive brains were dissociated and sorted on a FACS- cell sorter device. As a last step, RNA was extracted from these sorted GFP-positive cells.

Unfortunately, the results of this experiment were not available before the submission of this thesis and therefore, I will discuss possible outcomes in the discussion section.

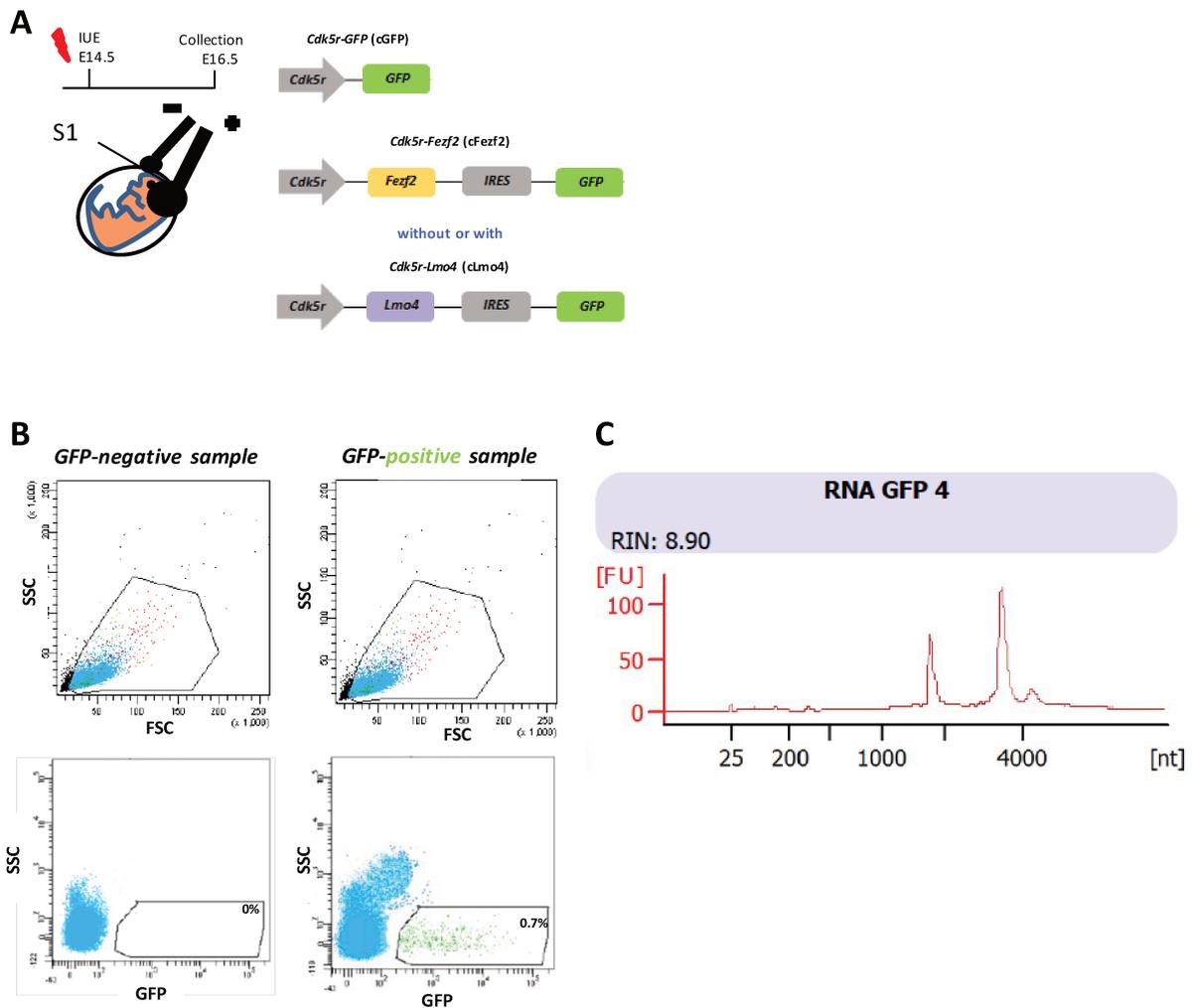


Figure 30: RNA extraction of UL neurons electroporated with cGFP, cFezf2 or cFezf2 and cLmo4 to screen for downstream targets via RNA-sequencing

A. Schematic representation of experimental procedure and used vectors. Embryos were electroporated at E14.5 and their brains collected at E16.5. Then, GFP-positive brains were dissociated and subsequently, GFP-positive cells FACS-sorted. After RNA-extraction, RNA was sent for RNA-sequencing. **B.** Representative FACS results of a GFP-negative sample and a GFP-positive sample after FACS-sorting. Green dots reveal GFP-positive cells. **C.** Representative analysis report of BioAnalyzer for RNA-integrity (RIN) after extraction. RINs higher than 8 are considered as qualitatively sufficient for subsequent RNA-sequencing.

DISCUSSION

I. Induction of *Lmo4* alone fails to efficiently convert post-mitotic UL neurons

At first, we explored whether *Lmo4* alone has the potential to reprogram UL neurons into cells showing characteristic features of CFuPNs.

LMO4 is expressed during early cortical development and is implicated in controlling the diversity of motor cortex projection neuron subpopulations, establishing their area-specific identity and specialized connectivity (Cederquist *et al.*, 2013). Furthermore, it is involved in the delineation of post-mitotic SCPNs and CPNs in L5 of the motor cortex (Azim *et al.*, 2009). More specifically, LMO4 is initially expressed in both post-mitotic SCPNs and CPNs during cortical development. CPNs and SCPNs are two different sub-types of projection neurons in the cortex, defined by their axonal projections. CPNs (mainly located in upper layers, but also present in fewer numbers in lower layers) project within the cortex, while SCPNs (located in L5) project outside the cortex to subcerebral targets. During mid to late differentiation, expression of LMO4 is absent in SCPNs but remains expressed in L5 CPNs, indicating a role in specification of PN-subtypes during development. CPNs and SCPNs have distinct molecular identities, which for instance are revealed by expression of SATB2 or CTIP2, respectively. SATB2 has been shown to be a transcriptional repressor of CTIP2, thereby inhibiting its expression and promoting CPN molecular identity (Alcamo *et al.*, 2008; Britanova *et al.*, 2008). However, our lab has recently demonstrated that both proteins, mostly mutually exclusive during pre-natal stages, are co-expressed in a subpopulation of lower layer neurons from birth to adulthood. This co-expression is enabled via mediation of LMO4, whose expression gradually increases in somatosensory cortex after birth and which sequesters histone-deacetylase HDAC1, thereby de-repressing the SATB2-regulated repression of the *Ctip2*-locus (Harb *et al.*, 2016). Furthermore, overexpression of LMO4 in early post-mitotic L5 neurons increased the number of cells expressing CTIP2, whereas reduction of LMO4 strongly reduced CTIP2-expressing neurons in L5 (Harb *et al.*, 2016). Thus, LMO4 is involved in the epigenetic regulation of *Ctip2* in post-mitotic L5 neurons.

As seminal work demonstrated that upper layer early post-mitotic CPNs can be reprogrammed into CFuPNs by *Fezf2*, including upregulation of CTIP2 expression (Rouaux and Arlotta, 2013), and that *Lmo4* was also able to induce CTIP2 expression in lower layer neurons (Harb *et al.*, 2016), we wondered whether *Lmo4* alone would allow UL neurons (including CPNs and IT

neurons) to subcerebral reprogramming in UL neurons. Overexpression of *Lmo4* revealed neither significant changes in expression of UL neurons marker CUX1 nor in expression of CFuPN markers CTIP2 or PCP4 (see Fig.17). It did however induce FOG2 and DARPP32 in a significant proportion of UL neurons. The number of cells expressing these CFuPN markers was low compared to published data upon *Fezf2* ectopic expression (Rouaux and Arlotta, 2013). Thus, *Lmo4* showed a slight change in the molecular identity in UL neurons but its reprogramming efficiency was low.

Since LMO4 is not a transcription factor binding directly to target genes, but a co-adaptor acting on the epigenetic regulation of chromatin structure, we hypothesized that its transcriptional co-regulation activity was not sufficient to induce an efficient change of molecular identity in post-mitotic UL neurons. Since *Lmo4* enabled de-repression of *CTIP2*-locus and, most probably other targets, the system might need a major determinant factor allowing *Ctip2* induction. The lack of transcription factors promoting lower layer CFuPNs is indeed the case for upper layer neurons born at E14.5, when for instance, FEZF2 or SOX5 transcription factors are not expressed. This could be a possible explanation of why *Lmo4* overexpression in post-mitotic neurons at 13.5, and not at E14.5, leads to an increase of neurons expressing CTIP2. At E13.5 most layer 5 neurons are born and FEZF2 promotes *Ctip2* expression in these neurons. Thus, overexpression of *Lmo4* leads to de-repression of the *Ctip2*-locus in a subpopulation of neurons where *Satb2* is active. Consequently, FEZF2 might take advantage of these open loci and induce expression of *Ctip2*. Another explanation could be that an intermediate reprogramming state, molecular changes are not stable until P7 and thus, cells revert to their original molecular identity. To investigate this possibility, sequential IHC analysis after electroporation could reveal whether a transient reprogramming upon ectopic expression of *Lmo4* is occurring and at what stage it possibly reverts.

In conclusion, *Lmo4* ectopic expression in post-mitotic UL neurons is not sufficient to convert neither UL neurons molecular identity nor their axonal projections.

II. *Lmo4* synergize with *Fezf2* to trigger efficient reprogramming of post-mitotic UL neurons into CFuPNs

As described above, *Lmo4* does not possess the capacity of reprogramming UL neurons on its own, probably due to a lack of TFs acting on opened loci of CFuPN determination genes. Thus, we wondered whether the addition of FEZF2 would enable UL neurons conversion, and, whether FEZF2 could take advantage of LMO4-mediated de-repression of corticofugal determination genes. Our electroporation experiments revealed that *Lmo4* synergizes with *Fezf2* to convert UL neurons into neurons showing molecular identity characteristic of CFuPNs. Almost none of the *Fezf2* & *Lmo4*-electroporated cells showed expression of the upper layer marker CUX1, whereas the majority of UL neurons expressing *Fezf2* & *Lmo4* induced expression of CFuPN markers (**see Fig.19**). These results clearly reflect the developmental role of FEZF2 during newly post-mitotic PNs specification and are in accordance with reported studies about developmental function of FEZF2 (Shimizu and Hibi, 2009; Shimizu *et al.*, 2010; C. Guo *et al.*, 2013; McKenna *et al.*, 2015). The differentiation of these neurons is regulated *via* competing molecular programs. In newly post-mitotic PNs, FEZF2 is inhibiting SATB2, a transcription factor that promotes upper layer neurons. These neurons express CUX1 downstream of SATB2 (McKenna *et al.*, 2015). Thus, ectopic expression of *Fezf2* & *Lmo4* might have led to SATB2 repression, loss of callosal identity, as in the case of *Satb2* KO mice (Leone *et al.*, 2015; McKenna *et al.*, 2015) and, consequently, CUX1 downregulation and CTIP2 upregulation. Thus, *Fezf2* and *Lmo4* ectopic expression in post-mitotic UL neurons allowed CFuPN marker induction. In particular, CTIP2-expression was visible in over 60% of double-electroporated UL neurons, whereas overexpression of only *Fezf2* led to an induction of around 25% (Rouaux and Arlotta, 2013)). During development, *Fezf2* acts upstream of CTIP2, promoting its expression in layer 5 SCPNs (Arlotta *et al.*, 2005; Chen *et al.*, 2008). Thus, ectopic expression of FEZF2 and LMO4 leads to an increased induction of CTIP2 in electroporated UL neurons.

Another CFuPN induced marker upon *Fezf2* & *Lmo4* overexpression in UL neurons was FOG2. This protein is known to be specifically expressed by L6 CThPNs at early postnatal stages (Galazo, Emsley and Macklis, 2016). This was not unexpected, since FEZF2 not only promotes SCPN differentiation, but also has a role in CThPN specification (Shimizu *et al.*, 2010). FEZF2 is

expressed at low levels in CThPNs, contributes to the formation of reciprocal connections between thalamus and cortex but is most probably not highly involved in the specification of CThPNs (Komuta *et al.*, 2007; Chen *et al.*, 2008; Tantirigama *et al.*, 2016). Our data show that *Fezf2* induces the CThPN marker FOG2 in electroporated cells, even though in fewer numbers than CTIP2. This observation could be explained by the minor role FEZF2 plays during CThPN development, compared to SCPN specification. It is known that FOG2 controls CThPN molecular differentiation and axonal targeting, but not cell specification, in part by decreasing CTIP2 expression levels in L6 (Galazo, Emsley and Macklis, 2016). Thus, induced expression of *Fog2* in electroporated cells might counteract *Ctip2* induction in upper layers and might explain why not all cells express CTIP2 upon *Fezf2* or *Fezf2 & Lmo4* misexpression. It is known that ectopic expression of *Fezf2* can induce *Fog2* expression in striatal neurons (Rouaux and Arlotta, 2010) most probably as part of its role in axon guidance, as previously reported (Lodato *et al.*, 2014).

Another CFuPN marker we found ectopically induced in *Fezf2 & Lmo4* electroporated UL neurons is PCP4. The role of PCP4 in the cortex is still not well understood, but its expression profile has been mentioned in some studies. PCP4 has been described to be expressed in deep layer neurons (Watakabe *et al.*, 2012), and to be a late determination gene of corticospinal motor neurons, a sub-type of SCPNs (Arlotta *et al.*, 2005). Interestingly, FEZF2 has been shown to directly bind on the proximal promoter region of *Pcp4* (Lodato *et al.*, 2014), suggesting a regulative role of FEZF2 on *Pcp4* expression. In this work, we show that upon ectopic expression of *Fezf2* and *Lmo4*, a large number of electroporated UL neurons show PCP4 induction, most probably mediated by the activation of *Fezf2*, even if this population increases in the presence of high *Lmo4* expression. We thus propose that the PCP4-induced neuronal population represents a mixture of SCPN and CthPN subpopulations.

At last, induced DARPP32 expression could be observed in reprogrammed UL neurons upon *Fezf2 & Lmo4* overexpression. As for PCP4, DARPP32 expression and function in the cortex is not extensively described in the literature, whereas it is highly expressed in the striatum (Anderson, 1991). It has been reported to be expressed in lower layer 6 at postnatal stages by a *Tbr1*-negative CThPN subpopulation in the mouse somatosensory cortex (McKenna *et al.*, 2011) and in the rat cingulate cortex (Ouimet, 1991). In *Fezf2 KO*, DARPP32 is downregulated in layer 6 (Molyneaux *et al.*, 2005), in agreement with our results in which ectopic *Fezf2*

expression induces DARPP32 expression. However, 41% of electroporated UL neurons co-express CTIP2 and DARPP32 upon *Fezf2 & Lmo4* overexpression in upper layers, similarly to what happened in layer 6 of *Tbr1 KO* mice (McKenna et al., 2011), which acquired a layer 5 SCPN-like fate and suggesting that the presence of *Tbr1* might inhibit DARPP32 in CTIP2+ neurons. However, CTIP2 is weakly expressed in layer 6 CThPNs and ectopic expression into upper layer 2-3 neurons results in the extension of axons to the thalamus (Chen *et al.*, 2008). Thus, ectopic expression of DARPP32 upon *Fezf2 & Lmo4* overexpression might label both layer 5 and 6 sub-populations. Moreover, since DARPP32 is highly and specifically expressed by MSN in the striatum and some PN in lower layers specifically project to the striatum (Shepherd, 2013), we would like to propose that DARPP32 expression might be correlated to PNs projecting to the striatum. Further studies on DARPP32 would be beneficial to better understand its role in layer 6 development during corticogenesis. For example, retrograde labeling of axons in the striatum followed by IHC of DARPP32 in the cortex could give a hint on whether DARPP32 is indeed expressed in corticostriatal PNs.

Taken together, *Fezf2 & Lmo4* possess the capacity of reprogramming UL neurons into CFuPN as revealed by analysis of the molecular identity of electroporated cells. The resulting molecular identity appeared to be heterogenous, with expression of markers known to be specific for different sub-types of SCPNs and CThPNs.

III. *Fezf2 & Lmo4* trigger significant higher numbers of UL neurons to express CTIP2 and PCP4 compared to single *Fezf2*

Ectopic expression of *Fezf2 & Lmo4* in postmitotic UL neurons downregulates the upper layer marker CUX1 expression, while CFuPN markers CTIP2 and FOG2 are induced in electroporated cells. The observations could also be seen in single *Fezf2* ectopic expression in UL neurons. In this study, single *Fezf2* has been used as a positive control, which has been described to induce SCPN molecular identity in post-mitotic UL neurons with an efficiency of around 25% (De La Rossa *et al.*, 2013; Rouaux and Arlotta, 2013). Interestingly, the number of *Fezf2 & Lmo4*-electroporated UL neurons expressing CTIP2 or PCP4 was significantly higher than in single *Fezf2*-electroporated UL neurons increasing to an efficiency of over 60% (see Fig.19) An

explanation could be the function of LMO4 as de-repressor of the CTIP2 locus (Harb *et al.*, 2016), whereby FEZF2 took advantage and induced expression of CTIP2 more efficiently than when *Fezf2* is ectopically induced in the absence of *Lmo4*. It has not been described whether *Lmo4* acts on the PCP4 locus, but our results indicate that LMO4 is implicated in the regulation of PCP4, which could be similar to the de-repressive effect described for CTIP2. This could be examined by chromatin-immunoprecipitation (ChIP) of brain nuclear extracts after electroporation with *Fezf2* & *Lmo4*.

Differently from CTIP2 and PCP4 induction, the number of reprogrammed UL neurons expressing the CThPN markers FOG2 or DARPP32 in *Fezf2* & *Lmo4*-electroporated cortices was similar and not significantly different to electroporation of single *Fezf2*. A possible explanation could be, that *Lmo4* does not have an effect on the induction of factors expressed in CThPNs, in line with its role in driving SCPN from the motor cortex towards the brainstem region (Cederquist *et al.*, 2013). However, the percentages of single *Fezf2*-reprogrammed UL neurons expressing CUX1, CTIP2 or FOG2 in my study differ from published data, obtained by the same experimental strategy (Rouaux and Arlotta, 2013). In our study, the percentage of *Fezf2*-reprogrammed UL neurons expressing CUX1 was three-times less than in Rouaux and Arlotta (2013), while the percentage of *Fezf2*-reprogrammed UL neurons expressing CTIP2 and FOG2 were 1.5-times and almost 8-times higher compared to Rouaux and Arlotta (2013). Most probably, this is due to methodological differences and/or differences in analyzing co-expressions of IHC revealed markers in electroporated cells. This is also why we compared our reprogramming efficiency among our experimental conditions.

Taken together, our study shows that the synergistic combination of *Fezf2* & *Lmo4* triggers CFuPN marker expression in electroporated upper layer post-mitotic UL neurons, revealing conversion of these neurons. In addition, the efficiency of UL neurons inducing CTIP2 or PCP4 was significantly higher compared to single-*Fezf2* reprogramming.

IV. *Fezf2* & *Lmo4* overexpression drive UL neurons to re-route their axonal projections towards corticofugal targets

In addition to the investigation of molecular identity of reprogrammed electroporated UL neurons, it was necessary to follow their axonal projections. After electroporation of the above-mentioned plasmids containing GFP, we could follow their trajectories thanks to the presence of the GFP (see Fig.18, 20). In accordance with the data obtained with the molecular markers, axonal projections of *Lmo4*-electroporated cells maintained projections through the CC and the Striatum (Str), similarly to *Gfp* control-electroporated cortices. This connectivity feature is characteristic of CPNs, the most abundant cell-type of UL neurons. Furthermore, no GFP-positive axons were found in targets of CFuPNs. Thus, *Lmo4* did not alter the molecular identity and axonal trajectories of UL neurons.

This situation differs after induction of *Fezf2* & *Lmo4* electroporated UL neurons, which showed clear projections towards corticofugal targets, with no axons projecting towards the CC. Specifically, reprogrammed UL neurons revealed axonal projections to the Str, the internal capsule (IC), the thalamus (Th), the cerebral peduncle (CP) and the spinal cord (SC). However, we also noticed that some electroporated cells displayed an ectopic location in the lower layers. Since overexpression of *Fezf2* & *Lmo4* expression occurred in post-mitotic neurons during migration, these cells most probably experienced a migratory deficit. Nevertheless, the majority of electroporated cells properly migrated to their location in the upper layers, revealing that *Fezf2* & *Lmo4* led to migration deficits in only a subset of cells. This phenotype was also observed in cortices electroporated with single *Fezf2* and has been reported in literature (Rouaux and Arlotta, 2013), indicating that high expression of *Fezf2* can induce a migratory defect without altering the differentiation process, since abnormally located neurons do express appropriate differentiation markers (Lodato et al., 2011).

Interestingly, the axonal projections of *Fezf2* & *Lmo4* electroporated UL neurons seemed to split up into two separate populations at caudal positions, where Th and IC are visible. Some GFP-positive axons were directed towards the Th, whereas other axons projected within the IC. Whether these are collaterals of the same neuron, or are independent neurons remains to be elucidated. It is known that SCPN have multiple projection targets, including the Th, the striatum, the brainstem region and the spinal cord (Spheperd, 2013). Since the GFP-signal in

even more caudal parts of the brain, at the level of the CP, is similarly strong to the signal in the Th and IC, we propose that they might represent collaterals of GFP-positive axons directed to the Th. This could be examined by lowering the concentration of the plasmids, which would lead to fewer numbers of electroporated cells or combining electroporation with retrograde double tracing in CP and Th. In this way, both populations could be independently labeled and reveal whether projections to the Th are collaterals or independent axons.

Another aspect that should be further investigated is, why there are only few GFP-positive axons reaching the SC. Does the majority of GFP-positive axons stop at a certain level before the SC and if yes, which structure is it? The brainstem is a target of SCPN (Arlotta *et al.*, 2005; Shepherd, 2013) and is a structure that lies just rostral to the spinal cord. It is possible that axons of reprogrammed cells stop at the level of the brainstem and only few continue to the SC. This would imply that the majority of reprogrammed UL neurons turn into SCPNs that project to the brainstem, in line with the role of *Lmo4* driving layer 5 neurons to the brainstem region (Cederquist *et al.*, 2013). Another possibility is, that the GFP signal diminished by distance and only some GFPs are maintained in the very fine axonal endings of electroporated UL neurons in the spinal cord.

The alteration of axonal projection patterns in UL neurons with high *Fezf2* & *Lmo4* expression is similar to CPNs with single *Fezf2*, as also observed by Rouaux and Arlotta (2013). However, the presence of high *Lmo4* expression definitely increased the efficiency of reprogramming, since for example, no contralaterally callosal axons observed in the CC was observed upon *Fezf2* & *Lmo4* electroporation, which is not the case with control GFP-positive and *Fezf2*-electroporated. The above-mentioned results suggest two interpretations. First, *Fezf2* & *Lmo4* have a higher efficiency in reprogramming UL neurons than single *Fezf2*. In line with the observations that the induction of certain CFuPN markers are found in higher numbers of *Fezf2* & *Lmo4* reprogrammed UL neurons than single *Fezf2*, we propose that *Lmo4* drastically enhances the *Fezf2*-driven conversion. Second, the finding that no GFP-positive axons were found in the CC could possibly indicate that all UL neurons underwent reprogramming or partial reprogramming. Possibly, all UL neurons retracted their axonal projections through the CC, but it does not necessarily mean that all converted into CFuPN, resulting in a partial reprogramming. In my study, I could examine the molecular identity of converted UL neurons by using one or maximum two markers in GFP-positive cells. Most primary antibodies used in

this study were produced in the same host (apart from anti-CTIP2) and thus, IHC of multiple CFuPN was in most cases not feasible. Co-expression studies of *Fezf2* & *Lmo4* electroporated cells however indicated that CTIP2 & PCP4, as well as CTIP2 & DARPP32 are not homogeneously expressed in all electroporated cells. Therefore, it is possible that virtually all electroporated UL neurons underwent reprogramming, resulting in expression of distinct pattern of CFuPN markers and different axonal projection re-wiring.

V. The cellular environment of the motor cortex has an impact on reprogramming efficiency of *Fezf2* & *Lmo4*

As a following step, we wondered whether the cellular environment of the motor cortex would impact the reprogramming efficiency of UL neurons into CFuPNs. The motor cortex has a distinct cytoarchitecture with a thick L5 containing a large number of CSMN, whereas the somatosensory cortex possesses a thick L4 where neurons that receive relayed sensory inputs are located and a thin L5 (Dehay and Kennedy, 2007; Tomassy *et al.*, 2010). These differences in the cytoarchitecture can also be seen by the expression of FEZF2 and CTIP2. In the motor cortex, both proteins delineate a much broader and denser L5 than in the somatosensory cortex (Tomassy *et al.*, 2010). Thus, we sought whether the cellular environment of the motor cortex was more instructive for converting UL neurons into CFuPNs. Quantification of the percentage of *Fezf2* & *Lmo4* electroporated cells in the motor cortex revealed a higher number of GFP-positive cells expressing CTIP2 compared to the somatosensory cortex (**see Fig.21**). In addition, also DARPP32 was present in a higher number of *Fezf2* & *Lmo4* electroporated UL neurons in the motor cortex compared to the somatosensory one. Interestingly, the percentage of CUX1-, FOG2- and PCP4-expressing neurons did not differ between motor and somatosensory cortex upon *Fezf2* & *Lmo4* electroporated UL neurons. Furthermore, cortices electroporated with single *Fezf2* did not show significant changes of marker expression in GFP-positive UL neurons when comparing motor and somatosensory cortex. Also, electroporation of *Lmo4* into UL neurons of the motor cortex did not induce any significant CFuPN marker expression as it was the case in the somatosensory cortex. Thus, the cortical environment had only an effect on *Fezf2* & *Lmo4* mediated reprogramming regarding CTIP2+ and DARPP32+ induced neurons. This result was not confounded by a probable higher number of upper layer

cells expressing CTIP2 in the motor cortex, since control GFP electroporated UL neurons did not reveal significant changes in CTIP2 expression.

Taken together, the motor cortex with its thick L5 and its broad CTIP2 expression had a limited, but statistically significant impact on the efficiency of *Fezf2* & *Lmo4* reprogrammed UL neurons, showing higher numbers of reprogrammed UL neurons expressing CTIP2 or DARPP32, while expressions of other markers were not affected. Thus, it could be that the motor area is more instructive to reprogram UL neurons into CFuPNs, indicating an impact of the cellular environment on reprogramming capacities.

VI. Reprogrammed post-mitotic UL neurons maintain expression of CFuPN markers at juvenile-adult stages

It might be possible that reprogrammed UL neurons were not stable and reverted to their original identity or might have died over time. My results showed that this is most likely not the case, since at P35, reprogrammed single *Fezf2* or double *Fezf2* & *Lmo4* UL neurons revealed stable expression of the majority of CFuPN markers (**see Fig.22**). However, DARPP32 was clearly downregulated in both conditions at P35 and maintained its expression in a drastically lower number of converted cells compared to P7. Since other markers remained stably expressed in reprogrammed UL neurons, and since DARPP32 is co-localized with CTIP2 in almost half of the electroporated UL neurons and, since cell death has not been reported for *Fezf2*-reprogrammed CPNs (Rouaux and Arlotta, 2013), it is unlikely that the DARPP32-expressing neurons died or reverted to their original identity. A possible explanation could be either that *Darpp32* was simply downregulated over time, since its function was not needed anymore, or that the axons expressing DARPP32 were pruned over time, or that *Fezf2* and *Lmo4* expression was unable to maintain DARPP32 expression at advanced differentiation stages.

It would also be important to investigate the axonal projections of reprogrammed UL neurons at P35, in order to see whether altered axonal projections can be maintained with time. Unfortunately, the GFP-signal of reprogrammed UL neurons was faint at P35 and thus, axonal trajectories could not be followed anymore. An additional tracing molecule that is stable for long times could overcome this issue and could reveal whether axonal projections of reprogrammed UL neurons remain stable over time.

VII. Postnatal UL neurons retain the capacity to convert their molecular identity

It became important to understand whether reprogramming of UL neurons was possibly not only at pre-natal stages, when neurons are still immature, but also at postnatal ages when neuronal maturation progressively takes place. Thanks to the use of tamoxifen (TAM)-inducible vectors, we were able to overexpress *Fezf2* or *Fezf2&Lmo4* at different postnatal stages (see Fig.24).

Our data show that at P3, induction of *Fezf2* & *Lmo4* in UL neurons of the somatosensory cortex led to expression of FOG2, PCP4 or DARPP32, while CUX1 was downregulated (see Fig. 5B). Surprisingly, at P7, the stage we analyzed the TAM-induced neurons, CTIP2-expression was not induced in electroporated UL neurons. According to the literature, *Ctip2* is an early developmental gene in CSMN, a subgroup of SCPN (Arlotta *et al.*, 2005). Thus, at P3, when UL neurons have positioned in upper layers, the *Ctip2*-locus might not be more accessible in these cells. *Lmo4* has been shown to de-repress SATB2-repressing *Ctip2*-locus, a mechanism that might be limited to early post-mitotic lineage specifications. This was also observed in the study of Rouaux and Arlotta, 2013, upon *Fezf2* overexpression, but the authors did not discuss this issue in their paper. Interestingly, the number of PCP4-positive neurons in *Fezf2* & *Lmo4* targeted UL neurons resulted even higher than after embryonic induction. As a late developmental gene for CSMN (Arlotta *et al.*, 2005), its *mRNA*-expression reaches its highest peak at P3. Thus, the induction of PCP4 in P3 UL neurons might be facilitated due to a more favorable gene accessibility.

Moreover, the efficiency of *Fezf2* & *Lmo4* reprogrammed UL neurons at P3 resulted also higher than single *Fezf2*-driven reprogramming, as observed by the reduced number of CUX1-positive neurons in electroporated UL neurons, as well as by the increased number of PCP4-positive neurons. On the contrary, FOG2- and DARPP32-induced expression diminished and was only expressed in few neurons. These data suggest, that either UL neurons gradually lose their ability to reprogram their molecular identity or, that at P3, reprogramming of UL neurons is restricted to a more limited subpopulation of CFuPNs. Additional CFuPN molecular identity markers, especially the ones that reveal high expression at early postnatal stages, could provide a broader picture at this stage.

1. Addition of inducible *Ctip2* to inducible *Fezf2* & *Lmo4*

Since reprogrammed UL neurons at P3 failed to show any CTIP2-expression, we wondered whether inducing *Ctip2* additionally to *Fezf2* & *Lmo4* would facilitate reprogramming (**see Fig.25A**). Ectopic embryonic CTIP2 expression has been demonstrated to alter axonal projections of upper layer neurons towards corticofugal targets, including Th, CP and pons (Chen *et al.*, 2008). Thus, since CTIP2 ectopic expression showed a similar reprogramming at embryonic stages to *Fezf2* & *Lmo4*, we hypothesized that maintenance of CTIP2 would improve reprogramming. Unfortunately, this was not the case and addition of *Ctip2* to the reprogramming cocktail did not lead to a lower number of CUX1-expressing UL neurons, nor to an increased number of UL neurons expressing PCP4 when compared to *Fezf2* & *Lmo4* reprogramming. It is not known whether other genes are indeed induced, as only a limited number of molecular markers were examined here. Unfortunately, GFP-positive axons were not visible and therefore changes in axonal projections could not be examined. Thus, *Ctip2* does not seem to have a facilitating effect on the reprogramming efficiency induced by *Fezf2* & *Lmo4* at postnatal stages, despite its downregulation.

2. Addition of inducible PBX1 to inducible *Fezf2* & *Lmo4*

We wondered whether other factors would improve reprogramming efficiency at postnatal stages and tested *Pbx1*, as a candidate reprogramming gene. PBX1 is implicated in homeostasis of adult neurogenesis in the SVZ (Grebbin *et al.*, 2016), and has been shown to have features of pioneer factors (Grebbin and Schulte, 2017). Pioneer factors can normally access transcriptionally inactive genomic loci. We hypothesized that *Pbx1* could transcriptionally open inactive genes, allowing a better chromatin accessibility of FEZF2 & LMO4 in inducing CFuPN related genes. The data indicated that the addition of *PBX1* to the reprogramming cocktail did not reveal any drastic changes in expression of molecular markers in electroporated UL neurons (**see Fig.25B**). Again, whether other CFuPN markers were induced is not known from these data. Also, GFP-positive axons were not visible and therefore changes in axonal projections could not be examined. Thus, *Pbx1* does not seem to increase the reprogramming efficiency induced by *Fezf2* & *Lmo4*.

At P7, UL neurons that received *Fezf2* & *Lmo4* still retained the ability to change their molecular identity (see Fig. 6B, C and D). The number of CUX1-expressing neurons in *Fezf2* & *Lmo4* induced UL neurons was significantly lower compared to the control, whereas PCP4-expressing neurons was significantly higher. However, the number of reprogrammed UL neurons was lower compared to *Fezf2* & *Lmo4* induction at P3, suggesting that the cellular plasticity of UL neurons diminishes over time. Not surprisingly, CTIP2-expression was absent and FOG2 expression almost non-detectable in reprogrammed UL neurons. Overall, UL neurons can alter their molecular identity even at later postnatal stages and reveal lower expression of CUX1 and elevated expression of PCP4.

VIII. UL neurons retain their ability to change their axonal projections towards corticofugal targets from P3 to P21

To improve axonal trajectories of reprogrammed neurons, we made use of a high-performance tracer, named 'spaghetti monster' fluorescent protein, which has been reported to be well distributed in neurons and to reveal signals even in few axons (Viswanathan *et al.*, 2015). The protein was encoded by the vector *pCAG_smFP FLAG*, containing multiple FLAG epitopes, which can be revealed by an anti-FLAG antibody (see Fig.26, 28, 29). Induction of *Fezf2* & *Lmo4* at P3 in upper layer UL neurons revealed at P10 FLAG+ callosal axons as well as FLAG+ signal in axons of subcortical structures. This was the case as well for single *Fezf2* induction in P3 UL neurons but with fewer FLAG axons at the level of the IC and CP, indicating a lower efficiency compared to *Fezf2* & *Lmo4*. We noticed that at P3 fewer UL neurons than at E14.5 were re-wired to corticofugal targets. However, it is unclear whether conversion of UL neurons was completed or whether these neurons revealed dual projections, one to the contralateral side and one to corticofugal targets. Throughout development CPNs extend exuberant projections with the maximal number of UL neurons with dual projections occurring at early to late postnatal stages (Innocenti and Price, 2005; Mitchell and Macklis, 2005). These dual projections are refined until approximately P21, when the adult projection pattern for CPNs is established (Fame, MacDonald and Macklis, 2011). It has been reported for layer 5 CPNs that until completion of refinement, some CPNs still extend a bifurcated axon that projects both to the midline and IC, which is then retracted later during development

(approx. P11) (Garcez *et al.*, 2007). Thus, it could be that *Fezf2* or *Fezf2 & Lmo4*-induced UL neurons at early postnatal stages mimic this transient state of L5 dual projection neurons.

1. *Fezf2 & Lmo4* induction at P7 and P10 still enable partial re-routing of UL neuron axons

By inducing *Fezf2 & Lmo4* at P7 or at P10, we still observed FLAG signal in the CC and in all examined corticofugal structures at P14 and P21, respectively, as previously described for P3. Interestingly, single *Fezf2* induction at P7 failed to reveal any FLAG signal in the SC, indicating that single *Fezf2* overexpression is not sufficient to re-route UL neuron axons to the SC. We noticed that the number of FLAG axons steadily decreased from P3 to P7 and then to P10, suggesting that UL neurons gradually lose their capacity of re-wiring. However, it cannot be excluded that these axons might need longer to reach the SC. Increasing the time window from the onset of TAM administration and brain analysis could resolve this question.

2. *Fezf2 & Lmo4* induction at P21

Induction of *Gfp*, single *Fezf2* or *Fezf2 & Lmo4* at P21 revealed a different observation than the previous experiments. In all conditions, FLAG axons were present in almost all corticofugal structures. Presumably, at P35 the complexity of axonal branching is higher and thus, easier to visualize than at earlier postnatal stages. Nevertheless, the FLAG signal in these subcortical structures was clearly higher in single *Fezf2* or *Fezf2 & Lmo4* conditions. This indicates that at P21, some UL neurons still retain the capacity of re-wiring their axonal projections.

To conclude, until P21, UL neurons still retain the capacity of re-routing their axonal projections.

IX. RNA extraction of early post-mitotic electroporated UL neurons

As a last step, we aim to further investigate the molecular changes that occur in reprogrammed UL neurons at embryonic stages by using high throughput *RNA* sequencing (see Fig.30). E14.5 electroporated brains with control *GFP*, *Fezf2*, or *Fezf2 & Lmo4* were dissociated and FACS-sorted at E16.5 in order to identify early and most probably direct molecular changes upon induction. Bulk *RNA*-sequencing is the method of choice for analyzing transcriptomes of disease states or of defined biological processes by quantifying gene

expression of different biological samples. Thus, mRNA-sequencing of our samples, *Gfp*, *Fezf2* or *Fezf2 & Lmo4*-electroporated neurons, will give us a more complete picture of changes in gene expression upon reprogramming at this early stage.

I expect to see elevated levels of genes that are specifically expressed by CFuPNs and downregulated gene-levels that are specifically expressed by UL neurons. Furthermore, interesting genes could be discovered, for example, genes that are unknown for their role in CFuPN specification. Additionally, *mRNA*-sequencing could help us to find downstream targets of *Fezf2* in synergy with *Lmo4*. I would then like to use some interesting genes and try to find out whether induction of these genes would lead to reprogramming of UL neurons. Lastly, it would be interesting to see which genes are differently expressed in *Fezf2 & Lmo4* UL neurons compared to single *Fezf2*. These discoveries could help us to find out why *Lmo4* facilitates *Fezf2*-reprogramming and thus could reveal targets or pathways that might be combined with *Fezf2 & Lmo4* for even higher reprogramming efficiency.

CONCLUSION

The work of my thesis demonstrates that callosal intratelencephalic neurons in cortical upper layers can be reprogrammed into neurons, showing molecular identity, as well as connectivity features, characteristic of corticofugal projection neurons, at both embryonic and early postnatal developmental stages in the mouse. Upon induction of *Fezf2*, - a transcription factor known for its role in specification of lower layer 5 SCPN and layer 6 CThPNs during cortical development - the number of UL neurons expressing CUX1 is reduced at the expense of neurons expressing lower layer markers. Additionally, at embryonic and early postnatal stages, reprogrammed UL neurons also show axonal projections towards corticofugal targets, such as the thalamus and spinal cord. Addition of *Lmo4* - a nuclear co-adaptor that is implicated in the epigenetic regulation of gene transcription and specification of post-mitotic neurons – to *Fezf2* drastically increased the number of cells expressing a subset of lower layer markers and re-routing to corticofugal targets. Furthermore, *Fezf2* & *Lmo4* induction in UL neurons allows these cells to project to the spinal cord until postnatal day P10. This was not the case for single *Fezf2*-induction, as induction in UL neurons did not lead to axonal projections to the SC after P3. Partial re-wiring of reprogrammed neurons was observed until P21, indicating a higher plasticity of cortical neurons than previously expected.

Overall, this work characterized a potent reprogramming cocktail, *Fezf2* & *Lmo4*, that unveiled efficient conversion of UL neurons into CFuPNs and extended the temporal window of UL neuron reprogramming, compared to previous published data. Thus, these results contribute to a deeper understanding of cortical neuron plasticity which can be exploited in direct neuronal reprogramming *in vivo*.

PERSPECTIVES

I. To further characterize reprogrammed UL neurons

These data showed changes in molecular identity and alterations in axonal projections of reprogrammed UL neurons. It would be crucial to further investigate neuronal and molecular properties of these cells to obtain a more thorough characterization of these reprogrammed cells. For instance, analysis of the morphology, electrophysiological properties and functional integration of these neurons could give us more information about their cell identity. L5 neurons possess an apical dendrite that bifurcates in L2/3, a feature which is different from upper layer PNs (Lefort *et al.*, 2009). Thus, investigating whether the morphology of reprogrammed UL neurons resemble such a feature could broaden our identity characterization. Furthermore, investigating a functional input onto reprogrammed UL neurons could reveal whether these neurons are communicating with other neurons. This could be performed by stimulating neighbouring upper layer neurons and recording possible responses in reprogrammed UL neurons.

II. To investigate reprogramming in other cell-types

It would be interesting to explore whether our factors could possibly reprogram other cell types such as astroglia or fibroblasts. This would give us a hint whether *Fezf2* & *Lmo4* are able to convert cells from a less related lineage and would open up further possibilities. Reprogramming neurons into other neurons of a specific cell-type is challenging and come with drawbacks. Upon converting a neuron, the subsequent function of the original cell is lost and thus, finding other cell-types such as NG2-cells or astrocytes could circumvent loss of neuronal function upon reprogramming. Moreover, conversion of cells *in vitro* into CFuPNs could pave the way for subsequent transplantation studies.

III. To increase reprogramming efficiency at postnatal stages

It has been reported by other studies that small molecules or chromatin remodelling factors could facilitate reprogramming approaches (Torper and Gotz, 2017). Thus, it would be interesting to examine whether the addition of interesting candidate could improve the reprogramming efficiency of late postnatal or mature UL neurons. Reprogramming of cortical PNs in the adult brain has not been reported so far. However, it is a goal for regenerative

medicine, as most patients suffering from e.g. ALS are adult (ALS Association). Thus, reprogramming neurons at adult stages would be a beneficial feature that could possibly serve as an alternative approach in restoring CMNs in patients in the future.

IV. To apply possible downstream targets of FEZF2 revealed by *mRNA-sequencing*

As soon as we will obtain our data from the *mRNA-sequencing* experiment, possibly interesting candidates could be used for further applications revealing a possible enhancement of UL neuron reprogramming. Furthermore, we could identify other downstream targets of FEZF2 and discover, which genes are particularly targeted by LMO4 in combination with FEZF2. Another possibility could be that we find an interesting molecule that could serve as a potential drug to specifically convert somatic cells into CFuPNs. This would ease the application for studies on patient-derived cells without the necessity of genetically modifying cells. Furthermore, *Lmo4* is implicated in cancer formations (Visvader *et al.*, 2001; N. Wang *et al.*, 2004; Singh *et al.*, 2005) and has been reported as an oncogene. Finding a candidate that could replace LMO4 would therefore be beneficial, regarding any possible clinical studies.

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Abstract

The mammalian neocortex is tangentially organized into functional areas and radially subdivided into six layers of neuronal populations with distinct projections, morphology and expression patterns. While neurons in the upper layers project within the neocortex or towards the contralateral hemisphere, such as callosal projection neurons, most lower layer neurons, named corticofugal projection neurons, innervate subcerebral targets via the corticospinal tract. Injuries or loss of these neurons can lead to severe neurological disorders, such as Amyotrophic Lateral Sclerosis or other diseases and/or lesions implicating the corticospinal tract. Since the adult mammalian brain lacks a significant regenerative strategy and thus, damage or loss of neurons is permanent, methods for restoring neurons are urgently needed.

Recent studies revealed an unexpected plasticity of post-mitotic neurons, which can be converted into cell-types of other neuronal lineages. This approach, called direct neuronal reprogramming, bypasses an intermediate pluripotent state. Direct reprogramming is fast, likely to keep epigenetic hallmarks and it can be conducted *in vivo*. Forced expression of transcription factors, known to act as master regulators of cell fate during development, is one of the best strategies for directly converting one cell-type into another one. However, it is still not clear to which extent neurons, generated by direct reprogramming, acquire authentic molecular signatures of the desired cell type, down to the point of precise subtype specification. The transcription factor FEZF2 is known for its role in cell fate specification of layer 5 subcerebral projection neurons and high ectopic *Fezf2* expression can convert upper layer or striatal neurons into a corticofugal fate, even if at low efficiency.

During my PhD thesis, I used *Fezf2* and the nuclear co-adaptor *Lmo4* to efficiently reprogram upper layer projection neurons into corticofugal lower layer projection neurons, at both, embryonic and postnatal stages. The successful reprogrammed cells downregulated upper layer markers, while lower layer markers were drastically increased. Additionally, by using advanced tracing methods, we showed that reprogrammed neurons projected towards subcerebral targets, including the thalamus, cerebral peduncle and spinal cord. These data demonstrate an unexpected synergistic role of *Lmo4* with *Fezf2* in neuronal reprogramming and reveals an effective cocktail for the conversion of neuronal cells into corticofugal neuronal subtypes.