MOLECULAR AND CELLULAR DEVELOPMENT OF CORTICAL PROJECTION NEURONS: SPECIFICATION, DIVERSITY, AND DIRECTED DIFFERENTIATION FROM ENDOGENOUS PROGENITORS FOR FUNCTIONAL CIRCUIT REPAIR

by

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ABSTRACT

MOLECULAR AND CELLULAR DEVELOPMENT OF CORTICAL PROJECTION NEURONS: SPECIFICATION, DIVERSITY, AND DIRECTED DIFFERENTIATION FROM ENDOGENOUS PROGENITORS FOR FUNCTIONAL CIRCUIT REPAIR

Responsible for perception, integration of sensory information, cognitive function, motor control, and consciousness, the complex, yet highly organized, six-layered mammalian neocortex contains distinct classes of neurons. Specific subtypes of cortical projection neurons are selectively vulnerable in distinct neurodegenerative, developmental, and acquired diseases of the central nervous system (CNS), resulting in irreversible functional deficits. Evidence for the existence of progenitors in restricted regions of the adult brain, and integration of new neurons into preexisting neural circuitry, support the feasibility of cellular repair in the CNS. However, functional repair of diseased or injured neuronal circuitry requires detailed understanding of molecular controls over development of neuronal lineages, and manipulation of these controls in progenitors to direct the differentiation of functional neurons with appropriate identity, maturity and circuit connectivity. In this study, I target endogenous cortical progenitors present in postnatal and adult brain to direct their differentiation into corticofugal projection neurons. Application of a select combination of central and complementary transcriptional controls, Ngn2, VP16:Olig2 and Fezf2, in cultured cortical Sox6+/NG2+ progenitors directs acquisition of cardinal morphological, molecular, and electrophysiological features of corticofugal projection neurons. These findings demonstrate the feasibility of achieving subtype-specific differentiation of cortical projection neurons from a widely distributed *in vivo* neocortical progenitor population. Further, in the framework of this thesis, I describe the ongoing effort to identify key molecular controls over development, diversity and connectivity of corticostriatal projection neurons, which would serve as a solid step toward achieving a holistic view of the establishment of corticostriatal circuitry and its potential dysgenesis in disease.

ÖZET

KORTİKAL PROJEKSİYON NÖRONLARIN MOLEKÜLER VE HÜCRESEL GELİŞİMİ: ÖZELLEŞMELERİ, ÇEŞİTLİLİKLERİ VE SİNİR AĞLARININ İŞLEVSEL TAMİRİ İÇİN ENDOJEN ÖNCÜL HÜCRELERDEN YÖNLENDİRİLMİŞ FARKLILAŞMALARI

Algı, duyusal bilginin entegrasyonu, bilişsel işlevler, hareket kontrolü ve bilinçten sorumlu olan neokorteks, çok karmaşık olmasıyla birlikte son derece organize altı katmanlı yapısıyla birbirinden çok farklı nöron grupları içerir. Kortikal projeksiyon nöronlarının belirli alt-tipleri merkezi sinir sisteminin birçok nörodejeneratif, gelişimsel ve edinilmiş hastalıklarında seçici olarak zarar görerek beyinde kalıcı hasar bırakırlar. Yetişkin beyninin sınırlı bölgelerinde öncül hücrelerin varlığı ve bu öncül hücrelerden oluşan yeni nöronların mevcut sinir ağlarına entegrasyonu, merkezi sinir sisteminin hücresel tamirinin uygulanabilir bir yöntem olduğunu desteklemektedir. Bununla birlikte yaralanma ve hastalıklar dolayısıyla zarar gören sinir ağlarının işlevsel tamiri, özellikle farklı nöron gruplarının erken gelişimlerini düzenleyen moleküler kontrolleri derinlikli olarak anlamayı ve bunları öncül hücrelerde etkinleştirerek özgün kimlikleri olan olgunlaşmış ve sinir ağlarına bağlantı yapma kapasitesi olan işlevsel nöronlar oluşturmayı gerektirir. Bu tez çerçevesinde, postnatal ve yetişkin beyninde bulunan kortikal öncül hücrelerin kortikospinal projeksiyon nöronları da kapsayan kortikofugal projeksiyon nöronlara deneysel olarak dönüştürülmesi üzerinde çalıştım. Özgün seçilmiş merkezi ve birbirini tamamlayıcı bir dizi transkripsiyon kontrolünün (Ngn2, VP16: Olig2 ve Fezf2), kültürdeki kortikal Sox6+/NG2+ öncül hücrelere uygulanması, bunların kortikofugal hücrelerin çok önemli morfolojik, moleküler ve elektrofizyolojik özelliklerini kazanmasını sağlamaktadır. Bu bulgular, neokortekste yaygın olarak bulunan öncül hücrelerin belirli kimlikleri ve özgün alt-tipleri olan kortikal projeksiyon nöronlara dönüştürülebilir olduğunu göstermektedir. Ayrıca, bu tez çerçevesinde, kortikostriatal projeksiyon nöronların gelişimi, çeşitliliği ve mevcut sinir ağlarına bağlantı kapasitelerini oluşturmak için gerekli ana moleküler kontrol mekanizmaları üzerinde süregelen çalışmalarımı da anlatıyorum. Bu bulgular kortikostriatal sinir ağlarının oluşumu ve hastalık durumunda olası disgenezine bütüncül olarak bakabilmek için sağlam bir zemin oluşturacaktır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS/ABBREVIATIONS

2A	2A co-translational cleavage peptide
3-D	Three Dimensional
5-HT	5-hyrdoxytryptamine (Serotonin)
A1	Primary Auditory Cortex
AAV	Adeno-Associated Virus
ABI	Allen Brain Institute
ACSF	Artificial Cerebrospinal Fluid
ACTB	Actin, beta
ALS	Amyotrophic Lateral Sclerosis
APV	DL-2-amino-5-phosphonopentanoic acid
B27	culture medium supplement
BAC	Bacterial Artificial Chromosome
Bcl11a	B-Cell CLL/Lymphoma 11A (=Ctip1)
Bcl11b	B-Cell CLL/Lymphoma 11B (=Ctip2)
BDNF	Brain-Derived Neurotrophic Factor
BGEM	St. Jude Brain Gene Expression Map
bHLH	basic Helix-Loop-Helix
Bhlhb5	Basic Helix-Loop-Helix Domain Containing, Class B, 5
BMP	Bone Morphogenetic Protein
BMP4	Bone morphogenetic protein 4
BrdU	Bromo-deoxy Uridine
Brn2a	Brain-Specific Homeobox/POU Domain Protein 2a
BSA	Bovine Serum Albumin
C57BL/6	C57 black 6 mouse strain
$CaCl_2$	Calcium Chloride
CalB	Calbindin (Calb1)
CalR	Calretinin (Calb2)
Cas9	CRISPR associated protein 9 (RNA-guided DNA endonuclease)
Cb	Cerebellum
CBD	Corticobasal degeneration

CBuPN	Corticobulbar Projection Neurons
CA1-3	Cornus Ammonis Area 1-3 (portion of the hippocampal formation)
CalB	Calbindin neurons
CalR	Calretinin neurons
CC	Corpus Callosum
CCD	Charge-Coupled Device
CD1	Caesarean Derived-1 (mouse line)
CD44	CD44 antigen, a cell-surface glycoprotein
CFuPN	Corticofugal Projection Neurons
Cited2	Cbp/P300-Interacting Transactivator, With Glu/Asp-Rich Carboxy-Terminal Domain, 2
CNS	Central Nervous System
CO_2	Carbon dioxide
COUPTF1	Chicken Ovalbumin Upstream Promoter-Transcription Factor 1
CPN	Callosal Projection Neurons
CR	Cajal-Retzius Neurons
CRB	Cerebellum
Cre	Cre recombinase from bacteriophage P1
Crim1	Cysteine Rich Transmembrane BMP Regulator 1
CRISPR	Clustered regularly interspaced short palindromic repeats
Crym	Crystallin, Mu
CSMN	Corticospinal Motor Neurons
Cspg4	Chondroitin Sulfate Proteoglycan 4
CST	Corticospinal tract
CStrPN	Corticostriatal Projection Neurons
CStrPNi	Corticostriatal Projection Neurons (intra-telencephalic subtype)
CStrPNp	Corticostriatal Projection Neurons (pyramidal subtype)
CTB	Cholera Toxin, B subunit
CThPN	Corticothalamic Projection Neurons
Ctip1	COUP-TF-Interacting Protein 1
Ctip2	COUP-TF-Interacting Protein 2
Ctsz	Cathepsin Z
Ctx	Cortex

Cux1/2	Cut-Like Homeobox 1/2
Cxcl12	Chemokine (C-X-C Motif) Ligand 12
Cxcr4	Chemokine (C-X-C Motif) Receptor 4
DAPI	4′, 6-diamidino-2-phenylindole
DARPP-32	Dopamine And CAMP-Regulated Neuronal Phosphoprotein 32
Dbx1	Developing Brain Homeobox 1
Dcx	Doublecortin
Deptor	DEP Domain Containing MTOR-Interacting Protein
DG	Dentate Gyrus
DIV	Days-in-vitro
Dlx1/2	Distal-Less Homeobox 1/2
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribo Nucleic Acid
DNAase	Deoxyribonuclease
DPI	Days-Post-Induction
DPT	Days-Post-Transfection
DsRed	Discosoma species Red Fluorescent Protein
E	Embryonic Day
EGF	Epidermal Growth Factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Emx1/2	Empty Spiracles Homeobox 1/2
ENVOF	EGFP-2A-Ngn2-2A-VP16Olig2-2A-Fezf2
EPL	External Plexiform Layer
ES	Embryonic Stem Cells
etc	et cetera (and other things)
F12	Culture medium nutrient mix
FACS	Fluorescence-Activated Cell Sorting
Fezf2	Forebrain embryonic zinc finger-like protein 2
FGF	Fibroblast Growth Factor
Fgf2	Fibroblast Growth Factor 2
Fog2	Friend of GATA protein 2
Foxg1	Forkhead Box G1
GABA	gamma-Amino Butyric Acid

GAD1/GAD67	Glutamate Decarboxylase 1 (Brain, 67kDa)
GC	Granule Cells of the olfactory bulb
GENSAT	Gene Expression Nervous System Atlas Project
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GL	Glomerular Layer
GlutaMAX	Culture medium supplement, alternate to L-glutamine
GN	Granular Neurons
GRL	Granular Layer of the Olfactory Bulb
Gsx2	Genetic-screened homeobox 2
HA tag	Human influenza hemagglutinin amino acids 98-106 (epitope tag)
HCN	Hyperpolarization-activated cyclic nucleotide-gated
HD	Huntington's Disease
HDAC	Histone Deacetylase
HEK293T	Human Embryonic Kidney 293 Cells, with SV40 large T-antigen
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGFAP	human Glial Fibrillary Acidic Protein
HMGA2	High-Mobility Group A
HSP	hereditary spastic paraplegia
HTT	Huntingtin protein
ICC	Immunocytochemistry
IGF-1	Insulin-Like Growth Factor 1
Igf2bp1-3	Insulin-Like Growth Factor 2 mRNA Binding Protein1-3
Ih	current through HCN channels
iNs	induced Neurons
IP	Intermediate Progenitors
IPL	Internal Plexiform Layer
iPS	induced Pluripotent Cells
ISH	in situ hybridization
Isl1	ISL LIM Homeobox 1
KCl	Potassium Chloride
Klh14	Kelch-like family member 14
kHz	kilo Hertz

Ki67	antigen Ki67, marker of proliferation
$KMeSO_3$	Potassium Methanesufonate
LGE	Lateral Ganglionic Eminence
LGN	Lateral Geniculate Nucleus
Lhx2	LIM Homeobox 2
LIM	Lin11, Isl1, Mec3
Lmo4	LIM Domain Only 4
lv	lateral ventricle
M1	Primary Motor Cortex
MAP2	Microtubule-Associated Protein 2
Mash1	Mammalian Acheate-Scute Homolog 1
Mg-ATP	Adenosine 5′-triphosphate, magnesium salt
MgCl2	Magnesium Chloride
MGE	Medial Ganglionic Eminence
MgSO4	Magnesium Sulphate
ML	Molecular Layer
mM	millimolar
modRNA	Synthetic chemically modified mRNA
mRNA	messenger Ribonucleic Acid
ms	milliseconds
MSN	medium Spiny Neurons
mV	millivolt
Myt1l	Myelin Transcription Factor 1-Like
N2	culture medium supplement
N2 Max	culture medium supplement
Na-GTP	Guanosine 5′-triphosphate, sodium salt
NaCl	Sodium Chloride
Na_2SO_4	Sodium Sulfate
NaH_2PO_4	Sodium Dihydrogen Phosphate
$NaHCO_3$	Sodium Bicarbonate
NE	Neuroepithelial cells
NeuN	Neuronal Nuclei Antigen
NeuroD1	Neuronal differentiation 1

NF-M	Neurofilament, Medium Polypeptide
NG2	Neuron-glial antigen 2
Ngn2/Neurog2	Neurogenin 2
Nhlrc1	NHL Repeat Containing E3 Ubiquitin Protein Ligase 1
Nkx2.1	NK2 Homeobox 1
NSC	Neural Stem Cells
O2	Oxygen
OB	Olfactory Bulb
Olig2	Oligodendrocyte lineage transcription factor 2
OPC	Oligodendrocyte Progenitor Cells
oRG	outer Radial Glia
OT	Optic Tectum
Otx1	Orthodenticle Homeobox 1
P	Postnatal day
pA	pico Ampere
Pax6	Paired-box 6
PBS	Phosphate Buffered Saline
pCBIG	pCL-CMV beta-Actin IRES EGFP vector
PCP4	Purkinje Cell Protein 4
PDGF-A	Platelet-Derived Growth Factor Alpha Polypeptide
PDGFRβ	Platelet-Derived Growth Factor Receptor, Beta Polypeptide
PDL	Poly D-lysine
PGC	Periglomerular Cells
PLS	primary lateral sclerosis
PN	Projection Neurons
Po	Pons
PSA-NCAM	Polysialylated-Neural Cell Adhesion Molecule
PSB	Pallial-Subpallial Boundary
rAAV2/1	recombinant AAV, serotype 2/1
Rbp4	Retinol Binding Protein 4, Plasma
RG	Radial Glia
RMS	Rostral Migratory Stream
RNA	Ribonucleic Acid

ROSA	Reverse Orientation Splice Acceptor (genomic locus)
Ryk	Receptor-Like Tyrosine Kinase
S1	primary somatosensory cortex
SD	Standard Deviation
s.e.m	standard error of mean
S100A10	S100 Calcium Binding Protein A10
S100B	S100 Calcium Binding Protein B
Sag	Sagittal
Satb2	Special AT-Rich Sequence-Binding Protein 2
SC	Spinal cord
SCPN	Subcerebral Projection Neurons
SGZ	Subgranular Zone
SHH	Sonic Hedgehog
shRNA	small hairpin Ribonucleic Acid
SMN	spinal motor neurons
SOD1	Superoxide dismutase 1
Sox10	SRY (Sex Determining Region Y)-Box 10
Sox2	SRY (Sex Determining Region Y)-Box 2
Sox5	SRY (Sex Determining Region Y)-Box 5
Sox6	SRY (Sex Determining Region Y)-Box 6
SP	Subplate
SPN	Subplate Neurons
Spon1	Spondin 1, Extracellular Matrix Protein
Str	Striatum
SVZ	Subventricular Zone
T3	Triiodothyronine
Tbr1	T-Box, Brain, 1
Tbr2	T-Box, Brain, 2
tdTomato	tandem dimer of Tomato red fluorescent protein
TGF	Transforming Growth Factor
TH	Tyrosine Hydroxylase
Tlx3	T-cell leukemia homeobox 3
Tox	Thymocyte Selection-Associated High Mobility Group Box

UTR	Untranslated Region
V1	Primary Visual Cortex
VEGF	Vascular Endothelial Growth Factor
vGlut1	Vesicular Glutamate Transporter 1
VL	Ventral Lateral (thalamic nuclei)
VP	Ventral Posterior (thalamic nuclei)
VP16	Herpes Simplex Virus virion protein 16
VZ	Ventricular Zone
Wnt	Wingless-type MMTV integration site family member
WM	White matter
ZsGreen1	Zoanthus species green fluorescent protein
μm	micrometers
μg	micrograms
μl	microliters

1. INTRODUCTION

1.1. Neuronal Diversity in Mammalian Neocortex

The mammalian cerebral cortex contains diverse populations of distinct neuronal and glial cells that collaboratively organize the complex cognitive and behavioral activity of the organism. There are two broad classes of neurons in the cortex; 1) glutamatergic projection neurons, which transfer information across distant regions within and outside of the cortex by long-distance axonal connections, and 2) GABAergic inhibitory interneurons, which modulate neuronal activity by local connections (Figure 1.1) (Molyneaux *et al.*, 2007; Greig *et al.*, 2013).

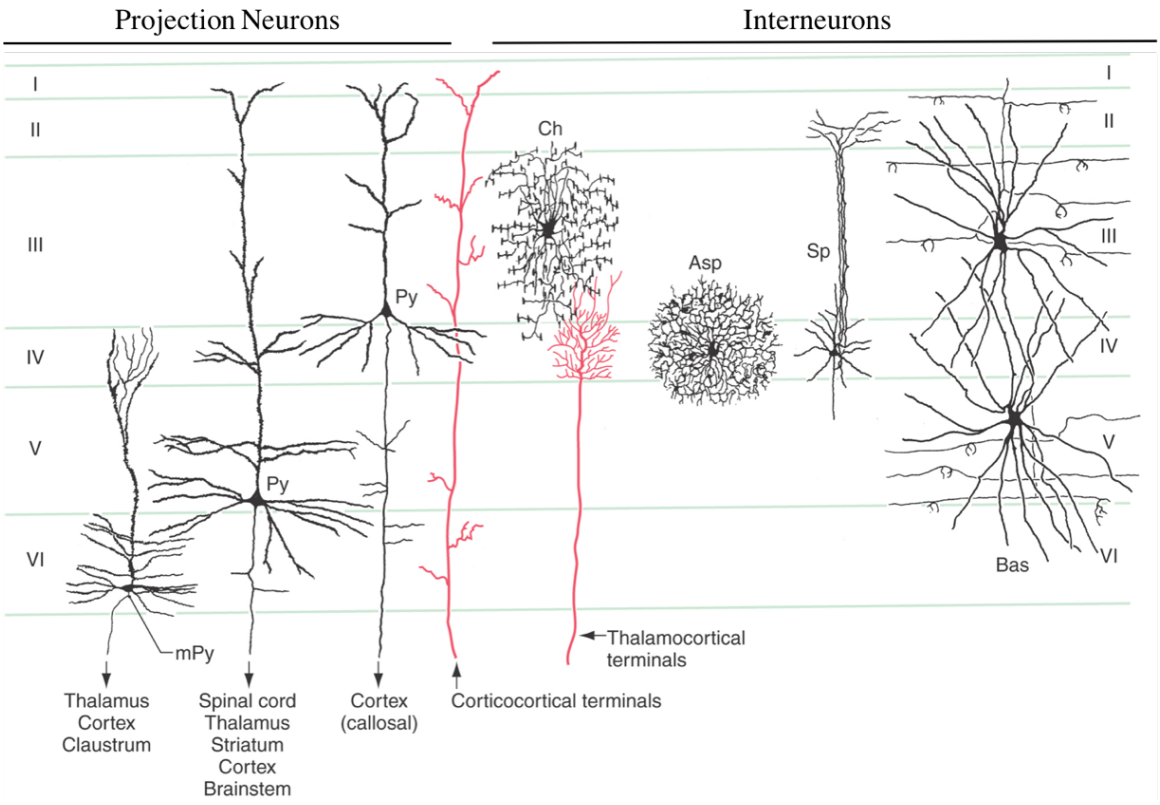

Figure 1.1. Representative images of two neuron types in the cerebral cortex; projection neurons with long axons conveying information to distant targets, and distinct subclasses of interneurons, modulating neuronal activity with local connections (Lynch, 2012).

Cortical interneurons, which are not the focus of this thesis study, are highly heterogeneous by any categorization, but are usually classified either based on specific expression of neurotransmitters and other functional molecules including parvalbumin, somatostatin, neuropeptide Y, calretinin, vasoactive intestinal peptide, etc., and/or by morphology (Wonders and Anderson, 2006).

Projection neurons differ from one another in various features including cellular morphology, laminar and anatomical position, patterns of connectivity, electrophysiological and neurochemical properties, and ultimately the function they serve. Though such diversity makes any classification challenging, 'hodology', the study of paths taken by axons to reach their targets, is the most practical and widely used approach for categorizing cortical projection neurons, in part because hodology can also be regarded as a manifestation of biological function (Migliore and Shepherd, 2005; Woodworth *et al.*, 2012; Greig *et al.*, 2013; Wichterle *et al.*, 2013).

Based on hodology, neocortical projection neurons are classified into three broad groups: associative, commissural, and corticofugal (Figure 1.2). 1) Associative projection neurons extend their axons unilaterally, either to the same or adjacent cortical columns, or to the neighboring cortical areas within the same hemisphere. 2) In contrast, commissural (callosal) projection neurons (CPN) extend their axonal projections to the homotopic regions of the contralateral hemisphere via the corpus callosum or the evolutionarily older tract, the anterior commissure. 3) As a divergent population, corticofugal projection neurons (CFuPN), also called cortical output neurons, extend axons away from the cortex and make long distance projections to subcortical targets. CFuPN is a broad class of neurons that include corticothalamic projection neurons (CThPN) that project to the thalamus; and subcerebral projection neurons (SCPN) that project below the cerebrum to the midbrain (e.g. corticotectal PNs), the brainstem (e.g. corticopontine), and to different levels of the spinal cord (corticospinal motor neurons, CSMN) (Greig *et al.*, 2013).

Within these main groups, researchers have identified neuronal subgroups with multiple long projections that fall into more than one category (Figure 1.2). These include interhemispheric corticostriatal projection neurons (CStrPNi), which extend axons contralaterally to both cortex and striatum of basal ganglia; callosal projection neurons with

secondary ipsilateral forward or backward projections; and subcerebral projection neurons with secondary forward or backward branches to caudal areas of cortex ipsilaterally (Mitchell and Macklis, 2005; Fame *et al.*, 2011; MacDonald *et al.*, 2013; Sohur *et al.*, 2014).

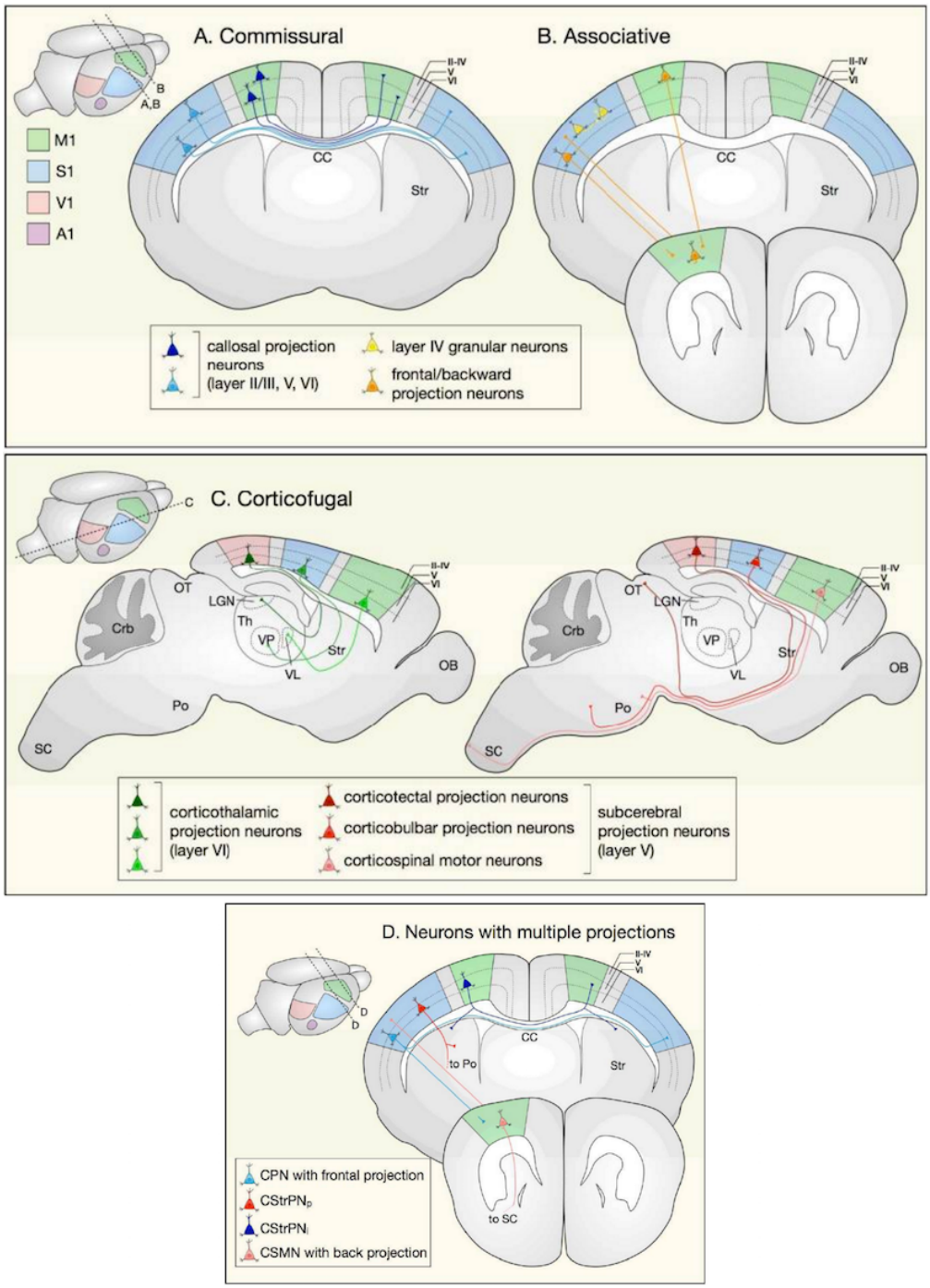

Figure 1.2. Major subtypes of projection neurons of the neocortex. Callosal PN project to the contralateral cortex, associative PN with ipsilateral cortical axons, corticofugal PN with axons to subcortical targets, and neurons with multiple projections (Greig *et al.*, 2013).

1.2. Cortical Development

1.2.1. Patterning of Telencephalon

During central nervous system (CNS) development, a diverse range of neuronal and glial subtypes with distinct functional, morphological, and biological properties are generated via synchronous action of extracellular morphogen gradients as well as intracellular molecular programs, some of which are induced/maintained by these gradients. During early embryogenesis, overlapping dorso-ventral and rostro-caudal gradients of diffusible morphogens (e.g. SHH, FGFs, BMPs, Wnts) pattern the neural tube. Around embryonic day (E) 9, the anterior region of the neural tube becomes the telencephalon or the 'end-brain', from which neocortex will later develop. Subsequently, by combinatorial and cross-repressive interactions of many transcription factors, the telencephalon is patterned into two progenitor domains (Figure 1.3); the pallium (dorsal domain) and the subpallium (ventral domain), which will eventually give rise to excitatory projection neurons and inhibitory interneurons, respectively (Jessell and Sanes, 2000; Rallu *et al.*, 2002; Rash and Grove, 2006; Azim *et al.*, 2009a).

During the patterning process (Figure 1.3), the pallial transcription factors Pax6 and Sox6 specify the dorsal progenitor identity, and via cross-repressive interaction with their ventral counterparts Gsx2 and Sox5, establish the pallial-subpallial boundary (Azim *et al.*, 2009a). In addition, proneural genes Ngn2 (a transcription factor activated by Pax6) and Mash1 (a transcription factor activated by Gsx2) suppress each other's expression in pallial and subpallial progenitors, respectively, and thereby further help to establish the molecularly distinct pallial and subpallial proliferative zones. Establishment of pallial and subpallial domains with progenitors specified to generate neurons with distinct fates is a crucial step towards generating of a diverse range of excitatory and inhibitory neuronal subtypes thereafter (Casarosa *et al.*, 1999; Yun *et al.*, 2001; Parras *et al.*, 2002; Schuurmans and Guillemot, 2002; Hevner, 2006; Quinn *et al.*, 2007; Azim *et al.*, 2009a) .

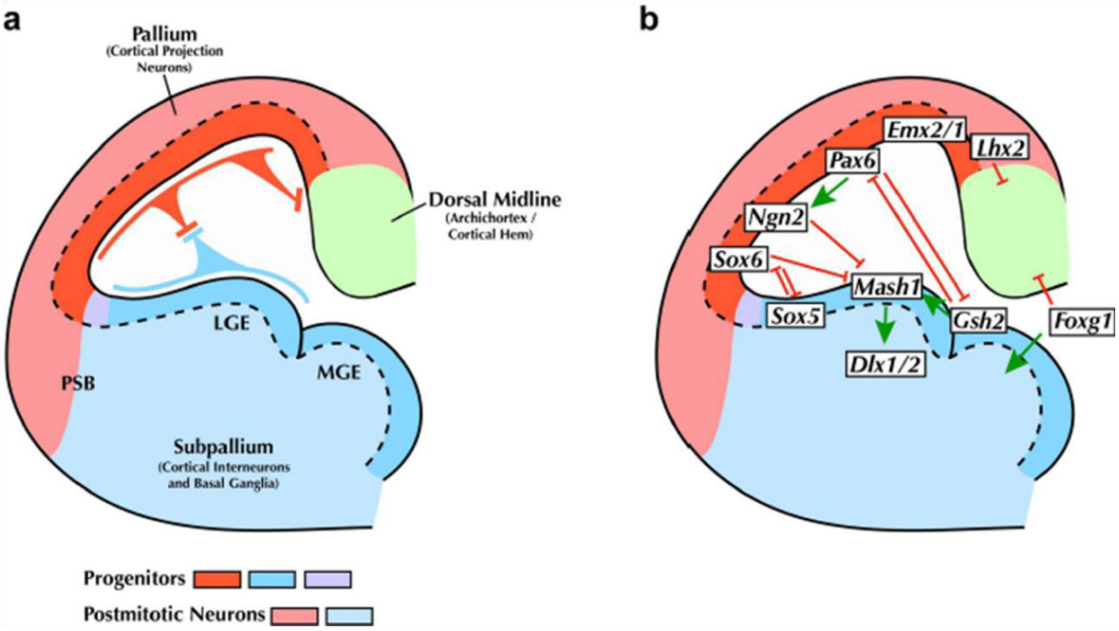

Figure 1.3. Establishment of distinct progenitor zones during patterning of telencephalon. Combinatorial and cross-repressive interactions specify the pallium and subpallium, which generates projection neurons and interneurons, respectively (MacDonald *et al.*, 2013).

1.3. Cortical Neurogenesis

Patterning of the telencephalon is followed by neurogenesis, during which projection neurons arise from dorsal ventricular zone (VZ), migrate radially toward their final positions, and form the six-layered structure seen in mammalian neocortex; meanwhile, inhibitory neurons arise ventrally from the lateral and medial ganglionic eminences (LGE and MGE), and tangentially migrate long distances to populate the cortex (Tan *et al.*, 1998; Ware *et al.*, 1999; Gorski *et al.*, 2002; Wonders and Anderson, 2006) (Figure 1.4).

After the specification of dorsal and ventral proliferative domains, neuroepithelial cells in the VZ transform into so-called radial glial cells, which are progenitors of all neurons and glia (Malatesta and Gotz, 2013) (Figure 1.5). Radial glia extend long ascending processes to the pial surface, which act as a structural scaffold for newborn migrating neurons, and guide them to their final position in the developing cortex. They also extend a short ‘apical’ process towards the ventricle (Rakic, 1971; Rakic, 2003). Around the time neurogenesis starts, radial glia proliferate and generate additional progenitors; outer radial

glia (lack apical process) and intermediate progenitors (multipolar cells with no apical or basal processes) which together form the second germinal layer, called the subventricular zone (SVZ). Progenitors within these two domains divide symmetrically to maintain the progenitor pool or asymmetrically to produce new projection neurons (Kriegstein and Alvarez-Buylla, 2009).

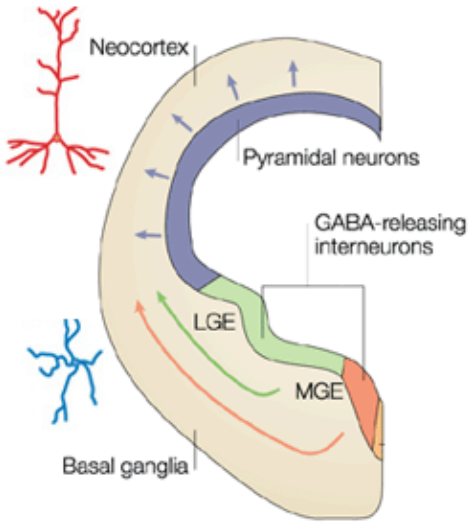

Figure 1.4. Cortical projection neurons and interneurons are specified at distinct progenitor domains of the developing telencephalon (modified from (Rowitch, 2004; Rakic, 2009)).

In mice, the earliest neurons are born around embryonic day (E) E11, and migrate radially to form a new layer called the preplate, which subsequently is split into the marginal zone and the deeper subplate by later-born neurons. Later, the cortical plate, which will eventually give rise to the mature six-layered cortex, forms between the marginal zone and subplate. For the next ~6 days, under very tightly controlled and temporally dynamic molecular programs, a diverse range of cortical projection neuron subtypes are born in sequential, but overlapping waves (Figure 1.5). Newborn projection neurons migrate radially and populate the cortical plate in an inside-out manner, such that earlier born neurons are located in the deeper layers, and later born neurons (migrating past the earlier born neurons) form the superficial layers. At around E12.5, neurons that are destined to reside in layer VI and become corticothalamic (CThPN) are born. Subsequently, subcortical projection neurons, including CSMN and subpopulations of callosal projection neurons (~%15 of all CPN) are born at ~E13.5, and migrate to layer V. In subsequent days, initially layer IV

neurons, which function in processing of thalamocortical inputs are born, and thereafter, between E15.5 and E17.5, superficial layer neurons, which are heterogeneous populations of callosal and associative projection neurons, are generated. Concurrently with dorsal neurogenesis, diverse categories of interneurons are generated from ventral subpallial proliferative zones and migrate, first tangentially toward the cortex, and then radially to their final destinations (Tan *et al.*, 1998; Ware *et al.*, 1999; Gorski *et al.*, 2002; Wonders and Anderson, 2006; Azim *et al.*, 2009a).

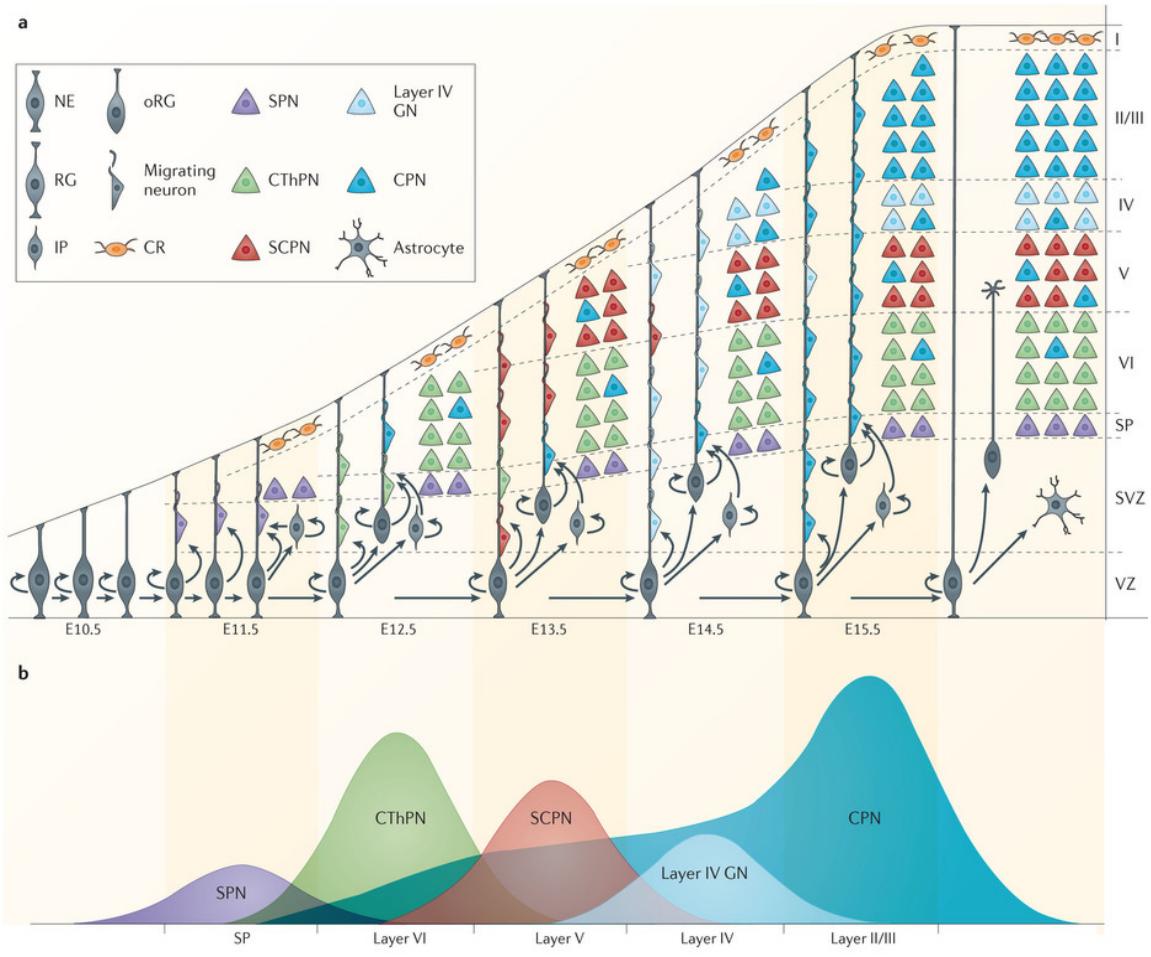

Figure 1.5. Distinct subtypes of neocortical projection neurons are sequentially generated in an 'inside-out' fashion by progenitors located in the VZ and SVZ (Greig *et al.*, 2013).

1.3.1. Molecular Controls over Neuronal Subtype Specification in Cortex

Until recently, elucidation of molecular programs controlling specification of neuronal subtypes was hindered because of the complexity of the system. Challenges arise due not only to the difficulty of identifying distinct neuronal subtypes from highly heterogeneous cell populations within and across cortical layers, but also due to the involvement of each transcriptional regulator in multiple aspects of differentiation, such as progenitor proliferation and fate commitment, or neuronal migration, fate specification, morphological maturation, areal specialization, etc. (Hevner, 2006; Molyneaux *et al.*, 2007; Greig *et al.*, 2013). Within the last decade, a number of high-throughput projects have been performed to identify cortical area- and layer-specific expression of genes during development and adulthood, including: generation of comprehensive digital atlases of mRNA *in situ* hybridization of thousands of transcripts (Gray *et al.*, 2004; Visel *et al.*, 2004; Magdaleno *et al.*, 2006; Lein *et al.*, 2007); the creation of transgenic mouse lines expressing GFP under the control of promoters of lineage or layer restricted genes (Gong *et al.*, 2007); and gene expression studies comparing micro-dissected regions of neocortex (Liu *et al.*, 2000; Zhong *et al.*, 2004; Bedogni *et al.*, 2010). Although these open-access databases have been immensely helpful as reference resources and catalyzed numerous studies in the field, the extremely heterogeneous nature of neurons, even within individual layers of the cortex, require gene expression analysis at finer spatial resolution in pure cell populations to be able to formulate hypothesis-driven questions towards identification of key set genes that regulate distinct aspects of neuronal subtype differentiation.

One particular approach devised by Macklis lab members has been especially productive towards the elucidation of subtype-specific molecular controls over neuronal development (Arlotta *et al.*, 2005). By fluorescence-activated cell sorting (FACS) of retrogradely labeled distinct neuronal populations, Arlotta et al. achieved isolation of specific cortical projection neuron subtypes, namely corticospinal motor neurons, callosal projection neurons and corticotectal projection neurons. Then, by comparative transcriptomic analysis of these purified cell populations, they identified candidate molecular controls acting at key stages of differentiation of these neurons. This approach was first applied to compare corticospinal motor neurons with callosal and corticotectal projection neurons during development, and it was later extended to other neuron subtypes

including corticothalamic and corticostriatal projection neurons; and finally to distinct subpopulations of corticospinal motor neurons that project to different levels of spinal cord. Subsequent hypothesis-driven functional studies built on these screens revealed combinatorial molecular programs unique to each projection neuron subtype, controlling specificity and precision of differentiation and connectivity of distinct subtypes of projection neurons in the neocortex (Woodworth *et al.*, 2012; Greig *et al.*, 2013).

The last decade of research has shown that, during development of diverse lineages of projection neurons, there are series of cellular events in which distinct sets of transcriptional controls act in stage-, dose- and lineage-dependent manners to specify individual projection neuron subtypes (Figure 1.6). Specification starts very early at the progenitor stage when there is initial parcellation of germinal zones; progenitors located dorsal to the ventricles (pallial zone) are specified to give rise to glutamatergic projection neurons, while ventrally located (subpallial zone) progenitors are programmed to give rise to interneurons. In parallel, within the pallium, neocortical progenitors are delineated from cortical hem progenitors, which give rise to Cajal-Retzius neurons. Further, although still an issue of debate, there seems to be heterogeneity among neocortical progenitors that give rise to callosal versus corticofugal projection neurons, two main lineages of cortical projection neurons with quite divergent axonal trajectories. Next, there are lineage-specific post-mitotic controls that direct distinct cell fates while suppressing alternative subtype identities. Lastly, there are areal controls that mostly act both at the progenitor level and post-mitotically to instruct further specialization and refinement of each lineage including collateralization and pruning decisions. Importantly, many of these subtype-specific molecular controls are initially co-expressed by multiple cell populations (especially by closely related cell populations, e.g. CThPN and CSMN or CPN and CStrPN), but get refined progressively as competing transcription factors cross-repress each other during the course of development. In summary, at each of these multiple stages described above, there are positive additive transcriptional regulators and negative exclusionary transcription factors that control the sequential generation of next stage for each cell lineage. This overall process of order- and dose- dependent nature of projection neuron identity specification can be collectively termed the “molecular logic of neocortical development” (Greig *et al.*, 2013; Lodato and Arlotta, 2015).

Here, I highlight selected molecular controls/transcriptional regulators over corticofugal projection neuron development that are especially relevant for this thesis study (Figure 1.6). During early corticogenesis, Sox6, Pax6, and Ngn2 collectively establish dorsal progenitor identity by repressing subpallial programs of gene expression. Concomitantly, Lhx2 determines cortical identity in early neuroepithelial cells (at E8.5-E10.5) by repressing hippocampal and subpallial identity (Monuki *et al.*, 2001; Mangale *et al.*, 2008). Fezf2, expressed both by a subpopulation of Pax6+/Sox6+ dorsal progenitors, and post-mitotically by subcerebral projection neurons, regulates the specification of broad subcerebral projection neuron identity, including CSMN and CThPN (Arlotta *et al.*, 2005; Chen *et al.*, 2005; Molyneaux *et al.*, 2005; Chen *et al.*, 2008; Lodato *et al.*, 2014; McKenna *et al.*, 2015). Sox5 is expressed post-mitotically by all corticofugal projection neurons, and ensures the sequential birth of corticothalamic projection neurons first, and subcerebral projection neurons later, probably via de-repression of genes that required for differentiation of later-born subtypes, like Ctip2 (Lai *et al.*, 2008). Ctip2 controls another important aspect in proper differentiation of CSMN; it functions downstream of Fezf2 and controls post-mitotic CSMN axon outgrowth and fasciculation (Arlotta *et al.*, 2005). Lmo4, expressed in the motor cortex by both callosal and subcerebral projection neurons, regulates the diversity of both populations in multiple ways, including extension of backward axonal collaterals to caudal cortex by both populations, as well as the ratio of brainstem-to-spinal cord-projecting SCPN in the rostral motor cortex (Cederquist *et al.*, 2013). Otx1 acts at later stages of subcerebral projection neuron development, and regulates corticotectal identity in the visual cortex over corticospinal fate by controlling elimination of axonal collaterals to the caudal pons and spinal cord (Weimann *et al.*, 1999). Klhl14 and Crim1 control the segmental specificity of corticospinal motor neurons that project to cervical versus lumbar levels of spinal cord, respectively (Sahni et al. in preparation). Igf-I specifically enhances the extent and rate of CSMN axon outgrowth, both *in vitro* and *in vivo* (Ozdinler and Macklis, 2006). Cell surface receptor RYK regulates the directional growth of CSMN axons along the corticospinal tract (Liu *et al.*, 2005).

Compared to corticofugal projection neurons, relatively little is known about molecular controls over callosal projection neuron specification. Satb2 and Cited2 are the only transcriptional regulators identified so far that control CPN specification and precision. In the absence of Satb2, presumptive CPN axons do not project to contralateral cortex, but

instead take the axonal trajectory of corticofugal projection neurons (Alcamo *et al.*, 2008; Britanova *et al.*, 2008; McKenna *et al.*, 2015). Cited2 regulates neocortical layer II/III generation and somatosensory callosal projection neuron development and connectivity (Fame *et al.*, 2016). Cux1 and Cux2 regulate dendrite branching, spine development, and synapse formation of upper layer CPN (Nieto *et al.*, 2004; Cubelos *et al.*, 2010). Recently, a set of other genes has been identified to be expressed by distinct subsets of CPN, but functions of these genes in CPN differentiation remain to be elucidated (Molyneaux *et al.*, 2009).

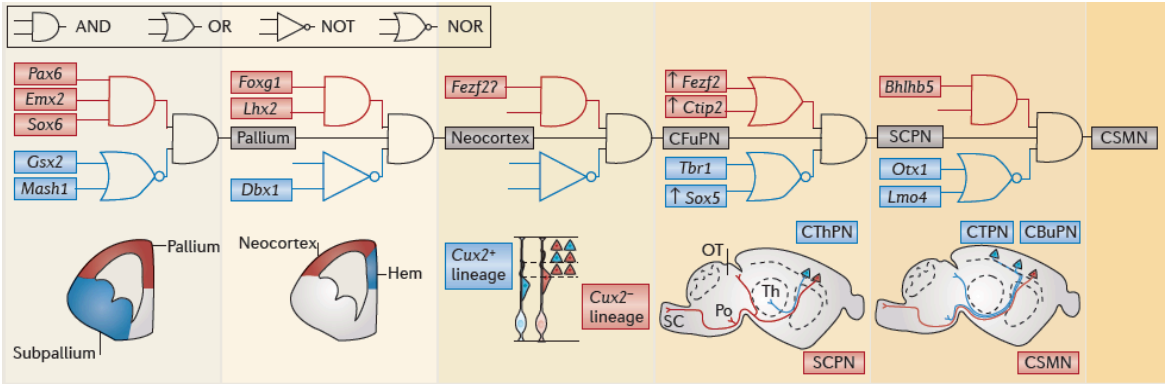

Figure 1.6. Specification of corticospinal motor neurons (CSMN) via order- and dose-dependent coordinated action of multiple transcriptional regulators and chromatin modifying proteins (Greig *et al.*, 2013).

Diversity in the neocortex is further enhanced by formation of functionally distinct cortical areas through the action of area-specific transcriptional regulators. These post-mitotic regulators transform diffuse morphogen gradients generated at the early progenitor stages into sharp boundaries postnatally, and establish distinct functional cortical areas that specialize in motor output, sensory, auditory, and visual processing etc. Cellular cytoarchitecture, connectivity, and laminar composition is different across these areas, which further imparts a unique functional capacity to the neocortex. Lmo4, Ctip1, Blhlb5 and CoupTF1 are a few examples of such post-mitotic controls that are crucial for the acquisition of areally appropriate input and output connectivity patterns (Joshi *et al.*, 2008; Tomassy *et al.*, 2010; Cederquist *et al.*, 2013).

1.4. Discovery of Neurogenesis in Adult Mammalian Brain

At the beginning of the 20th century, Ramon y Cajal stated *"Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree."(Cajal, 1913).* As declared by Cajal, integration of new neurons into fully formed, mature neuronal circuitry has long been thought to be impossible. According to this historical argument, given the complexity and specificity of connections between neurons, potential plasticity of the circuitry to accommodate new neurons or regrowth of cellular processes after injury (for example, regeneration of CSMN axons after spinal cord injury) would disrupt the existing circuitry and the encoded information within. This teleological notion relied on centuries-long clinical observations, as well as decades-long meticulous investigation by Cajal and others at the beginning of 20th century, and ultimately the failure to find evidence for 'morphologically' young-looking neurons in mature neural circuitry that exhibits immense structural complexity at various scales.

In his series of reports in the 1960's, Joseph Altman challenged this dogma of neuroscience for the first time. Using radioactive tritiated thymidine, Altman reported evidence for birth of new neurons in the hippocampal dentate gyrus, olfactory bulb, and neocortex of young and adult rat brain (Altman, 1962; Altman and Das, 1965; Altman, 1969). 15 years later, Michael Kaplan used electron microscopy to further report that thymidine incorporated newborn cells in the adult rat dentate gyrus are indeed genuine neurons, rather than glial cells (Kaplan and Hinds, 1977). However, relying on little evidence, both Altman's and Kaplan's claims were too ambitious at the time, and not welcomed in the field. A decade and a half later, the discovery that cells cultured from adult rodent brain can give rise to neurons and astrocytes was a turning point in the field (Reynolds and Weiss, 1992; Richards *et al.*, 1992). Also, the advancement of cell labeling techniques such as more reliable DNA synthesis indicator, thymidine analog-BrdU, and immuno-labeling of cell-type specific antigens has made it possible to unequivocally confirm the existence of progenitors and new neuron birth in the adult mammalian brain (Nowakowski *et al.*, 1989; Lee *et al.*, 1990; Honig *et al.*, 1996; Kuhn *et al.*, 1996; Gross, 2000).

Although it took more than three decades for people to commonly accept the occurrence of neurogenesis in the adult brain, today there are two commonly recognized "neurogenic niches" that continuously generate new neurons in adult mammalian brain: the subgranular zone (SGZ) of the dentate gyrus in the hippocampus; and the subventricular zone (SVZ) lining the walls of the lateral ventricles (Figure 1.7). Progenitors in the SGZ gives rise to glutamatergic granule cells that mature locally within the dentate gyrus, and SVZ progenitors generate diverse set of GABAergic, dopaminergic, and glutamatergic interneurons that migrate long distances rostrally to the olfactory bulb. In addition to these two canonical neurogenic regions, the hypothalamus (surrounding the third ventricle) is recognized as a third active neurogenic site in adult rodent brain. However, the rate of neurogenesis in hypothalamus is relatively lower than the SVZ and SGZ. Under physiological conditions, there is no neurogenesis occuring in other areas of the adult brain (Lois and Alvarez-Buylla, 1994; Magavi *et al.*, 2000; Sohur *et al.*, 2006; Chojnacki *et al.*, 2009; Feliciano *et al.*, 2015).

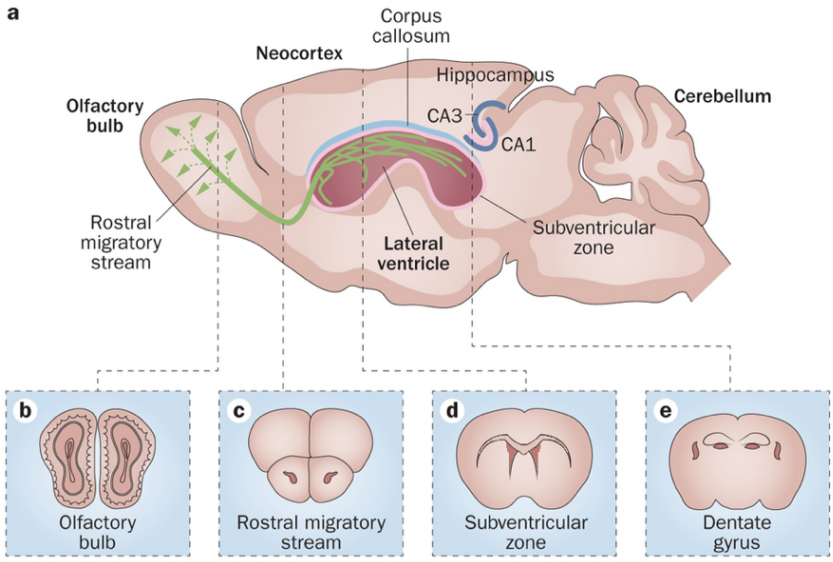

Figure 1.7. Schematic representation of the two main constitutively neurogenic regions in the adult rodent brain. SVZ wall of the lateral ventricles, and SGZ in dentate gyrus of the hippocampus (Ziegler *et al.*, 2015).

Importantly, the SVZ and SGZ neurogenic niches have been well conserved in humans, although the presence or extent of neurogenesis in the SVZ after infancy is a matter

of debate (Sanai *et al.*, 2011). Strikingly, the striatum emerges as a new site of neurogenesis in humans, though whether the origin of new neurons in the striatum is SVZ- or striatum-resident precursors is yet to be determined (Ernst *et al.*, 2014; Bergmann *et al.*, 2015).

1.5. Induction of Neurogenesis in Non-Neurogenic Regions of the Adult Brain

The discovery that progenitors do exist in the adult brain, and the demonstration that new neurons are continuously born throughout life, can migrate long distances, and functionally integrate to preexisting complex circuitry have aroused great optimism for repair of neuronal circuitry in neurodegenerative diseases and CNS injuries such as spinal cord injury.

To replace the diseased neurons with new ones in non-neurogenic areas of the brain (particularly in cortex), two approaches has been conceived in the field: First, assessing the regenerative potential of progenitors in the SVZ neurogenic niche, which would include assessment of their responsiveness to injury, migration of their progeny to pathologic sites, and differentiation into local circuit neurons in damaged areas. Secondly, exploration of the existence of quiescent, residual progenitor cells in non-neurogenic regions and their activation locally. Additionally, use of exogenous sources of neurons generated either from pluripotent ES or IPS cells, or directly from other somatic cells (such as fibroblasts) has been an alternative cellular replacement approach in the field.

1.5.1. Regenerative Potential of SVZ-resident Progenitors

It is very well documented that, under physiological conditions, SVZ- and SGZ-resident neural precursors in adult rodent brain are tightly fate-restricted to generate particular neuron subtypes destined to their corresponding local circuitry. However, a large number of studies have demonstrated that experimentally induced ischemia leads to a marked increase in proliferation of SVZ progenitors, and triggers new neuron recruitment to the lesion site, particularly to striatal areas near the SVZ (Figure 1.8). Therefore, manipulation of SVZ precursors towards cellular repair of striatal or cortical circuitry has been extensively explored in the field (Arvidsson *et al.*, 2002; Parent *et al.*, 2002; Zhang *et al.*, 2007; Ohira, 2011). It has been reported that both progenitor proliferation and ectopic

neuron migration can be considerably augmented with intra-ventricular administration of trophic factors, such as BDNF (Benraiss *et al.*, 2001; Pencea *et al.*, 2001; Chmielnicki *et al.*, 2004; Mohapel *et al.*, 2005), EGF (Teramoto *et al.*, 2003; Ninomiya *et al.*, 2006), FGF2 (Yoshimura *et al.*, 2001; Leker *et al.*, 2007), VEGF (Wang *et al.*, 2007b), and TGF-alpha (Fallon *et al.*, 2000). Chemokines like Cxcl12 and its receptor Cxcr4 regulate the migration of neuroblasts to injury sites (Thored *et al.*, 2006).

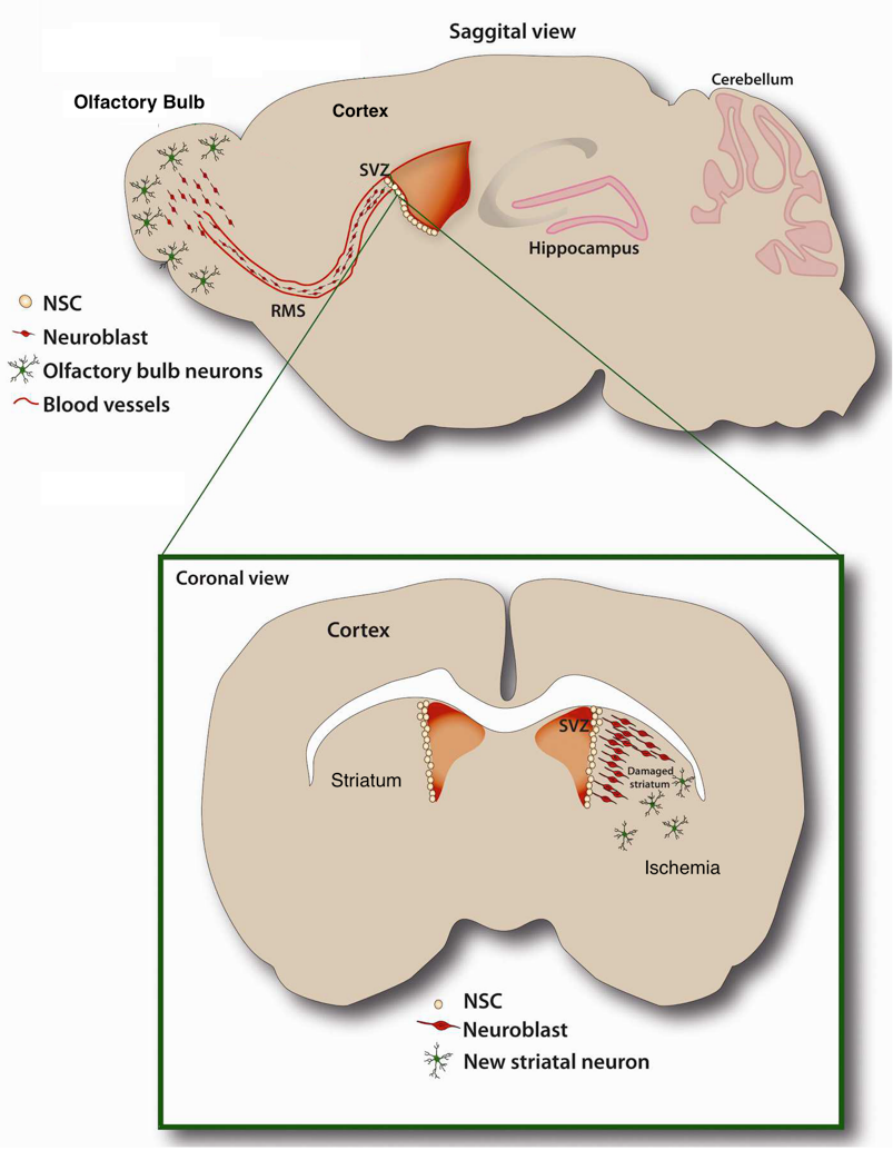

Figure 1.8. Transient focal ischemia increases proliferation of SVZ progenitors, and triggers new neuron recruitment to the infarct site in the striatum (M. Guerra-Crespo, 2012).

Importantly, though numerous studies consistently found evidence for recruitment of some young neurons to the lesion site in the striatum, the overall process is still extremely inefficient. A majority of young neurons die before maturation, and surviving neurons can replace only ~0.2% of neurons that are lost to injury within the peri-infarct region (Arvidsson *et al.*, 2002; Li *et al.*, 2010). Also, the few studies that properly assess differentiation and identity of ectopic neurons in striatum report conflicting findings (Chmielnicki *et al.*, 2004; Collin *et al.*, 2005; Tonchev *et al.*, 2005; Yamashita *et al.*, 2006; Hou *et al.*, 2008; Liu *et al.*, 2009; Benraiss *et al.*, 2013). For example, contrary to previous reports, in their rigorous analyses, Liu et al. found that newborn neurons recruited to the striatum after ischemic injury do not acquire striatal medium spiny neuron identity, but maintain the molecular characteristics of their olfactory neuron identity. The authors argue that this raises the theoretical possibility that SVZ precursors or their progeny might not actually be responding to injury, but that the disruption of vascular scaffold around their migration corridor results in passive drainage of neurons to the environment (Liu *et al.*, 2009). Indeed, in their elegant heterotopic transplantation experiments, the Alvarez-Buylla group identified that progenitors in SVZ are intrinsically coded based on their topographical location around the ventricles to produce only specific types of inhibitory neurons destined for unique circuits within olfactory bulb (Merkle *et al.*, 2007) (Figure 1.9).

Once isolated and grafted into neocortex, SVZ-derived neuronal progenitors form aggregates, do not migrate, stay as immature neurons, or differentiate into glia (Herrera *et al.*, 1999; Seidenfaden *et al.*, 2006). In line with these findings, recent fate mapping experiments with modern genetic tools showed that SVZ-derived cells in striatum after ischemia are mostly glial cells, and only around 5% of cells show the molecular characteristics of mature neurons (Li *et al.*, 2010).

In some rare cases, ectopic new neuron migration is observed in other brain regions proximal to the ventricles, such as the cortex, septum, thalamus and hypothalamus, but the extent of migration, survival, and maturation of new neurons in these areas is noticeably less efficient compared to the striatum (Jiang *et al.*, 2001; Pencea *et al.*, 2001; Yang *et al.*, 2004; Sirko *et al.*, 2009; Ohira, 2011).

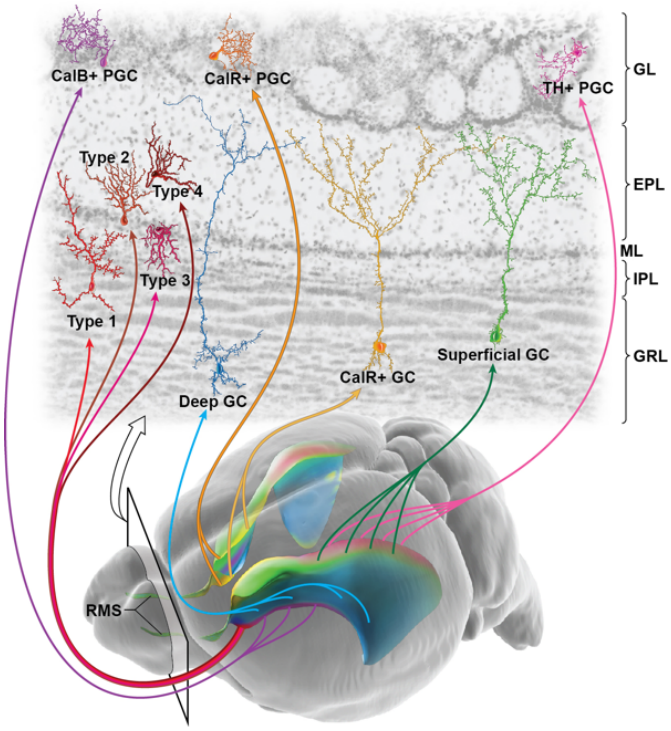

Figure 1.9. SVZ progenitors located in geographically distinct subregions of the ventricle walls produce unique types of interneurons in the olfactory bulb (Lim and Alvarez-Buylla, 2014).

Notably, compared to the striatum, ischemia-induced migration of SVZ-derived neurons to the cortical parenchyma is substantially less inefficient (Arvidsson *et al.*, 2002; Jin *et al.*, 2003; Ohab *et al.*, 2006; Sirko *et al.*, 2009; Osman *et al.*, 2011; Zhang *et al.*, 2011; Saha *et al.*, 2012). Multiple causes might underlie this observation: between the SVZ neurogenic niche and the cortex, intersecting axons of callosal and corticofugal projection neurons form highly myelinated, thick white matter tracts that might limit the penetration of new neurons into the cortex. As a matter of fact, SVZ-derived neuroblasts have an inherent capacity to travel long distances to reach their destination in the olfactory bulb; however, the path they take toward the olfactory bulb is a highly specialized corridor that is sheathed with a special glial framework and vascular scaffold (Nguyen-Ba-Charvet *et al.*, 2004; Sawamoto *et al.*, 2006; Whitman *et al.*, 2009; Kaneko *et al.*, 2010). The therapeutic potential of the SVZ niche is further limited in humans given the fact that neurogenesis in the SVZ after early childhood is found to be extremely limited or absent in humans (Bergmann *et al.*, 2012). If the SVZ niche is at all active in adult humans, the relative distance between the

ventricles and cortical gray matter that newborn cells would need to travel is orders of magnitude greater in humans than in rodents (Paredes *et al.*, 2016) (Figure 1.10), which further limits the potential of the SVZ niche for repair of specific cortical circuits.

Together, years of investigation has proven that, although ischemia induced signals are potent in activating SVZ progenitors and recruiting new neurons, the overall process is not robust enough for repair of diseased circuitry in any brain region, including the striatum.

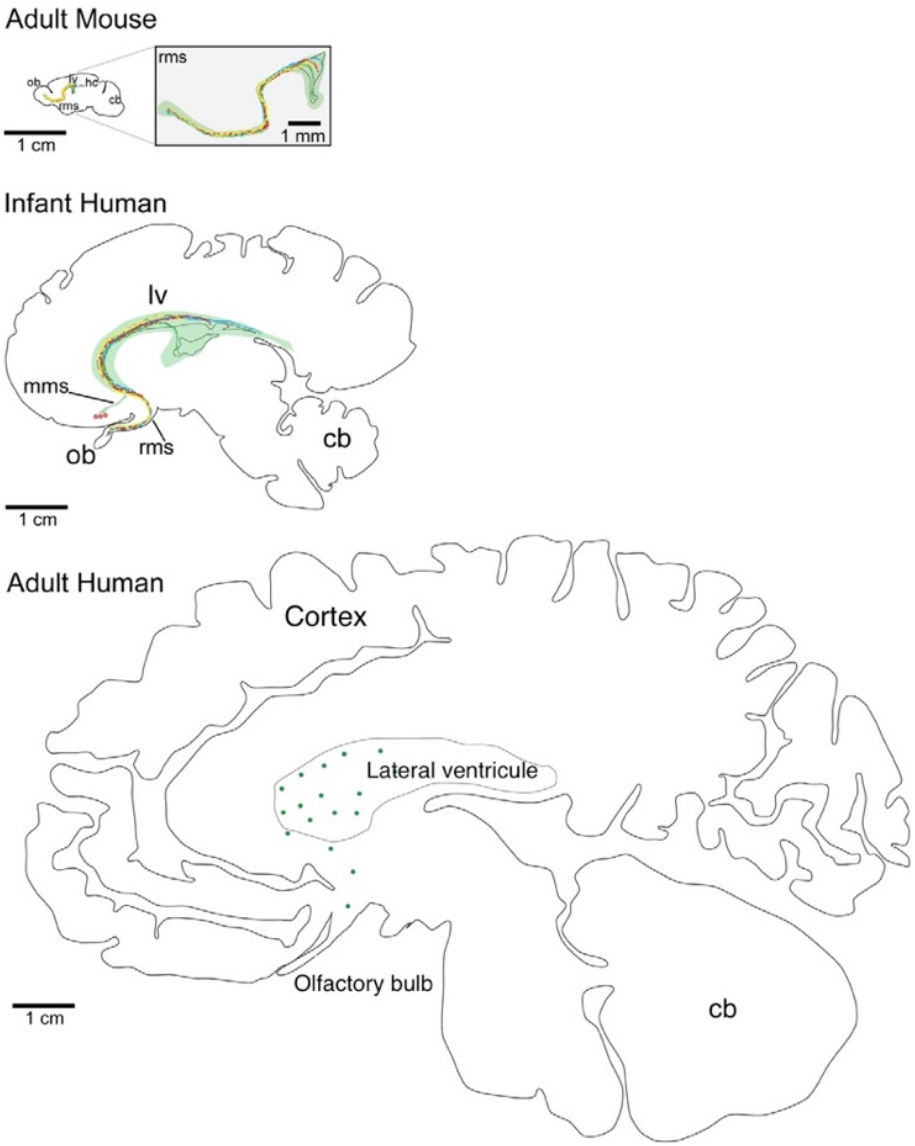

Figure 1.10. Sagittal representation of the SVZ neurogenic niche in the mouse and human brain. With expanded brain size, the spatial separation of neurogenic niches and potential brain targets is further increased (Paredes *et al.*, 2016).

1.5.2. Molecular and Genetic Manipulation of SVZ Progenitors

Observations that the natural response of SVZ-progenitors and their progeny to injury is not significant enough for circuitry repair in CNS disease or injury led the field to question the usefulness of SVZ-progenitors for circuitry repair. However, recent progress in viral and genetic gene manipulation tools have made direct molecular interventions possible, which has revived interest in SVZ progenitors.

Malin Parmar and her colleagues have over-expressed Islet-1 (Isl1) in SVZ-progenitors to mobilize the SVZ-derived cells toward the striatum. Isl1 is a LIM homeodomain transcription factor expressed in the SVZ only during embryonic development in cells that differentiate into striatal medium spiny neurons. Retroviral over-expression of Isl1 in postnatal SVZ recruited a significant number of cells to the striatum; however the overwhelming majority were glial cells (Rogelius *et al.*, 2008). In a subsequent study, they complemented Isl1 with Ngn2, seeking to increase the proportion of neurons relative to glial cells, as well as to redirect these neurons to other brain regions such as the cortex.

Ngn2 is a transcription factor that plays a critical role in glutamatergic neurogenesis from dorsal embryonic progenitors during cortical development (Fode *et al.*, 2000; Morrison, 2001). Though co-transduction of SVZ progenitors with Ngn2 and Isl1 induced a large number of ectopic neuroblasts in the striatum and external capsule, new neurons failed to acquire mature neuronal morphology, and did not reach the cortex (Rogelius *et al.*, 2008). Similarly, forced over-expression of Fezf2, a master transcriptional control over specification of subcerebral projection neurons (SCPN), in postnatal SVZ progenitors changed the neurotransmitter properties of these normally GABAergic neurons to a glutamatergic identity, but had no effect on axonal morphology or the destination of newborn neurons (Zuccotti *et al.*, 2014).

In a recent study performed in a mouse model of Huntington's disease, Benraiss et al. reported that AAV-mediated continuous high-level expression of BDNF and noggin in SVZ progenitors recruited new neurons to the striatum, where they matured and exhibited the molecular hallmarks of striatal medium spiny neurons. These new neurons delayed the

onset of motor deficits by 3-4 weeks and had a modest effect on the survival of the animals (Benraiss *et al.*, 2013). Of note, these results are not consistent with previous findings from Alvarez-Buylla's group, who observed no positive effect of BDNF on SVZ neurogenesis (Galvão *et al.*, 2008).

Recent studies indicate that progenitors in the SVZ niche are heterogeneous, and presumably distinct subsets of progenitors generate neurons and glial cells throughout life. The transcriptional regulators Pax6 and Olig2 are expressed by distinct subsets of SVZ progenitors, and instruct neuronal versus glial specification, respectively (Marshall *et al.*, 2005; Jang and Goldman, 2011). To increase the overall number of neurons generated, a number of studies sought to switch the fate of glia-generating SVZ-progenitors via over-expression of Pax6 or blocking Olig2 function. This idea attracted a fair amount of interest, partly because of the fact that SVZ-born glioblasts are capable of migrating dorsally to the corpus callosum and the cortical parenchyma in the postnatal and adult brains (Nait-Oumesmar *et al.*, 1999). Though this approach yielded a higher number of 'young neurons' produced in response to injury upon ischemia or stab wound, the overall process was still extremely inefficient, and young neurons failed to mature (Heins *et al.*, 2002; Marshall *et al.*, 2005; Kronenberg *et al.*, 2010; Jang and Goldman, 2011; Christie and Turnley, 2012; Klempin *et al.*, 2012; Faiz *et al.*, 2015).

In conclusion, based on the limitations that i) SVZ-derived new neurons leave their normal migratory path only upon severe hypoxia-induced inflammatory stimuli, and have extremely limited ability to migrate toward areas other than striatum, and ii) in humans, the SVZ niche is active only during early childhood and is at a considerable distance from the cortex, the data in the field support the interpretation that SVZ-progenitors holds a little or no therapeutic potential for injured or diseased cortical circuitry in humans.

1.5.3. Residual Quiescent Progenitors Might Exist in Non-Neurogenic Areas of the Adult Mammalian Brain

Since the discovery of active neurogenic niches in two specific regions of adult brain, tremendous effort has sought to identify potential residual neurogenic progenitors in other areas. Over the last two decades, an increasing number of studies have reported compelling

evidence for the potential existence of residual progenitor cells in non-neurogenic regions of the adult brain, including the neocortex. Formerly, evidence for these claims relied on conventional labeling methods such as BrdU birth dating analysis and immunocytochemical analysis of certain neuronal-specific molecules; however, more recent studies that use modern viral and genetic cell fate-mapping methods have reinforced these findings. According to the prevailing view, in their normal context, these local progenitors do not actively give rise to new neurons, but either stay dormant or undergo restricted proliferation to generate a subset of glial cells. Their innate neurogenic potential is revealed upon injury-induced environmental stimuli or via direct molecular manipulation (Sohur *et al.*, 2006; Ohira, 2011).

Members of the Macklis lab showed for the first time that endogenous progenitors can be induced to differentiate into mature neurons with appropriate connections in the adult neocortex (Magavi *et al.*, 2000). Upon synchronous targeted apoptosis of particular subtypes of projection neurons (i.e. corticothalamic or corticospinal projection neurons) in the anterior cortex, small numbers of new neurons were generated, which migrated to appropriate laminar locations and formed long distance connections with appropriate targets at distant sites (the thalamus or the spinal cord, respectively). The newly born neurons matured and survived for at least 6 months or more (Magavi *et al.*, 2000; Chen *et al.*, 2004). Though the source of newborn neurons was not identified in these studies, the distribution of BrdU-positive Dcx-expressing neuroblasts suggest that while a significant portion may have originated from the SVZ, some of them may have been derived from a pool of local, quiescent cortical progenitors. Using the same targeted, synchronous neuronal apoptosis approach for callosal projection neurons, Magdalena Goetz's group observed that a specific subset of neuroblasts derived from SVZ migrated towards the injury site, and differentiated into callosal projection neurons in rostral cortex (Brill *et al.*, 2009). It is conceivable that the rostral end of the cortex might be particularly conducive for integration of new neurons from the SVZ for multiple reasons: 1) neuroblasts pool together and form a larger chain rostrally as they migrate towards the olfactory bulb; 2) the thickness of cortical layers decreases rostrally, such that the distance for signals from dying cells to reach the SVZ is shorter, and a new neuron has less distance to travel; 3) the cytoarchitecture of the white matter that isolates cortex from the rostral migratory stream might be more amenable for a migrating

neuron to pass through; and, lastly 4) it is possible that a subpopulation of progenitors in the rostral SVZ might be more reactive to cortical injury.

In a recent report, using a fluorescent reporter-encoding retrovirus, Ohira et al. sought to characterize the short- and long-term progeny of the actively proliferating cells in adult rat cortex. They found that, while the great majority of proliferative cells produce glial cells, a certain subset located exclusively in the outermost layer of the cortex (layer I) display some molecular features of SVZ- and SGZ-localized progenitors, and produce functionally mature interneurons after mild ischemia (Ohira *et al.*, 2010). Ischemia-induced neurogenesis from local progenitors has been claimed by multiple groups, both in the striatum and cortex of juvenile or adult rodents (Sirko *et al.*, 2009; Xue *et al.*, 2009; Shimada *et al.*, 2010; Magnusson *et al.*, 2014; Duan *et al.*, 2015), and in the external capsule and lateral striatum of adult guinea pigs (Luzzati *et al.*, 2014). Similarly, in a quinolinic acid-induced excitotoxic model of Huntington's disease, a subset of local astrocytic cells in the adult mouse striatum were reported to become reactive, upregulate the neural progenitor marker Nestin, proliferate locally and generate Dcx+ immature neurons (Nato *et al.*, 2015).

Although still an issue of debate, limited "physiological neurogenesis" might be occurring in healthy postnatal or adult rodent cortex, in some of the so-called 'non-neurogenic' areas. Performing short- and long-term BrdU birth-dating and rigorous confocal microscopy analysis, Dayer et al. report that, though extremely limited in number (~4 cells/10mm^3), distinct subclasses of GABAergic interneurons are born in the cortex of healthy adult rats, and express the mature neuronal marker, NeuN. Intriguingly, they found that about one forth of the newly born yet differentiating neurons express NG2 chondroitin sulfate proteoglycan, (a trans-membrane molecule that is expressed by a group of proliferative cells found across the CNS) (discussed below), suggesting that neurons are produced locally from a subpopulation of NG2-expressing progenitors (Dayer *et al.*, 2005). Supporting this argument, a small subset of NG2-expressing progenitors was found to express the immature neuronal marker DCX in cortex of healthy adult rats (Tamura *et al.*, 2007).

These early observations regarding innate neurogenic potential in subset(s) of NG2-expressing cortical progenitors was initially solely based on immunocytochemical analysis,

and, therefore, initially did not receive broad attention in the field. However, recent cell fate-mapping studies using inducible Cre-expressing transgenic tools have kindled a substantial debate over innate neurogenic competence of a subset of NG2-expressing progenitors in certain areas of brain. Utilizing different Cre-driver lines, David Pleasure's and William Richardson's groups found that a subset of NG2-expressing progenitors found in the piriform cortex produce local neurons that functionally mature in the early postnatal and juvenile mouse brain (Rivers *et al.*, 2008; Guo *et al.*, 2009; Guo *et al.*, 2010) (Figure 1.11). These observations have been disputed by other groups utilizing different mouse lines: these groups attributed the results to non-specific expression of the transgenic promoter in pre-existing neurons (Dimou *et al.*, 2008; Kang *et al.*, 2010). Strikingly, DCX-positive cells were reported in the piriform cortex of adult rat brain almost a decade before these studies (Nacher *et al.*, 2001). Whether or not these studies eventually prove to be correct, these observations have already attracted broad attention to NG2-expressing progenitors as a potential *in situ* source of cells for cellular replacement therapies, and has motivated further research both to elucidate potential heterogeneity within them, and to direct their differentiation into local circuit neurons at different areas of the brain.

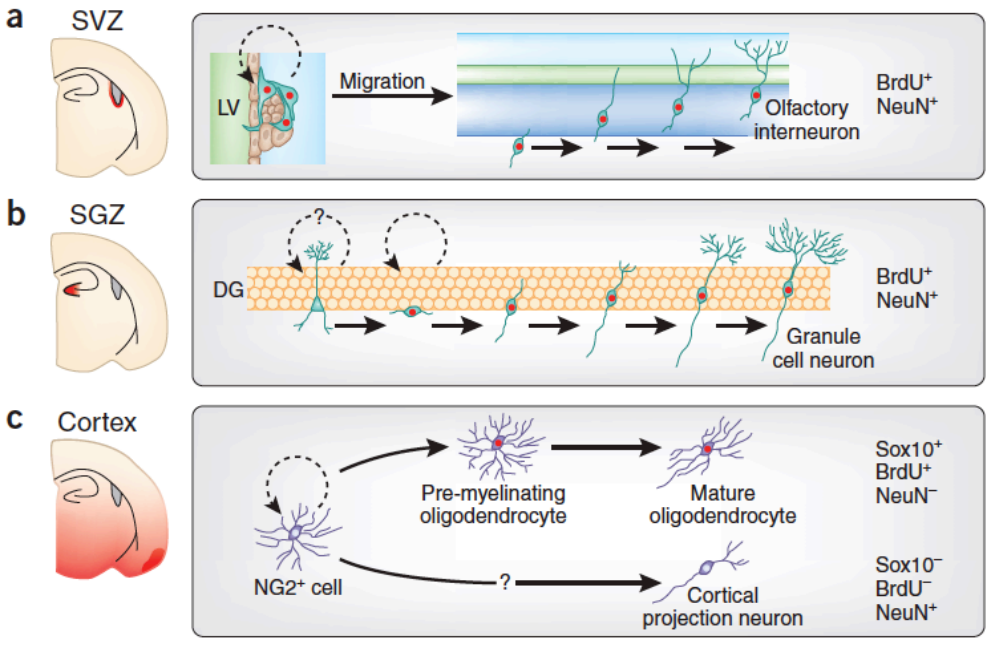

Figure 1.11. In addition to the SVZ and SGZ neurogenic sites (a, b), physiological neurogenesis might occur in other sites of the adult brain. In piriform cortex (c), subset of NG2-expressing progenitors give rise to new neurons (Kang and Bergles, 2008).

Of note, in addition to NG2-expressing progenitors, a subpopulation of local astroglial cells has also been claimed to have latent multi-lineage potential. In the juvenile mouse cortex, a subpopulation of glial cells identified with active transgenic hGFAP promoter was reported to generate a limited number of neurons (Ganat *et al.*, 2006).

Together, these studies provide evidence that there might be multipotent progenitors resident in at least some of the 'non-neurogenic' areas of postnatal and adult brain. Once the correct set of molecular programs are activated, their innate neurogenic potential might be exploited to generate specific subsets of neurons in the setting of CNS disease or injury.

1.6. Identification of Sox6+ Dorsal Progenitors Resident in Adult Neocortex

Upon completion of cortical neurogenesis during the late days of embryonic development, a subset of neural progenitors remains close to the ventricles, forms the SVZ-proliferative niche, and maintains neurogenic potential throughout postnatal stages and adulthood. On the other hand, another subset of progenitors departs from the VZ and SVZ niche around birth, and migrates to the cortical parenchyma. During postnatal development and adulthood, these progenitors present in the cortical parenchyma, divide both symmetrically to increase their pool, and asymmetrically to generate glial cells locally (Ge *et al.*, 2012; Gallo and Deneen, 2014).

The research that I summarized in the preceding section provides substantial evidence that some of these progenitors resident in the cortex might have latent neurogenic potential. These cortical progenitors and progenitors of cortical projection neurons share a common ancestry, specifically the VZ radial glia that were potentially exposed to the same morphogen gradients during embryonic development. It is highly likely that they have a more common epigenetic landscape (as compared to ventral SVZ-resident or exogenous sources of progenitors), and it is therefore conceivable that subsets of local progenitors in the cortex might be inherently more competent to differentiate into cortical projection neurons. Thus, it might be especially productive to identify and manipulate the correct subset of progenitors for induction of specific neuron subtypes faithfully in CNS diseases or injury.

In their unpublished work, members of the Macklis Lab, Dr. Hari Padmanabhan (post-doctoral fellow) and Dr. Eiman Azim (former graduate student) investigated whether progenitors resident in the postnatal and adult mouse cortex maintain their dorsal identity. They investigated whether key transcriptional controls that play a role in specification of dorsal progenitors earlier during embryonic development like Pax6, Tbr2, Sox6, and Fezf2 show retained expression in postnatal progenitors in the cortical parenchyma. Performing BrdU pulse chase experiments, and using rigorous 3-D confocal analysis, they identified that a subset of proliferative cells express Sox6+ in the caudal cortical SVZ and in the cortical parenchyma of postnatal and adult mice (Figure 1.12).

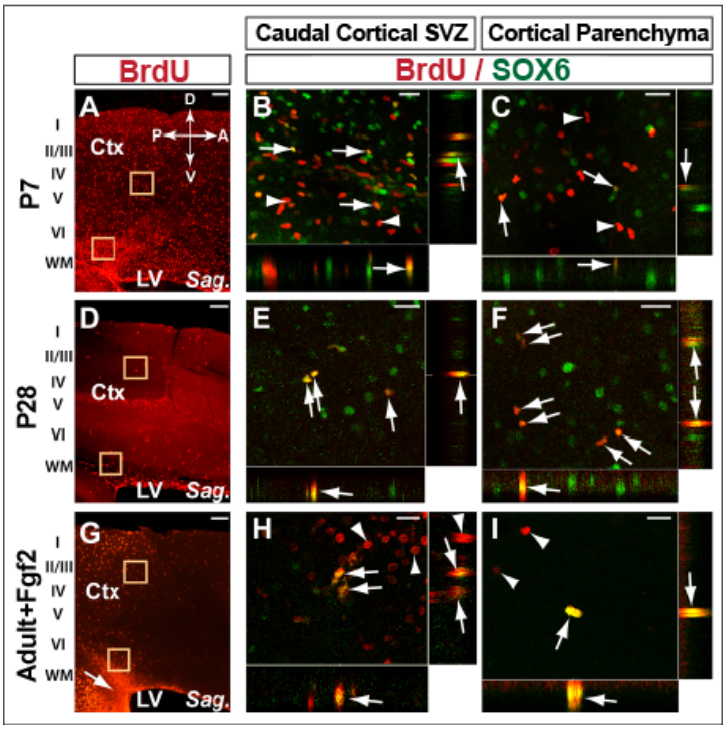

Figure 1.12. Identification of Sox6+ cortical/pallial progenitors in postnatal and adult neocortex (Padmanabhan and Azim, unpublished).

During parcellation of the telencephalon around mid-gestation, Sox6 plays a key role in proper establishment of dorsal identity, which is crucial to generate diverse sets of cortical projection neurons (Azim *et al.*, 2009a). Further characterization with cell-type specific markers revealed that Sox6+/BrdU+ cells do not express the mature neuronal marker NeuN, or the astrocytic marker S100B, or the SVZ astrocytic/radial glial marker GFAP. However, they found that Sox6+/BrdU+ cells do express NG2 (neuron/glial antigen 2), a trans-

membrane proteoglycan that is commonly expressed by a group of proliferative cells found across the CNS (Figure 1.13).

1.7. Identity of Sox6+/NG2+ Cortical Progenitors, and Their Potential for Cellular Repair of Cortical Circuitry

NG2-expressing progenitors are evenly distributed throughout both white and gray matter, and constitute ~5% of the total cells in the adult brain (Pringle *et al.*, 1992; Dawson *et al.*, 2003). Further, they are the primary proliferative cells of neural origin that exist in the adult mammalian brain. Within the adult cortex, ~93% of Ki67+ actively dividing cells express NG2-proteoglycan, whereas the remaining ~7% are endothelial cells (Mori *et al.*, 2009). They are historically termed simply as NG2-glia, or as oligodendrocyte progenitors cells (OPCs), because the majority give rise to oligodendrocytes throughout postnatal and adult ages (Levine and Stallcup, 1987; Horner *et al.*, 2000; Bu *et al.*, 2004). However, there is recent evidence indicating that so-called "NG2 cells" are not uniform. Genetic fate mapping studies showed that progenitor cells expressing NG2-proteoglycan generate a subpopulation (~40%) of protoplasmic astrocytes embryonically in the ventral forebrain (Zhu *et al.*, 2008a), ~10% of protoplasmic astrocytes in gray matter of the spinal cord (Zhu *et al.*, 2008b), a subset of reactive astrocytes upon injury (Tatsumi *et al.*, 2008; Sellers *et al.*, 2009; Zhao *et al.*, 2009), and, although still a matter of active debate, glutamatergic neurons in the postnatal and adult piriform cortex (Rivers *et al.*, 2008; Guo *et al.*, 2009; Guo *et al.*, 2010). Therefore, it is not surprising that a commonly expressed structural molecule like NG2 proteoglycan might be expressed by multiple diverse cell populations (see page 9).

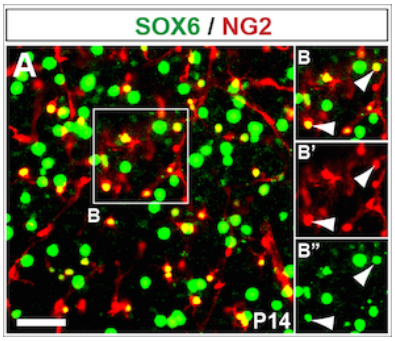

Figure 1.13. Sox6 is expressed by a majority of NG2+ progenitors in postnatal cortex (detected in an NG2-DsRed reporter mouse line).

Indeed, a broad population of NG2-expressing progenitors in the cortex arise from molecularly and anatomically distinct germinal zones at distinct embryonic times (Figure 1.14). During forebrain development, the first wave of NG2-expressing progenitors arises at about E12.5 from the medial ganglionic eminence (Nkx2.1 domain). Soon afterwards, a second population arises from the lateral ganglionic eminence (Gsx2 domain). These two ventrally generated populations tangentially migrate (together with interneurons) and populate the developing cortex around E16. Later, around birth, after completion of neurogenesis of cortical projection neurons, the dorsal progenitor zone (Emx1 domain) gives rise to the last wave of NG2-expressing progenitors. As dorsal progenitors invade the cortex, the majority of ventrally derived progenitors are gradually eliminated via unknown mechanisms, and 80% of the NG2-expressing progenitors that eventually remain in the cortex are from dorsal germinal domains (Tripathi *et al.*, 2011). Together, both heterogeneity in the developmental origins, and the distinct lineage potential of a subset strongly indicates that so called "NG2-progenitors" are not uniform.

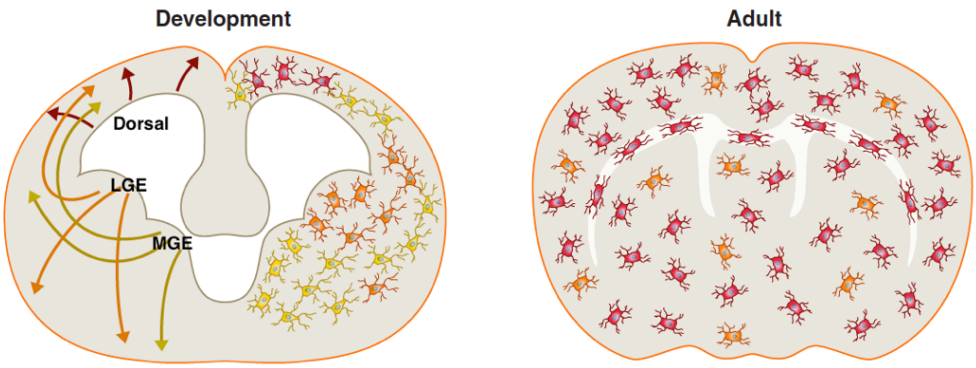

Figure 1.14. NG2-expressing progenitors arise from molecularly distinct dorsal and ventral progenitor domains during development (Dimou and Gotz, 2014).

In their parallel work, Drs. Azim and Padmanabhan have found that, quite strikingly, in the absence of Sox6, the proneural gene Ngn2 (otherwise SVZ-restricted) is ectopically expressed throughout the cortex at postnatal day 6 (P6), and the NG2 progenitors fail to acquire oligodendrogenic fate (Figure 1.15). These data, together with the multi-lineage cell potential of a subset of NG2-expressing progenitors discussed above, strongly indicate that a subset of progenitors resident in the cortex might still have neurogenic competence,

particularly for cortical projection neurons, and that Sox6 plays an active role in suppression of these neurogenic programs.

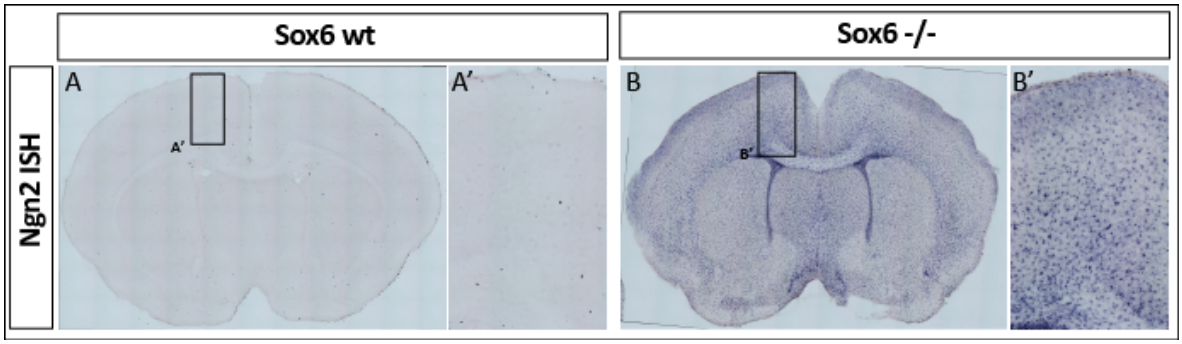

Figure 1.15. Loss of Sox6 function results in ectopic cortical Ngn2 expression (Padmanabhan and Azim, unpublished).

Importantly, a growing body of knowledge regarding molecular controls over the specification of NG2+/Sox6+ progenitors during development, and glial fate commitment at postnatal ages and during adulthood, provides substantial insight regarding how to manipulate NG2+/Sox6+ for their directed differentiation into a specific subset of cortical projection neurons. The basic helix-loop-helix transcription factor Olig2 plays a central role both in generating a broad population of cortical NG2+ progenitors, and in their glial fate commitment and differentiation. According to an emerging model (mostly based on studies in the spinal cord), during early neurogenesis, phosphorylated Olig2 homodimerizes and initiates neurogenic programs by inducing proneuronal genes, including Ngn2. As neurogenesis continues, the phosphorylation status of Olig2 gradually changes (i.e. by removal of phosphate from S147, probably regulated through cycling cascades), and Olig2 starts to counteract Ngn2 function via protein interactions and competition for shared DNA-binding sites. In addition to suppressing neurogenic programs by antagonizing proneural molecules, Olig2 forms heterodimers with gliogenic molecules, and thereby regulates the neurogenic to gliogenic transition (Gaber and Novitch, 2011; Li *et al.*, 2011; Sun *et al.*, 2011). Previous studies demonstrated that, during corticogenesis, for the neurogenic to gliogenic fate-switch to occur, the activity of Ngn2 must be down-regulated in progenitor cells. If expression is sustained ectopically, neurogenesis continues at the expense of gliogenesis (Cai *et al.*, 2000; Nieto *et al.*, 2001). After enabling sequential and proper generation of a broad NG2-progenitor population during development, Olig2 continues to

be expressed, and critically controls differentiation and maturation of oligodendrocytes in postnatal and adult brain. A number of studies have shown that antagonizing Olig2 function in NG2-progenitors shifts their fate toward the neurogenic lineage in adult brain (Buffo *et al.*, 2005).

Using an array of cell type- and developmental stage-specific Cre-driver lines, as yet unpublished work from our lab has found that Sox6 plays a pivotal role at multiple stages of NG2+/Sox6+ progenitor development. Sox6 initially controls the precise suppression of pro-neurogenic programs during generation of NG2+/Sox6+ progenitors. In the absence of Sox6 function, proneural gene expression is ectopically maintained in NG2+/Sox6+ progenitors, and myelination is severely disrupted due to the failure of oligodendrocyte differentiation. Together, deepening insight about molecular controls over specification and glial-fate commitment of NG2+/Sox6+ progenitors provides substantial insight toward effective experimental strategies to induce specific neuronal identities.

2. AIM

Specific classes of neurons are selectively vulnerable in distinct neurodegenerative, developmental and acquired diseases of the nervous system. In particular, for this thesis study, corticospinal motor neurons (CSMN) degenerate in amyotrophic lateral sclerosis (ALS) and other motor neuron diseases, and corticostriatal projection neurons (CStrPN), a cortical neuronal population, degenerate in Huntington's Disease.

Replacement of diseased neurons with new neurons via directed differentiation from endogenous local progenitors offers a potential therapeutic approach for functional repair of diseased or injured neuronal circuitry. Toward this aim, recent work has begun to identify central molecular controls over development of broad classes and specific subtypes of cortical projection neurons. In this Ph.D. thesis, I present two distinct projects that are complementary to each other. The first project aims to understand how to manipulate endogenous neural progenitors using developmental molecular controls, to enable directed population-specific neurogenesis and repair of complex neuronal circuitry. In the second project, I present initial efforts toward understanding of development, connectivity, and diversity of CStrPN.

In the first project presented in Chapter 4, I aim to target endogenous local NG2+ progenitors present in postnatal and adult cortex, and apply a select set of central and complementary developmental transcriptional controls to direct their differentiation into corticofugal (cortical output) projection neurons; a broad group of neurons that includes CSMN.

In the second project presented in Chapter 5, I aim to identify key molecular controls over subtype-specific development and diversity of CStrPN, which would serve as a solid step toward achieving a holistic view of the establishment of corticostriatal circuitry, and its potential dysgenesis in disease.

3. MATERIAL AND METHODS

3.1. Plasmids

To express multiple proteins from a single open reading frame, we cloned EGFP, Neurog2, VP16-Olig2, and Fezf2-HA coding sequences separated by 2A linker sequences into a pCBIG vector (ENVOF construct). In this system, genes linked to each other via viral 2A sites are transcribed as a single mRNA, but separate into individual polypeptides during translation (Donnelly *et al.*, 2001; Szymczak *et al.*, 2004; Trichas *et al.*, 2008; Tang *et al.*, 2009). Individual genes were also cloned into pCBIG to serve as single factor controls. We verified the expression of individual proteins by transfecting HEK293T cells, and conducting subsequent immunocytochemistry and western blotting.

3.2. Mice

All mouse studies were approved by the Harvard University and/or Massachusetts General Hospital Institutional Animal Care and Use Committee, and were performed in accordance with institutional and federal guidelines. The date of vaginal plug detection was designated E0.5, and the day of birth was designated as postnatal day 0 (P0). Wild-type C57BL/6 and CD1 mice were purchased from Charles River Laboratories (Wilmington, MA). The NG2.DsRed.BAC mouse line was generated by Nishiyama and colleagues (Zhu *et al.*, 2008a), and was procured from Jackson Laboratories (stock number: 008241). Emx1-Cre (RRID: IMSR_JAX:005628, stock number 005628), Rosa26R-ZsGreen-Ai6 (RRID: IMSR_JAX:007906, stock number: 007906) mice were purchased from Jackson Laboratories.

3.3. *In utero* Electroporation

Timed pregnant CD1 dams at embryonic day (E)15.5 of gestation were anaesthetized with isoflurane, and an incision was made in the abdomen. The uterine horns were exposed and gently positioned on a sterile piece of gauze. Approximately 1-2 micrograms of plasmid DNA (1.0 μg/ul) mixed with 0.005% Fast Green in sterile PBS was injected *in utero* into the

lateral ventricle of the embryos. Injections were performed using beveled glass micropipettes (tip diameter of 30–60 um) and by mouth pipetting with an aspirator tube assembly (Sigma, A5177). Electroporation of the plasmids was performed by placing a positive electrode (tweezer electrodes, 5mm diameter) above the cortex and a negative electrode behind the head, and applying five pulses of current at 40V for 50 milliseconds per pulse with 1 second intervals between pulses (CUY21Edit Electroporator, Bex Co. Ltd.). Brains were collected at P2 for CPN culture, and at P7 for ENVOF mis-expression analysis.

3.4. Purification and Culture of Cortical NG2-progenitors

NG2-DsRed pups at P2-P5 were anaesthetized in ice, meninges were removed from their brains, and neocortices were micro-dissected in ice-cold dissociation medium (20mM glucose, 0.8mM kynurenic acid, 0.05mM DL-2-amino-5-phosphonopentanoic acid (APV), 100U/ml penicillin, 100μg/ml streptomycin (Gibco, 15140122), 0.09M Na_2SO_4, 0.03M K_2SO_4 and 0.014M $MgCl_2$; pH=7.35), and enzymatically digested in dissociation medium (pH=7.35) containing 0.16 mg/ml DL-Cysteine hydrochloride, 10U/ml papain (Worthington) and 30 U/ml DNAse I at 37°C for 30min, followed by rinsing with ice-cold OptiMem (Gibco, 51985-034) containing 20mM glucose, 0.4mM kynurenic acid and 0.025mM APV to protect against glutamate-induced neurotoxicity (Catapano et al., 2001). Cortices were mechanically dissociated by gentle trituration using fire-polished Pasteur glass pipets to create a single-cell suspension. Dissociated cells were centrifuged at 200g for 5 minutes at 4°C, re-suspended (5-10x10^6 cell/ml), and filtered through a 40 μm cell strainer (Corning, 352235). All chemicals were purchased from Sigma-Aldrich, unless stated otherwise.

Cells were purified based on DsRed fluorescence intensity using the BD FACSAria II and III cell sorter on four-way purity mode. DsRed-positive cells from the NG2.DsRed BAC-transgenic mouse cortex consists of two distinct populations based on DsRed fluorescence intensity; dim and bright populations. Only the bright population, which yielded around 200-300K cells/brain, was purified for induced neurogenesis experiments. A previously published protocol was adapted to maintain cells in a proliferative progenitor state (Najm *et al.*, 2013). Purified cells were seeded on poly-D-lysine (PDL) (50 ug/ml) (Sigma, P0899) coated cover glasses (Fisher, 12-545-81) in 24-well plates (~10K cell/cm^2)

(Corning, 353047), and cultured in growth medium containing DMEM/F12 with GlutaMAX (Gibco, 10565018), 15mM HEPES (Gibco, 15630080), B27 without vitamin A (Gibco, 12587010), N2-max (R&D Systems, AR009), 100U/ml penicillin, 100μg/ml streptomycin (Gibco, 15140122), 10 ng/ml PDGF-A (Peprotech, 315-17) and 10 ng/ml Fgf2 (Peprotech, 450-33). One half of the growth medium in each well was replaced every other day, and transfection was performed at ~5 DIV using Fugene 6 (Promega) (1.2:1, lipofection reagent:DNA ratio). On the day following transfection, growth medium was replaced with neuronal induction medium (1:1 mix of DMEM/F12 (Gibco, 10565018) and Neurobasal (Gibco, 10888022) media that contains GlutaMAX, 15mM HEPES, B27 (with vitamin A) (Gibco, 17504044), N2 (Gibco, 17502-048), 100U/ml penicillin and 100μg/ml streptomycin (Gibco, 15140122). One half of the neuronal induction medium per well was replaced every third or fourth day-post-transfection until fixation.

For co-culture of NG2 progenitors/induced neurons with primary forebrain neurons, primary forebrain neurons were obtained from P0-P1 CD1 wild-type pups using the standard protocol described above, and were directly added onto NG2 cell culture with neuronal induction medium (25K/cm^2) 24-hr post-transfection of NG2-progenitors. One half of the medium per well was replaced once every three days.

3.5. Labeling and Culture of SCPN and CPN

Subcerebral projection neurons (SCPN) were labeled from their axonal projections in the cerebral peduncle bilaterally at P0-P1 by pressure injection (Nanoject II, Drummond) of Alexa Fluor 647-conjugated cholera toxin, subunit B (CTB) (Invitrogen) (six shots, 23nl/shot, 2μg/ul) using pulled and beveled glass micropipettes with a tip diameter of 30–50 μm. Injections were performed using a Vevo 770 ultrasound backscatter microscopy system (VisualSonics) to visualize the injection site. The fluorescent reporter line Rosa26R-ZsGreen-Ai6 (crossed to Emx1.Cre) was used for SCPN labeling and isolation for efficient identification for electrophysiology. ZsGreen and Alexa-647 double positive SCPN were FACS-purified and co-cultured with cortical primary neurons obtained from ZsGreen-negative littermates. Brains were collected at P2 for cell culture, and success of retrograde labeling was verified under a fluorescence-equipped dissecting microscope (SMZ-1500; Nikon), prior to dissociation.

Callosal projection neurons (CPN) were labeled via *in utero* electroporation (at E15.5, the time of peak superficial layer CPN production) of a tdTomato reporter driven by CMV-beta-actin promoter (modified from pCBIG; gift of C. Lois, Caltech). Brains were collected at P2, and the electroporated hemispheres were visualized with a fluorescence-equipped dissecting microscope and dissected out for culture. tdTomato fluorescence was used to FACS-purify labeled CPN after dissociation of cortical hemispheres. Purified CPN were co-cultured with dissociated cortical neurons from non-electroporated brains. Although sufficient numbers of labeled CPN for electrophysiology could be found when plated at comparable densities directly after dissociation (without FACS purification; unlike SCPN), we opted to FACS purify and plate CPN onto reporter negative neurons so that both SCPN and CPN experienced similar handling and culture conditions, prior to electrophysiology. CPN and SCPN labeled cortices were dissociated following the protocol described above for NG2 progenitors. For both CPN and SCPN culture, neurons are dissociated at P2, seeded at the density of $6x10^4$ cell/cm^2 in PDL coated 24-well plates, and cultured in the neuronal media described above.

3.6. Primary Culture of Cortical Astrocytes

We followed a previously published protocol for primary culture of cortical astrocytes (Heinrich *et al.*, 2011), together with advice from Pratibha Tripathi, HSCRB, Harvard University. Briefly, cerebral cortices were micro-dissected from wild-type P5-P7 CD1 pups and dissociated following the standard protocol described above. Dissociated cells were seeded in PDL-coated T75 flasks and cultured in astrocyte growth medium (DMEM/F12, 10% fetal calf serum, 5% horse serum, B27 (with vitamin A), 100U/ml penicillin and 100μg/ml streptomycin (Gibco, 15140122), 10ng/ml EGF (Peprotech, 315-09) and 10 ng/ml FGF2 (Peprotech, 450-33). Medium was fully changed 24-hr post-culturing, and half of the medium was again replaced three days-post-culturing. This culture consisted predominantly of astrocytes as revealed by their morphology and GFAP expression.

For preparing astrocyte-conditioned medium, astrocytes were passaged at ~5 DIV using trypsin (Gibco, 25200056), diluted ~1:5, re-seeded in PDL-coated T75 flasks containing astrocyte growth medium, and cultured for 24hrs. The growth medium was

subsequently replaced with neuronal induction medium (described above), and astrocytes were incubated for 10 days; then the conditioned medium was collected, and aliquots were made and stored at -80°C. One batch of astrocyte culture was used for two rounds of medium collection.

3.7. Immunocytochemistry and Antibodies

Immunocytochemistry (ICC) for tissue sections was performed following standard protocols. Briefly, mice were transcardially perfused with phosphate-buffered saline (PBS), then with 4% PFA, dissected, and post-fixed overnight at 4°C in 4% paraformaldehyde. Brains were sectioned at 50 μm on a vibrating microtome (Leica). Fixed tissues are stored in PBS with 0.025% sodium azide. Floating sections were blocked with 0.3% BSA (wt/vol) (Sigma, A3059), 0.3% Triton X-100 (Sigma, T8787), and 0.025% sodium azide (Sigma, S2002) in PBS for 30 min. Primary antibodies were diluted in the same blocking solution, and incubated with sections for 4 hours at room temperature (RT) or overnight at 4°C. Sections were rinsed three times with PBS for 10 minutes, and incubated with appropriate secondary antibodies diluted in blocking solution for 2-3 hours at RT. Sections were again rinsed three times with PBS, and mounted using Fluoromount with DAPI (SouthernBiotech, 0100-20) for image acquisition.

Immunocytochemistry for cultured cells was performed as follows: Cells were fixed in 4% paraformaldehyde at RT for 10 minutes, rinsed three times with PBS, and stored in PBS with 0.025% sodium azide at 4°C. Before primary antibody treatment, cells were blocked in the blocking solution for 15 minutes, incubated with primary antibodies for 2 hours, rinsed with PBS three times for 5 minutes, incubated with secondary antibodies for 45 minutes, rinsed with PBS three times for 5 minutes, all reactions at RT, and then mounted using Fluoromount with DAPI.

The following antibodies and dilutions were used: rat anti-BrdU, 1:500 (ACSC, OBT-0030); rabbit anti-CTIP2, 1:200 (Abcam, ab28448); rat anti-CTIP2, 1:200 (Abcam, ab18465); rabbit anti-Cux1, 1:200 (Santa Cruz Biotechnology, sc-13024); rabbit anti-FOG2, 1:250 (Santa Cruz Biotechnology, sc-10755); mouse anti-GABA, 1:200 (Sigma, A0310); mouse anti-GFAP, 1:1000 (Sigma, G3893); rabbit anti-GFAP, 1:1000 (Sigma, G9269);

chicken anti-GFP, 1:500 (Molecular Probes, A10262); rabbit anti-GFP, 1:500 (Molecular Probes, A11122); mouse anti-NeuN, 1:500 (Chemicon, MAB377); rabbit anti-NG2, 1:500 (Millipore, AB5320); mouse anti-h/rNGN2, 1:100 (R &D Systems; MAB3314); goat anti-OLIG2, 1:200 (R&D Systems, AF2418); mouse anti-SATB2, 1:200 (Abcam, ab51502); rabbit anti-SATB2, 1:500 (Abcam, ab34735); goat anti-Sox 10; 1:200 (Santa Cruz, sc-17342); rabbit anti-beta-tubulin (Tuj1), 1:1000 (Sigma, T2200); mouse anti-beta-tubulin (Tuj1), 1:1000 (Covance/Biolegend MMS-435P), rabbit anti-vGLUT1, 1:500 (Sigma, 052K4832); mouse anti-MAP2, 1:500 (Sigma, M1406); rabbit anti-Synapsin, 1:1000 (Synaptic Systems, 106002); rabbit anti-TBR1, 1:200 (Abcam, ab31940); rabbit anti-2A-peptide, 1:1000 (Millipore, ABS31); NF-M, 1:200 (Millipore, AB1987); rabbit anti-Sox6, 1:500 (Abcam, AB30455); mouse anti-Synaptophysin, 1:1000 (Millipore, MAB5258); rabbit anti-PDGF Receptor beta, 1:100 (Cell Signaling, 3169); chicken anti-Nestin, 1:2000 (Novus, NB100-1604); rabbit anti-DARPP-32, 1:250 (Cell Signaling Technology, 2306S); mouse anti-HA tag, 1:1000 (Covance, MMS-101R); rabbit anti-VP16 tag, 1:200 (Abcam, ab4808); rabbit PCP4, 1:500, (Proteintech, 14705-1-AP); rabbit anti-GAD1, 1:500 (Synaptic Systems, 198013); PSA-NCAM, 1:500 (Chemicon, MAB5324). Appropriate secondary antibodies from the Molecular Probes Alexa Series were used (1:1000, Invitrogen). A forebrain neuron primary culture control was included in immunocytochemistry experiments to verify that all antibodies and procedures were working as expected.

3.8. Imaging and Quantification

Wide-field image acquisition was performed with a Nikon 90i epifluorescence microscope equipped with a Clara DR-328G cooled CCD digital camera (Andor Technology). Images were assembled in Adobe Photoshop and Illustrator (CS5), with adjustments for contrast and brightness. Identical procedures were applied across different experimental conditions.

For cell quantifications, an area of 7mm^2 of cover glass was imaged using a 10x objective. The acquired image was binned as 1mm^2 boxes using NIS-elements software (Nikon), individual boxes were randomly selected, and all the GFP+ cells in each selected box were counted. Microsoft Excel was used for plotting graphs. All immunocytochemistry experiments were performed on a minimum of three independent biological replicates.

3.9. modRNA Synthesis

Fezf2-HA, EGFP, tdTomato, Neurog2, Neurog2-2A-VP16::Olig2, Cre open reading frames were cloned into pORFin or pORFinB vectors (from Rossi Lab, HSCRB and Boston Children's Hospital). pORFin vectors have appropriate 5' and 3' UTR sequences flanking the cloning sites and an upstream T7 promoter for in vitro transcription. modRNA was synthesized following a published protocol (Mandal and Rossi, 2013). The standard Fugene 6 (Promega) protocol described above was used for modRNA transfections.

3.10. Electrophysiology

Electrophysiological recordings were performed at 20-25°C on an Olympus BX51WI microscope. Cells were bathed in artificial cerebral spinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 4 $CaCl_2$, 4 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose. ACSF was continuously saturated with 95% O_2/5% CO_2. Intracellular recordings were obtained using glass micropipettes filled with an internal solution containing (in mM): 136 $KMeSO_3$, 17.8 HEPES, 0.6 $MgCl_2$, 1 EGTA, 4 Mg-ATP, and 0.3 Na-GTP. Traces were collected using a Multiclamp 700B amplifier (Molecular Devices), filtered with a 2 kHz Bessel filter, digitized at 50 kHz using a Digidata 1440A digitizer (Molecular Devices), stored using Clampex 10 (Molecular Devices), and analyzed off-line using customized procedures written in Igor Pro (WaveMetrics). Series resistance was monitored through the experiment. Cells at DPI/DIV 15-16 were identified visually by fluorescence. Action potentials were evoked by injection of current steps, ranging from –140pA to 400pA in 60-pA increments, with a duration of 600ms. Action potential parameters were quantified using the first action potential evoked at the lowest current injection that resulted in an action potential. Threshold was defined as the voltage at which dV/dt of the action potential waveform reached 10% of its maximum value, relative to a dV/dt baseline taken 10ms before the peak. Action potential amplitude was defined as the difference between the threshold value (in mV) and the maximum voltage at the peak of the action potential. Width was measured at half-maximum amplitude. Sag current was measured during a -140pA step current for a duration of 600ms.

4. DIRECTED DIFFERENTIATION OF CORTICOFUGAL PROJECTION NEURONS FROM ENDOGENOUS CORTICAL PROGENITORS

4.1. Summary

Neurons across the central nervous system (CNS) belong to various classes differing from each other in cardinal features including cellular morphology, patterns of connectivity, and electrophysiological and neurochemical properties (Sugino *et al.*, 2005; Usoskin *et al.*, 2014). This diversity, specifically within the cerebral cortex, underlies the tremendous repertoire of information processing and cognitive abilities of the mammalian brain (Fishell and Rudy, 2011; Greig *et al.*, 2013; Harris and Shepherd, 2015).

Specific neuronal subpopulations are selectively affected in distinct neurodegenerative, developmental, and acquired diseases of the CNS, resulting in irreversible functional deficits (Saxena *et al.*, 2011). Particularly relevant to this work, corticospinal motor neurons (CSMN) centrally degenerate (along with spinal motor neurons) in amyotrophic lateral sclerosis (ALS) and other motor neuron diseases, and loss of motor function in spinal cord injury results from damage to CSMN axons in the corticospinal tract (Rosler *et al.*, 2000).

Repair of damaged neuronal circuitry will involve integration of new relevant subtypes of neurons at the correct location, receiving circuit-appropriate inputs, and making efferent connections with their *in vivo* synaptic partners. The evidence that active and quiescent progenitors exist in restricted regions of the adult brain, and the demonstration that new neurons can integrate into preexisting neural circuitry, support the feasibility of cellular repair in the CNS (Kempermann *et al.*, 2015). Successful integration of new neurons, even at low levels, might help to partially restore function, and ameliorate disease symptoms.

In situ generation of neurons from local progenitors offer distinct advantages over transplantation of *ex vivo* generated iPS-derived neurons or recruitment of new neurons from

existing neurogenic niches in the brain: 1) it circumvents the requirement for new neurons to migrate to their site of incorporation after transplantation or recruitment, and 2) it potentially enables better integration at a single cell level, since transplantation often result in heterotopias that are likely harmful.

Recently, there has been substantial progress in reprogramming of injury-induced reactive glia *in vivo* (Gascon *et al.*, 2016), and proliferative glial cells *in vitro* into neurons (Heinrich *et al.*, 2010). Yet, functional repair of circuitry requires faithful generation of specific types of neurons in order to replace the function of the degenerated or injured neurons. Complementing these efforts, work from several labs has begun to identify central molecular controls over development of broad classes and specific subtypes of cortical projection neurons (Arlotta *et al.*, 2005; Leone *et al.*, 2008; Woodworth *et al.*, 2012; Greig *et al.*, 2013; Lodato and Arlotta, 2015). These molecular controls potentially enable induction of a more precise neuronal subtypes from progenitors, beyond a generic neurotransmitter identity, which can be then be used for the repair of neuronal circuitry.

Under physiological conditions, the primary proliferative neural progenitor population in the adult mammalian cortex consists of progenitors characterized by the shared expression of the proteoglygan NG2 (Chondroitin sulphate proteoglycan 4, Cspg4) (Mori *et al.*, 2009). Constituting ~5% of total cells in the adult rodent brain, NG2-expressing progenitors are evenly distributed throughout white and gray matter, and remain proliferative from early postnatal stages into adulthood and in the aged CNS (Bergles and Richardson, 2016). In the rodent cerebral cortex, NG2-progenitors are generated from diverse ventricular zone regions in distinct temporal waves, and efficiently tile the cortex early during postnatal life (Kessaris *et al.*, 2006). NG2-progenitor cells exhibit diversity in terms of their physiological properties and marker expression, suggesting that this progenitor pool may consist of several subpopulations, or it may reflect their distinct response to the regional heterogeneity of extrinsic signals (Hill and Nishiyama, 2014; Dimou and Gallo, 2015). Apart from generating oligodendrocytes throughout life during myelination and remyelination, a subset of NG2 progenitors also generate astrocytes in the embryonic ventral cortex (Zhu *et al.*, 2008a). Importantly, the density of NG2-progenitors in the adult brain is maintained via a homeostatic mechanism such that loss of progenitors through differentiation or cell death is compensated by local proliferation and migration of nearby progenitors (Hughes *et al.*,

2013). These cellular and molecular features of NG2 progenitors make them a rational target for neuronal replacement approaches in the CNS.

Here, we report the directed differentiation of corticofugal projection neurons (CFuPN), a broad group of clinically relevant neurons that includes CSMN, by the forced expression of a select set of developmental transcriptional controls in isolated mouse neocortical NG2 progenitors. Newly generated neurons acquire morphological, molecular and electrophysiological characteristics similar to their primary *in vivo* counterparts. We then apply synthetically modified RNA technology for precise control of expression dynamics of the transcriptional regulators, and show that even a single pulse of modRNA can induce neuronal identity in cortical NG2 progenitors, enabling the future work to improve functional maturation and faithful acquisition of CFuPN identity of induced neurons. Our proof-of-concept experiments demonstrate the feasibility of achieving subtype–specific differentiation of cortical projection neurons from a widely distributed *in vivo* cortical progenitor population, and has significant implications for efforts towards *in situ* repair of damaged cortical circuitry.

4.2. Results

4.2.1. Isolation and Culture of Cortical NG2-Progenitors

NG2-progenitors are distributed widely in the postnatal brain, and express transcriptional controls such as Sox6, Sox10, and Olig2 (Figure 4.1a, f, and data not shown). We isolated cortical NG2-expressing progenitors using Fluorescence Activated Cell Sorting (FACS) from a bacterial artificial chromosome (BAC) transgenic NG2-DsRed mouse line (DsRed, Discosoma species red fluorescent protein) (Zhu *et al.*, 2008a) from dorso-lateral cerebral cortex (neocortex) of postnatal pups (P2-P6) (Figure 4.1b-c). Cells were purified based on DsRed fluorescence intensity on high four-way purity precision mode. DsRed-positive cells from the transgenic mouse cortex appeared as two distinct populations based on DsRed fluorescence intensity: bright (DsRed++) and dim (DsRed+) populations (Figure 4.1d). Immunocytochemistry (ICC) characterization of the two populations showed that the DsRed++ population consists of almost exclusively of NG2 progenitors (>99.99%) (Figure 4.1e-g, and data not shown); whereas the DsRed+ population consists of a mix of PDGFR-

ß+ pericytes (which also express NG2 proteoglycan, and thus are DsRed positive) (Figure 4.1l), GFAP+/Nestin+/NG2+cells (Figure 4.1m), and NG2-progenitors (Figure 4.1n). The DsRed++ population was used for all the subsequent experiments for the induction of cortical projection neurons. Cortical NG2 progenitors proliferate robustly in culture in response to mitogens PDGF-A and FGF-2 (Figure 4.1h-k), and maintain expression of key transcriptional controls (Figure 4.1e-g). Previous work showed that Sox6 is expressed by proliferating NG2-progenitors, and is down-regulated upon differentiation (Stolt *et al.*, 2006; Baroti *et al.*, 2016). Under these culture conditions, FACS-purified cortical NG2 progenitors express Sox6 (Figure 4.1g), indicating maintenance of their progenitor identity.

4.2.2. Identification of Molecular Controls for Directed Differentiation of Corticofugal Projection Neurons (CFuPN) from Cortical NG2-expressing Progenitors

To direct the differentiation of CFuPN/SCPN from cortical NG2+ progenitors, we selected a set of transcriptional controls based on their developmental functions. To drive glutamatergic neuronal identity in cortical NG2 progenitors, we selected the pallial proneural transcription factor Neurogenin 2 (Ngn2), a critical regulator of embryonic neurogenesis in the neocortex (Schuurmans *et al.*, 2004; Mattar *et al.*, 2008). Previous data showed that forced expression of Ngn2 reprograms cultured postnatal glia and human ESC/iPSCs into synapse forming glutamatergic neurons *in vitro* (Heinrich *et al.*, 2010; Zhang *et al.*, 2013), and can induce immature neuron-like cells from injury induced reactive glial cells in the adult mouse brain (Gascon *et al.*, 2016).

Olig2, a bHLH transcription factor, is necessary for the specification of NG2-progenitors and for their differentiation into oligodendrocytes, and also functions in addition to its function in the development of spinal motor neurons (Li and Richardson, 2015). Intriguingly, antagonizing Olig2 function in reactive glial cells after injury results in a substantial number of immature neurons in the cortical parenchyma (Buffo *et al.*, 2005). In addition, Olig2 promotes gliogenesis against neurogenic functions of Pax6 in SVZ progenitors (Hack *et al.*, 2005; Marshall *et al.*, 2005), and has been shown to antagonize Ngn2 activity during neurogenesis to maintain progenitors for subsequent gliogenesis during spinal cord development (Lee *et al.*, 2005). Also, multiple lines of evidence indicate that Olig2 mediates anti-neurogenic effect by functioning as a repressor; an activator form of

Olig2 (VP16:Olig2, VP16 transactivation domain from herpes simplex virus fused to an Olig2 DNA binding domain) functions as a dominant negative to counteract Olig2 gliogenic function (Mizuguchi *et al.*, 2001; Novitch *et al.*, 2001; Zhou *et al.*, 2001). Hence, to overcome the predominant gliogenic program in NG2 progenitors, we decided to complement Ngn2 with VP16:Olig2.

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To induce CFuPN/SCPN fate in new neurons, we selected Fezf2, an upstream transcriptional regulator that controls specification and development of CSMN/SCPN during cortical neurogenesis (Chen *et al.*, 2005; Molyneaux *et al.*, 2005). Fezf2 is capable via single gene over-expression of generating SCPN/CSMN from alternate cortical progenitors (Molyneaux *et al.*, 2005), inducing glutamatergic SCPN-like neurons from progenitors of striatal neurons *in vivo* (Rouaux and Arlotta, 2010), and of reprograming other classes of cortical projection neurons to CFuPN/SCPN fate post-mitotically in the early postnatal brain *(De la Rossa et al., 2013)*.

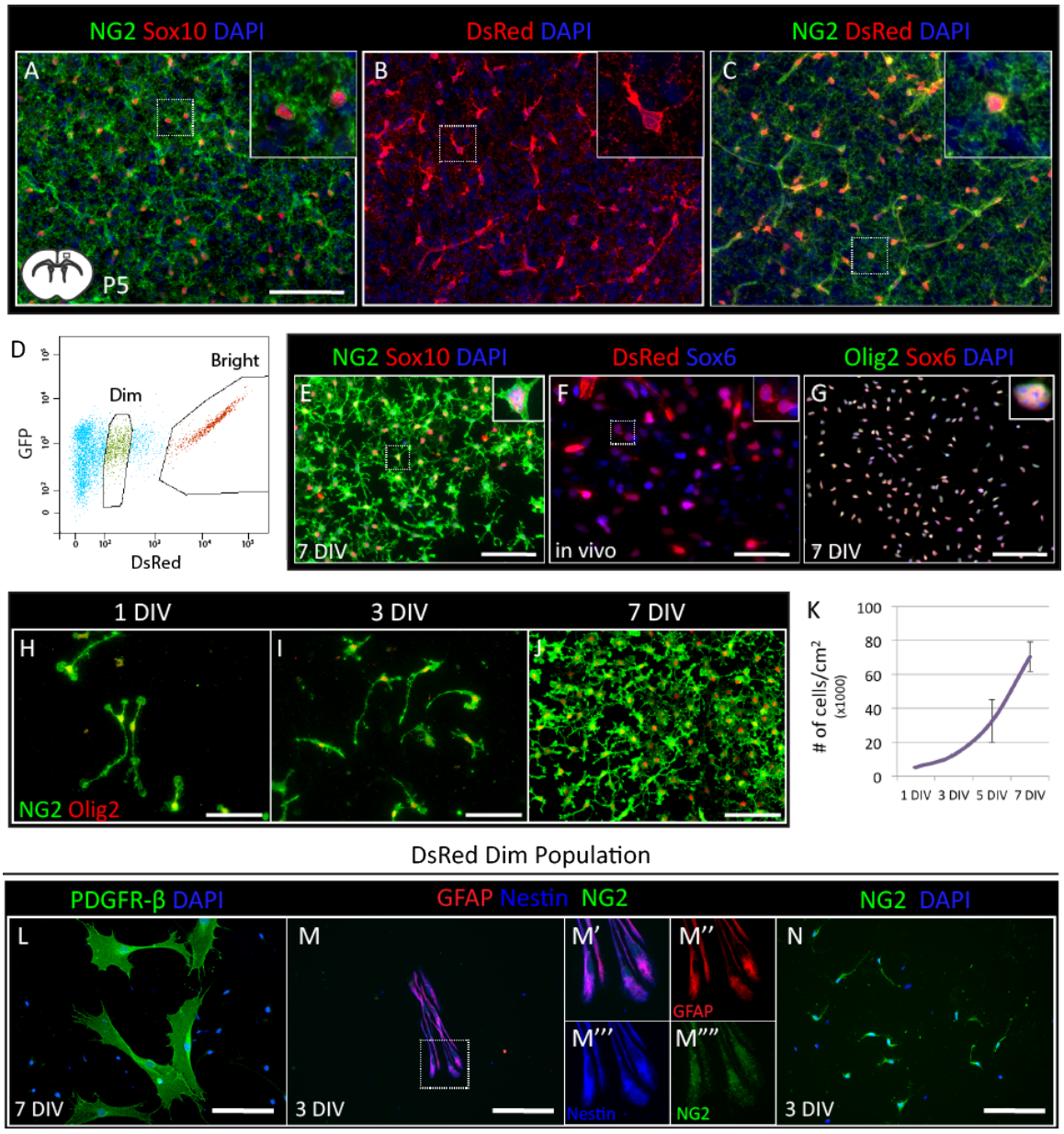

Figure 4.1. FACS-purified NG2+ cortical progenitors can be cultured with exceptional purity, and maintained *in vitro*.

Figure 4.1 (A) Immunocytochemical staining of NG2 and Sox10 showing the distribution of NG2 cortical progenitors in postnatal mouse brain (P5). Schematic in A depicts a coronal brain section identifying the region shown in A, B, C and F. (B) Distribution of DsRed-positive cells in the cortex of a postnatal NG2-DsRed transgenic mouse shows faithful expression of DsRed reporter in NG2+ progenitors (and pericytes on blood vessels) (P5). (C) Immunostaining for NG2 shows that NG2+ cortical progenitors

express DsRed. Inset shows the cell boxed in (C) with strong DsRed signal largely restricted to the cell body while NG2 proteoglycan is present both around the main cell body and in cellular processes. (D) DsRed-positive cortical cells consist of two distinct populations on a FACS plot based on DsRed fluorescence intensity. (E-G) The bright population exclusively consists of NG2 progenitors that express molecular controls Sox10 (E), Olig2 and Sox6 (G) (7 DIV). (F) Expression of Sox6 in NG2-DsRed+ progenitors *in vivo* (P5, cortex). (H-K) Culture conditions were optimized to promote proliferation of progenitors (bright population). NG2+ cortical progenitors proliferate robustly in culture and the number of cells expands over 7-fold in a week (K) (n=2). (L-N) Analysis of DsRed+ 'Dim' population shows that it consists of a mixture of pericytes (L, PDGFR-β+); GFAP, Nestin and NG2 positive clustered cells (M), and NG2+ progenitors (with relatively low NG2 expression compared to bright population) (N). All scale bars are 100 μm, except 1F (50 μm). Error bars show standard deviations.

We generated a polycistronic vector, driven by the CMV-β-actin promoter, containing 4 open reading frames: EGFP, Ngn2, VP16:Olig2, and Fezf2 with an HA tag (referred to here as ENVOF) utilizing 2A linkers from picornaviruses (Tang *et al.*, 2009) (Figure 4.2a). We first verified expression of individual proteins from the polycistronic construct in HEK cells by immunocytochemistry and western blotting (Figure 4.2b-d, and data not shown). To further assess functionality of this polycistronic construct, we tested it in cortical embryonic progenitors *in vivo*. Previous work has demonstrated that mis-expression of Fezf2 in embryonic cortical progenitors that give rise to callosal projection neurons (CPN), converts them to cortical output neurons that send their axons to subcortical targets (Molyneaux *et al.*, 2005). To test whether this function of Fezf2 was still intact in the presence of Ngn2 and VP16:Olig2, we electroporated ENVOF embryonic ventricular zone progenitors *in utero* at E15.5, during the peak production of CPN. We found that forced expression of ENVOF in CPN progenitors was sufficient to induce a corticofugal identity in electroporated neurons (Figure 4.3). Analyses of ENVOF electroporated brains at P7 show that, unlike control GFP-only expressing neurons (Figure 4.3a-b), large numbers of ENVOF+ axons descend through the internal capsule (Figure 4.3c'), many axons reach the thalamus (Figure 4.3c'''), and some reach the cerebral peduncle (Figure 4.3d). These data indicate that i) the multigene construct ENVOF is functional, and ii) the ability of Fezf2 to

re-route the axonal trajectory of upper layer neurons is not compromised by co-expression of Ngn2 and VP16:Olig2.

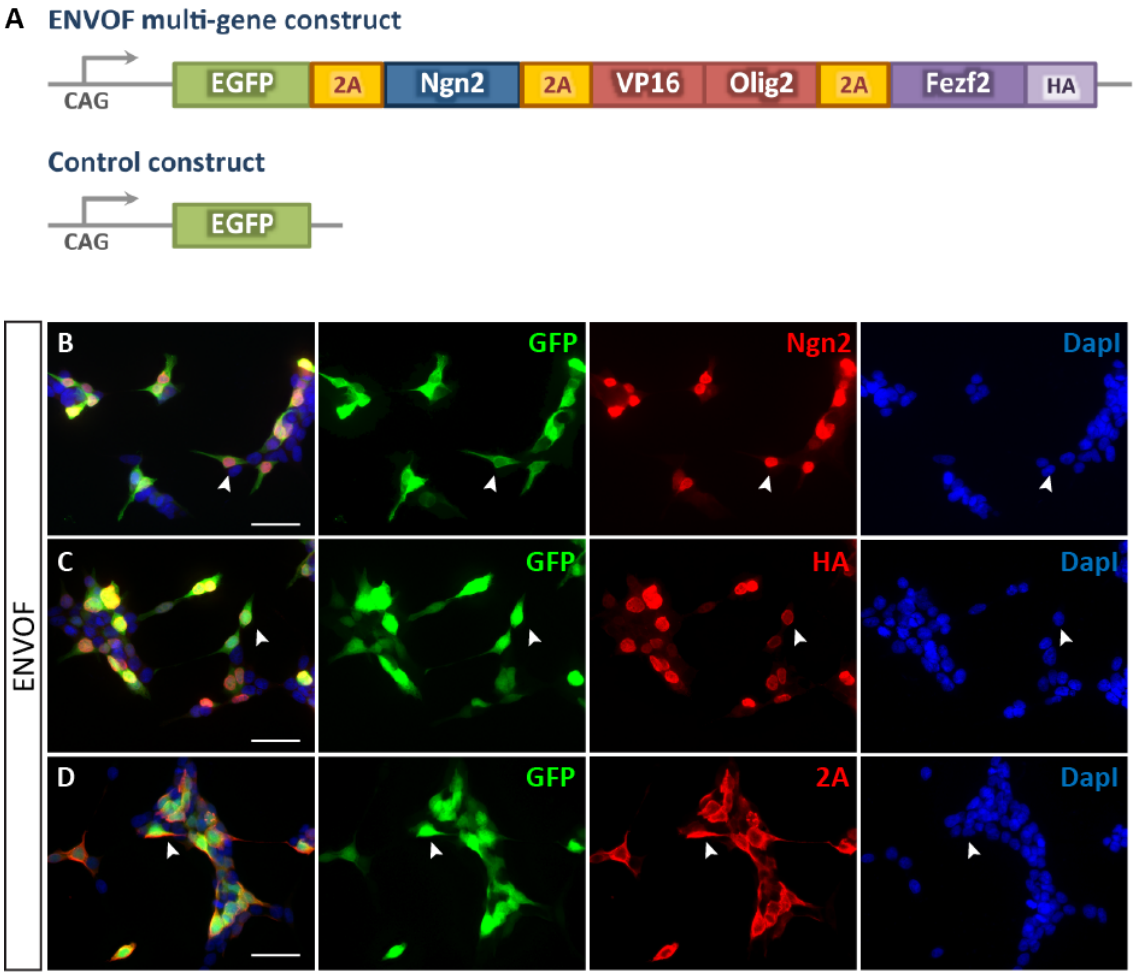

Figure 4.2. Individual molecular controls are expressed from the multigene construct, ENVOF.

Details of Figure 4.2: (A) Schematics of the multigene construct-ENVOF showing individual molecular controls: Ngn2, VP16:Olig2, Fezf2 and EGFP reporter, and control EGFP construct. (B-D) Immunofluorescence analysis of HEK293 cells transfected with ENVOF confirms that GFP, Ngn2 (B) and Fezf2 (with an HA tag) (C) proteins are expressed appropriately. Immunocytochemistry for 2A peptide reports expression of EGFP, Ngn2, and VP16:Olig2 (D). Scale Bars: 50 µm.

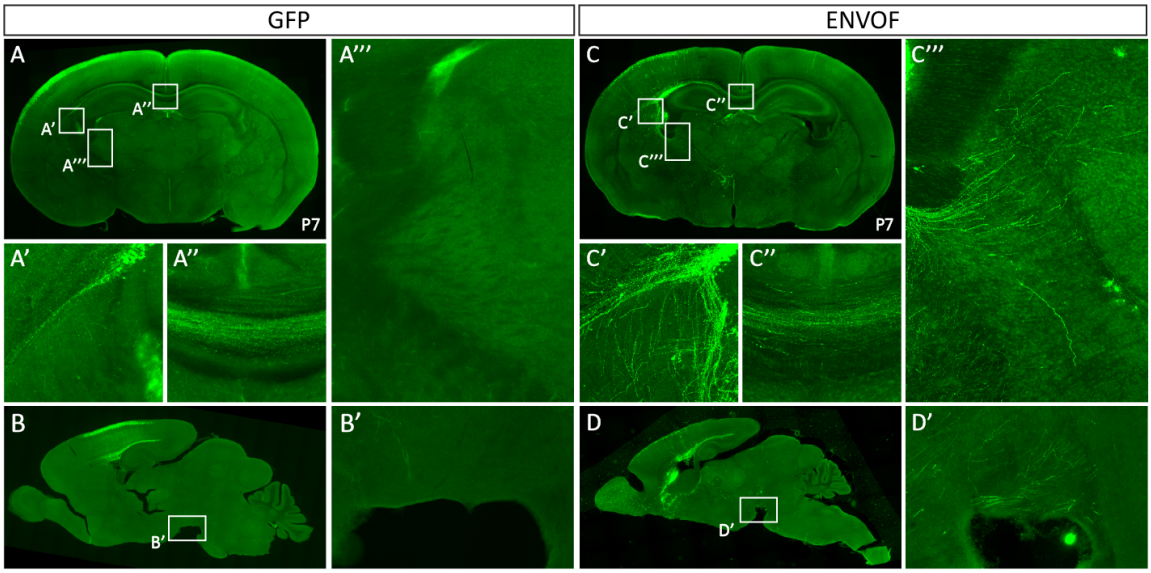

Figure 4.3. ENVOF multigene construct redirects the axons of later-born upper layer neurons to subcortical targets, similar to deep layer cortical output neurons.

Details of Figure 4.3: E15.5 embryos electroporated with control vector (EGFP) (A, B) and ENVOF multigene construct (C, D), and analyzed at P7. (A, B) Control vector electroporated GFP-positive neurons migrate to the cortical plate (A') and project to the contralateral cortex (A''), but not to subcortical targets (A''', B, B'). (C, D) In ENVOF electroporated mouse brains, a large number of GFP+ axons descend through the internal capsule (C') toward the thalamus (C''') and a few of these axons even reach the cerebral peduncle by P7 (D, D'). Some ENVOF electroporated neurons still send their axons to the contralateral hemisphere through the corpus callosum (C"). n=4.

4.2.3. ENVOF Induces Neuronal Identity in Cortical NG2-Progenitors

Next, we tested ENVOF in cultured cortical NG2 progenitors. Upon transfection with the ENVOF construct, progenitors begin to lose their multipolar morphology within 24-hours (Figure 4.4a vs. 4.4b), and by 72-hours post-transfection, extend a single long process (Figure 4.4c vs. 4d), and express the immature neuronal marker Tuj1 (42%) (Figure 4.4i, 4.5c-d). This morphological transformation is coupled with loss of progenitor markers such as NG2, Sox10, and Sox6 (Figure 4.5a-b vs. 4.5c-d, and data not shown). By 7 days-post-transfection (DPT), more than 70% of ENVOF-transfected progenitors express Tuj1,

and acquire bipolar neuronal morphology (Figure 4.4i and 4.4l) with dendrite-like features and a single prominent axon-like process (Figure 4.4f and 4.4l). In contrast, progenitors transfected with control GFP constructs displayed glial morphology throughout the period of culture, and none of them were Tuj1-positive (Figure 4.4i and 4.5e-f) (n=4).

The primary axon-like process of ENVOF-directed neurons undergoes significant extension between 3 and 7 days, with many cells (>10%, n=3) extending a primary process of length greater than 700μm (Figure 4.4j-k). By 16 DPT, the morphology of neuron-like cells becomes more elaborate; while preserving the overall bipolar morphology pattern with a single long axon-like neurite, dendrite-like structures become more tufted, and axonal branches of neighboring cells become intercalated (Figure 4.4h and 4.4m).

Next, we asked whether these morphologically neuron-like Tuj1-positive cells truly exhibit the appropriate molecular features of neurons. Our analyses at 7DPT showed that ENVOF-induced Tuj1+ cells express the somato-dendritic marker MAP2 (>90%, n=4, 130-200 cell) and the somato-axonal marker NF-M (Figure 4.6a-b), demonstrating polarization and dendritic compartmentalization. Confirming this finding, high-power imaging showed that the neurons have a single axon-like primary process, and dendrite-like processes with numerous filopodial protrusions, while these structures were absent from the presumptive axons (highlighted with red arrows) (Figure 4.6c).

Furthermore, ENVOF-induced neurons express polysialylated neural cell adhesion molecule (PSA-NCAM) (Figure 4.6d-e), neuronal nuclear antigen (NeuN) (66 ± 16%, n=4, >100 cell) (Figure 4.6f), and a majority of cells express the presynaptic molecule synapsin (Figure 4.6g), and albeit at a lower number, synaptophysin in the axonal branches and at the tips of axonal protrusions (Figure 4.6h), together indicating ongoing neuronal maturation at 7DPT. We have confirmed that ENVOF-directed neurons express vGlut1 (vesicular glutamate transporter 1) (Figure 4.6i), and do not express inhibitory neurotransmitter GABA (γ-aminobutyric acid), (Figure 4.6j), together indicating their glutamatergic identity. Taken together, these data indicate that ENVOF robustly induces neuronal identity in cortical NG2-progenitors *in vitro*.

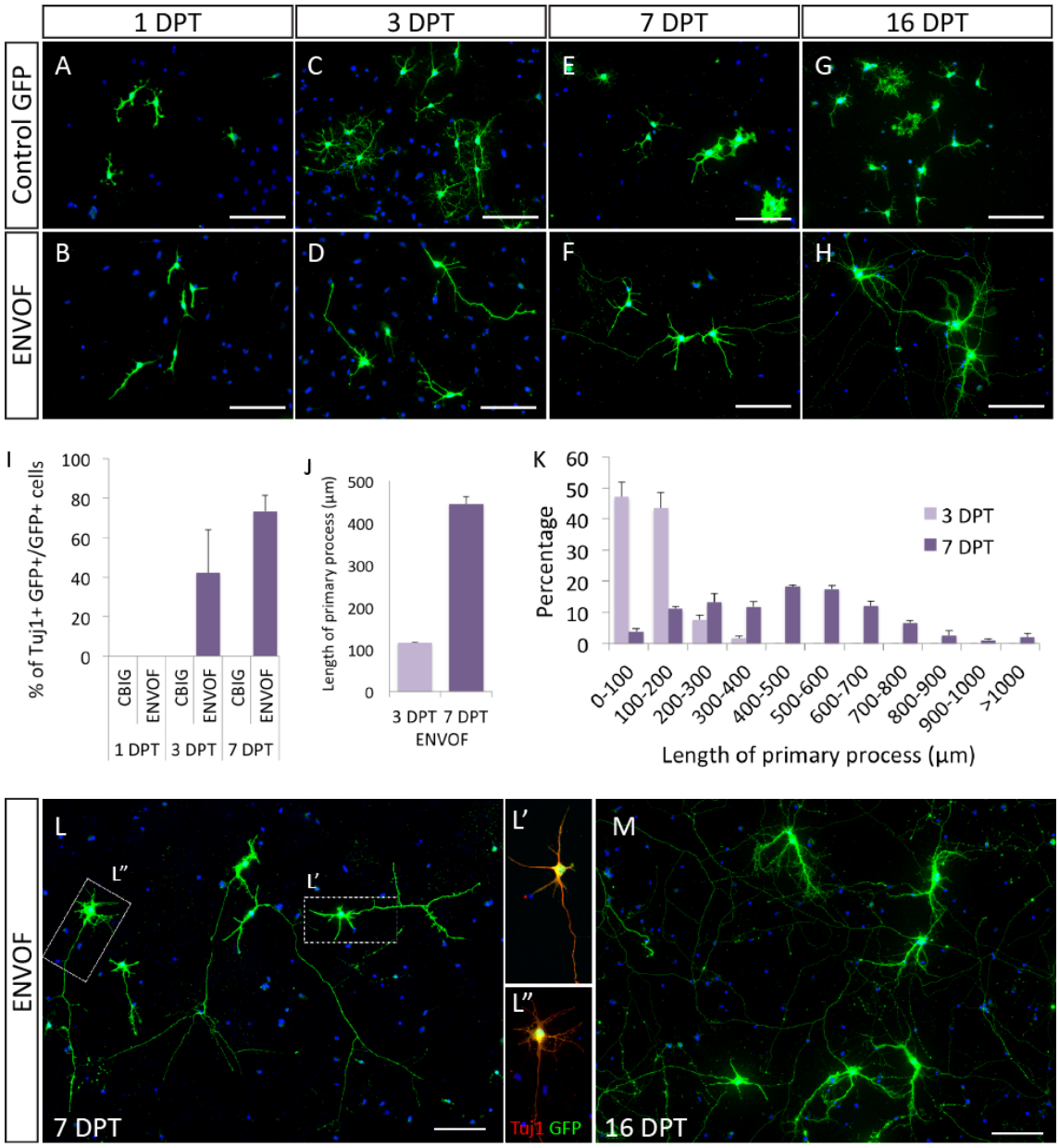

Figure 4.4. ENVOF-transduced NG2+ cortical progenitors acquire neuronal morphology *in vitro*.

Details of Figure 4.4: Unlike control vector transfected NG2+ progenitors (A, C, E, G), ENVOF multi-gene construct transfected cells lose progenitor morphology as early as 1 DPT (B), and gain increasingly complex neuronal morphology with a primary axon-like process and multiple dendrite–like processes, with time (D, F, H). At 16 DPT, ENVOF-induced neurons have elaborate complex morphology with neighboring cells having highly intercalated processes (H, M). (I) Graph shows the quantification of control and ENVOF

plasmid transfected cells with neuronal morphology and Tuj1 expression (42% ±22% at 3 DPT and 73% ±8% at 7DPT (I, L', L") (n=4, 250-300 cell). Length of the primary process of ENVOF-induced cells extends substantially between 3 DPT and 7 DPT, and many cells (>10%) have a primary process longer than 700um by 7 DPT (J, K). (n=3, 120-180 cell) Scale bars: 100 µm. Error bars show standard deviations.

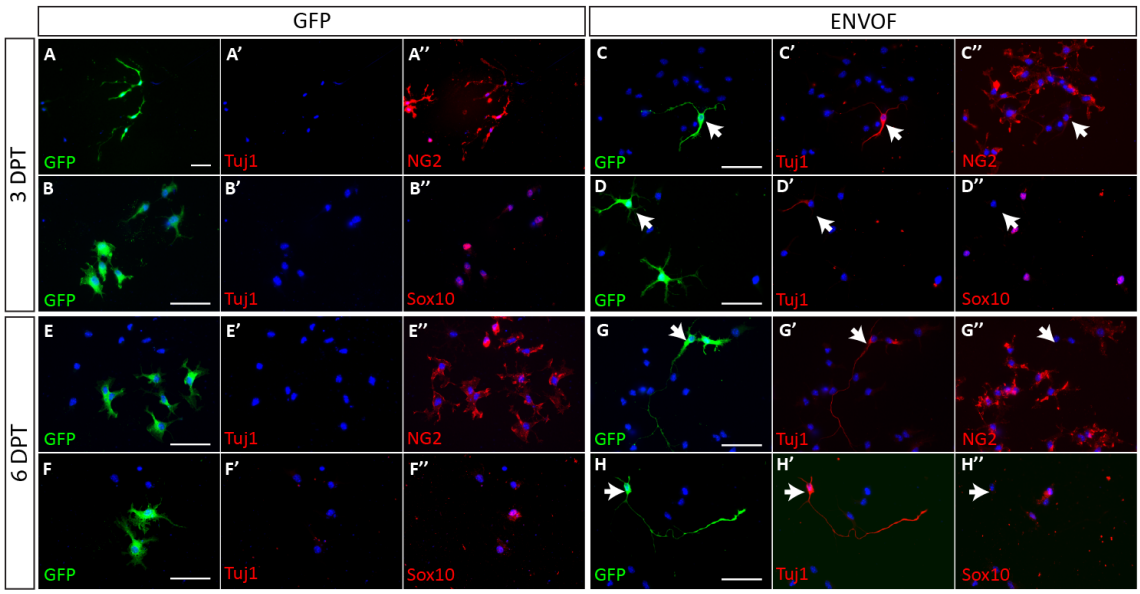

Figure 4.5. ENVOF-transduced NG2+ progenitors rapidly lose progenitor identity.

Details of Figure 4.5: As early as 3 DPT**,** unlike control vector transfected cells (A, B), ~40% of ENVOF transfected cells have started to acquire neuronal morphology (C, D), expressing Tuj1 (C', D') and down-regulated progenitor markers like NG2 and Sox10 (C", D"). At 6 DPT, while all control vector transfected cells still retain glial/progenitor morphology (E, F), ~70% of ENVOF transfected cells acquire a bipolar neuronal morphology (G, H), coupled with strong Tuj1 expression (G', H'), and completely lose expression of progenitor markers (G'', H''). Arrows indicate ENVOF-induced Tuj1-expressing differentiated cells. Scale bars: 100 µm.

Previous data and our own experience showed that neurons cultured below a critical density or in the absence of glial derived trophic factors survive poorly, and are impaired in their maturation (Pfrieger and Barres, 1997; Kaech and Banker, 2007). Therefore, we added primary forebrain cells from P0 postnatal mouse to cortical NG2-progenitor culture 1DPT

with ENVOF (Figure 4.7a). Indeed, we found that, upon co-culturing, ENVOF-induced neurons showed substantial morphological maturation with many having elaborate axonal branches, and an extended, thick dendrite (Figure 4.7b-c). Many ENVOF-induced neurons showed Synapsin1-positive puncta on their cell soma and on dendrites (though at a lower density than nearby primary neurons) (Figure 4.7d), indicating potential synaptic input from surrounding neurons.

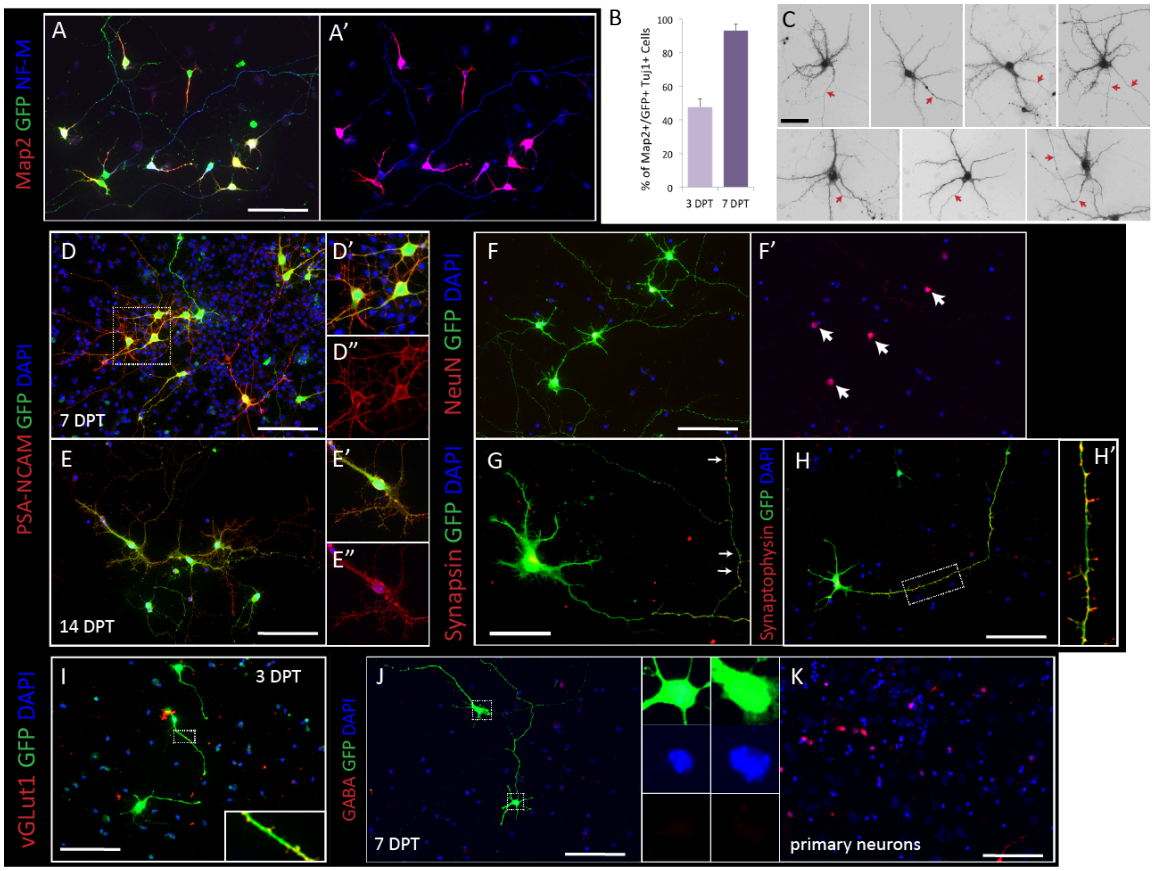

Figure 4.6. ENVOF induces neuronal identity from NG2+ cortical progenitors *in vitro*.

Details of Figure 4.6: (A) ENVOF-transduced cells show appropriately compartmentalized expression of the somato-dendritic marker MAP2, and the somato-axonal marker Neurofilament-M (A') (7 DPT). (B) The percentage of Map2 expressing Tuj1+ cells increases with time; 48% ±5% at 3 DPT (n=3, 60-250 cell) and at 7 DPT, 93% ±4% (n=4, 225-300 cell) of ENVOF-transduced cells express MAP2. (C) High-power imaging shows that ENVOF-induced neurons show complex dendrite-like morphology, and have a single primary axon-like process. Red arrows indicate potential axons (16 DPT). (D)

ENVOF-induced neurons express the cell adhesion molecule PSA-NCAM at 7 DPT (D), which shows punctate distribution at 14 DPT (E). ENVOF-induced neurons express mature neuron marker, NeuN (F, F') (66% ±16% at 7 DPT, n=4, >100 cell), presynaptic molecules Synapsin (G) and Synaptophysin on filopodial structures along their axonal compartments (G-H'). ENVOF-induced neurons express vGluT1 (I), a key molecular feature of glutamatergic neurons, and do not synthesize GABA (J). (K) GABA-positive cells in primary neuron culture act as a positive control in ICC procedures. All scale bars are 100 µm, except C and G (50 µm). Error bars show standard deviations.

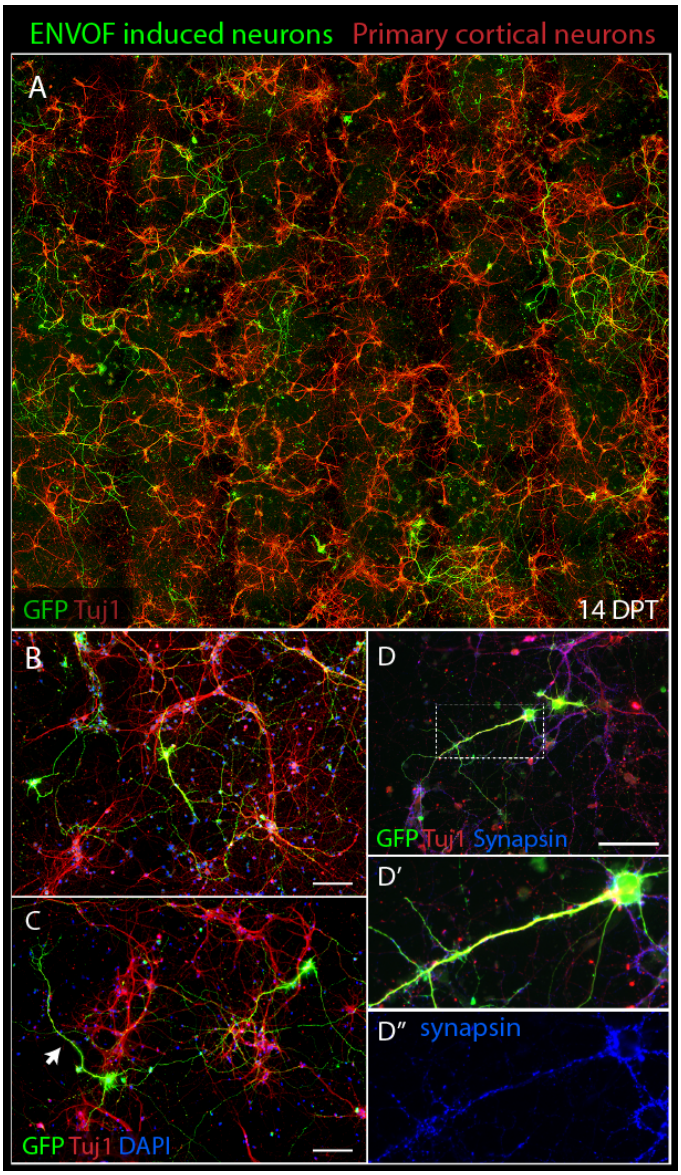

Figure 4.7. Co-culture with primary cortical neurons and astrocyte-conditioned medium improves the maturation of ENVOF-induced neurons.

Details of Figure 4.7: (A) ENVOF-transfected cells co-cultured with primary forebrain neurons isolated from wild-type postnatal mice (P0). Image shows low magnification representative view of ENVOF-induced neurons intercalated with primary neurons at 14 DPT. (B-D) Higher magnification images show considerable morphological maturation and axon elongation of ENVOF-induced neurons in co-culture. Arrow in (C) points to a highly elongated dendritic structure. (D) Representative ENVOF-induced neuron displays numerous Synapsin1/2-positive presynaptic contacts from nearby primary neurons (D'-D"). Higher magnification of the neuron in D, showing punctate synapsin staining. Scale bar: 100 μm

To assess functional maturation of ENVOF-induced neurons, we performed electrophysiological recordings from ENVOF-directed neurons at 10 DPT (without co-culture), and at 16 DPT co-culture with primary neurons. Consistent with the immunocytochemical characterization, ENVOF-directed cells possess several neuronal hallmarks, including action potentials (Figure 4.8a), HCN-channel currents (Isag) upon hyperpolarization (Figure 4.8d), and spontaneous synaptic currents (Figure 4.8k-l). Overall increases in the action potential threshold (Figure 4.8g), decreases in the width of the action potential (Figure 4.8i), and increases in Isag (Figure 4.8j) from 10 DPT to 16 DPT are in line with that of a maturing neuron. Moreover, control vector transfected cortical NG2 progenitors had membrane resistances and resting voltages that were inconsistent with those of neurons (Figure 4.8e-f).

4.2.4. Origin of New Neurons is Cortical NG2+ Progenitors

Though we have used stringent FACS-purification settings to fully eliminate potential contaminants, there is still a theorotical possibility (though remote) that these neurons might represent a "contaminant" population (i.e. endogenous neurons that might have been carried over along with DsRed-positive progenitors during FACS) or that they are derived by the selective expansion of a potential neurogenic progenitor population in culture. We thoroughly investigated these possibilities before performing an in depth characterization of their subtype identity. Our analyses revealed that, at the time of transfection (~5 DIV), only about 10 cells/well on average expressed Tuj1 (n=2) (out of a total of ~150,000 NG2+ progenitors per well of 24-well plate (200 mm^2/well), i.e.

<0.0001%). Analysis at 1-, 3- and 7 DPT of control GFP construct transfected wells showed that the number of these Tuj1+ cells did not increase with time (data not shown), suggesting that i) there are no neurogenic progenitors in our culture, and ii) cultured cortical NG2+ progenitors maintain their progenitor identity in these culture conditions. Additionally, the number of GFP- /Tuj1+ neurons is fewer by several orders of magnitude than the number of GFP+/Tuj1+ neurons that are induced by ENVOF; there are only ~5 GFP- Tuj1+ cells/well versus ~1,500 GFP+ Tuj1+ ENVOF-induced neurons per well in average, n=4) (data not shown). Beyond this, in control construct transfected cells, we did not find any GFP+ cells that express Tuj1 at any time point that we analyzed, ruling out preferential transfection of potential co-purified neurons or their progenitors (n=4, cells=250-350) (Figure 4.4i).

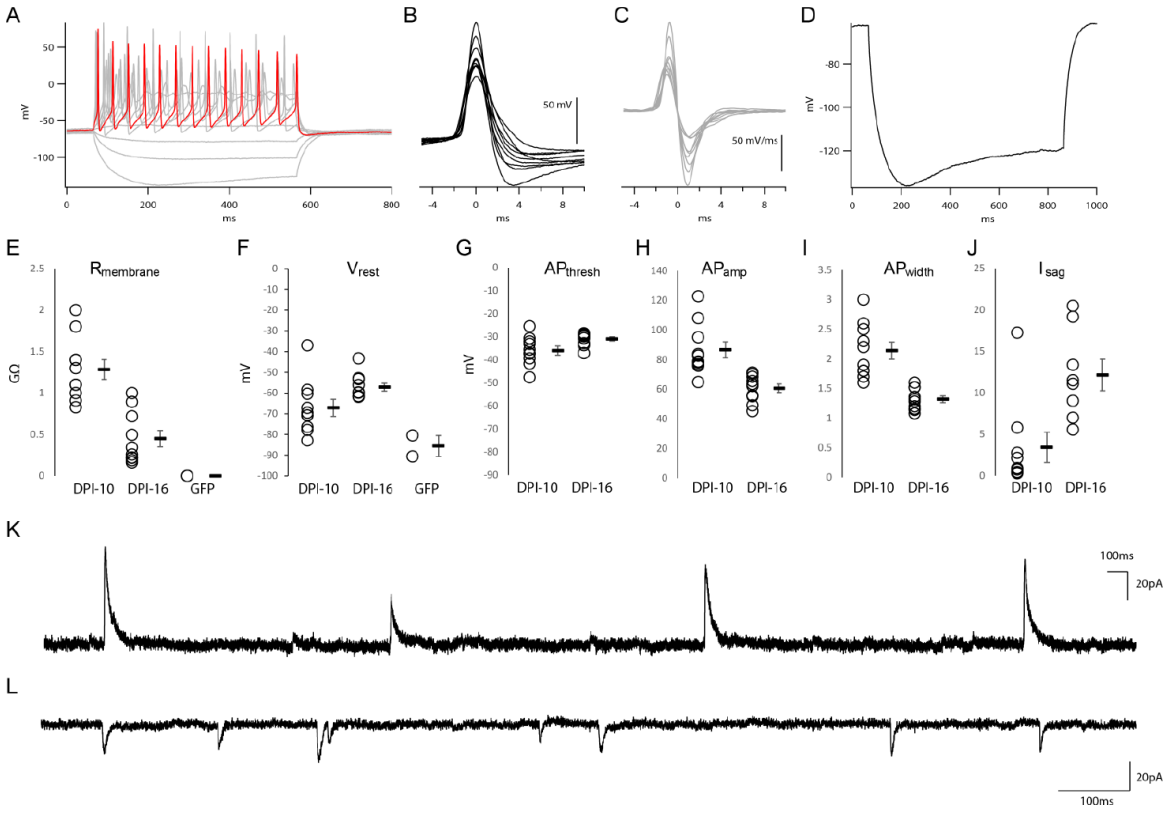

Figure 4.8. ENVOF-induced neurons are electrically active and show spontaneous synaptic currents, indicating functional synapses.

Details of Figure 4.8: (A) Panel A shows an example cell in which depolarizing steps evoke a train of action potentials (red highlighted trace: step 6, 50pA). (B) The first evoked action potential in response to positive current injections for ten individual cells, overlaid.

Waveforms are aligned at threshold for comparison (scale bar: 50 mV). (C) Corresponding dV/dt traces for the action potentials shown in B (scale bar: 50 mV/ms). (D) Representative sag current, indicating the presence of Ih, induced with a 500ms current injection of -40pA (average of 10 sweeps). (E) Resistance of the cell membrane, showing a decreased resistance with more time post-induction (DPI-10, n=10; DPI-16, n=10), and a substantially lower resistance in the absence of ENVOF (GFP, N=2). For all graphs E-J, open circles are individual cells, filled boxes are mean ± s.e.m. (F) Resting membrane voltage for each condition (DPI-10, N=10; DPI-16, N=10; GFP, N=2). (G-I) Action potential threshold, amplitude, and width for two conditions: DPI-10 (n=10) and DPI-16 (n=10). (J) Sag current for two conditions: DPI-10 (n=9) and DPI-16 (n=8). (K) Example of spontaneous outward synaptic currents recorded at -70mV in ENVOF+ cells at DPI-16. (L) Example of spontaneous inward synaptic currents recorded at -70mV in ENVOF-induced neurons at DPI-16.

4.2.5. Combinatorial Actions of Complementary Molecular Regulators Can Faithfully Execute Differentiation of Neuronal Subtype Specification

To dissect the contribution of each transcriptional regulator on the observed phenotype, we tested each of the three transcriptional controls (Ngn2, Fezf2, and VP16:Olig2) on cortical NG2+ progenitors individually. The capacity of Ngn2 to induce glutamatergic neurons from different cells, such as postnatal glial cells or induced pluripotent cells, has been well documented (Heinrich *et al.*, 2010; Zhang *et al.*, 2013). We found that expression of Ngn2 alone was sufficient to differentiate cortical NG2-progenitors to a form of neuron (Figure 4.9a-d). However, the morphology of neurons induced by Ngn2 was strikingly different from that of ENVOF-induced neurons; unlike ENVOF-induced bipolar neurons with a single primary axon, the majority of Ngn2-induced neurons have multiple long processes originating from the cell soma (Figure 4.9a-b vs. 9c-d, and 9e). Interestingly, cortical NG2-progenitors transfected with the single factor Fezf2 did not undergo neuronal differentiation, although there were occasional cells with a hybrid morphology – preserving a glia-like cell soma morphology while having a neuron-like single, long primary process suggesting incomplete neuronal induction (Figure 4.9f-g).

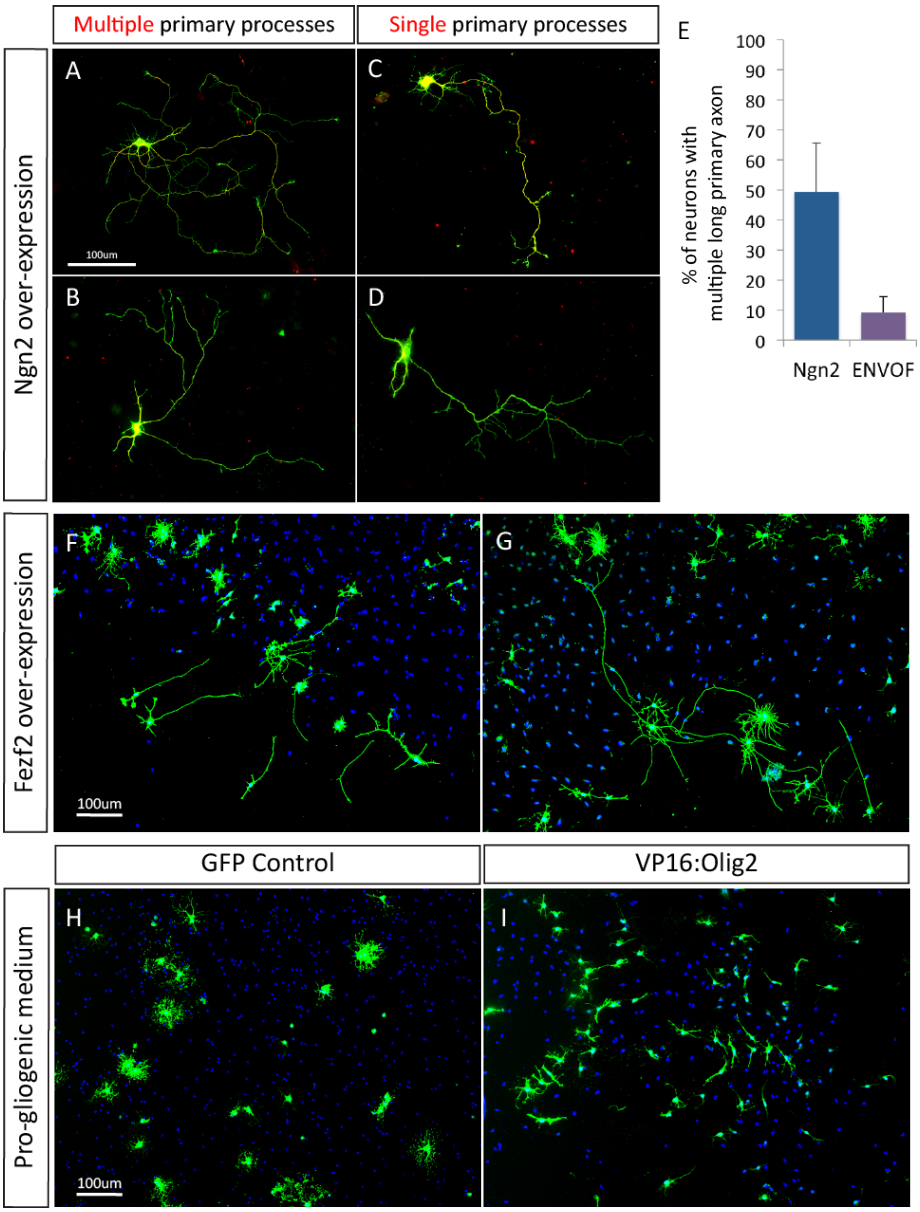

Figure 4.9. Combinatorial actions of complementary molecular regulators can faithfully execute differentiation of neuronal subtype specification.

Details of Figure 4.9: (A, D) Panel shows the representative images of Ngn2-induced neurons with multiple (A-B) versus single axon-like process (C, D). (E) Graph shows the percentage of neurons with single versus multiple long axons between Ngn2- and ENVOF-induced neurons. At 7 DPT, 49% ±16% of Ngn2-induced neurons have multiple, long axon-like process, whereas a very small number of such neurons exist among ENVOF-induced neurons (9% ±5%) (E) (n=5, >100 cell). Criteria for quantification: If a second primary process (originating from the cell soma) is at least half the length of the longest process, that

cell was considered multipolar. (F, G) Fezf2 expression in NG2+ cortical progenitors does not support differentiation into neurons, and many cells adopt a 'chimeric' morphology with a glial-cell-like soma structure and a long process. (n=2) (H, I) In contrast to control GFP transfected cells, over-expression of VP16:Olig2 alone represses T3-mediated differentiation of NG2-progenitors into oligodendrocytes, and transduced cells acquire the morphology of immature neuroblasts (H vs. I) (n=3). Scale bars: 100 μm. Error bars show standard deviations.

To test whether VP16:Olig2 is able to suppress glial differentiation of cortical NG2+ progenitors, we transfected progenitors with either VP16:Olig2 or control GFP constructs. At 1DPT, the cultures were treated with thyroid hormone (T3) to induce differentiation of cortical NG2-progenitors to oligodendrocytes. At three-days post-T3 treatment, as expected, almost all control cells had differentiated into oligodendrocyte-like cells, whereas VP16:Olig2 transfected progenitors had remarkably turned into neuroblast-like bipolar cells, indicating that VP16:Olig2 successfully blocks endogenous Olig2 function (Figure 4.9h vs. 9i).

These findings highlight the relative contribution of transcriptional controls in our strategy for induction of neurogenesis from cortical NG2-progenitors, Ngn2 induces a glutamatergic identity, VP16-Olig2 blocks glial differentiation potential, and Fezf2 refines the neuronal program to generate a bipolar neuron with a single prominent axon.

4.2.6. ENVOF Directs CFuPN/SCPN Identity in Neurons Derived from Cortical NG2-Progenitors

To genuinely replace the function of the diseased neurons *in vivo*, or to develop reliable disease models *in vitro*, newly generated neurons, whether via reprogramming of distant somatic cell types or directed differentiation of endogenous neural progenitors, will likely need to exhibit correct neuronal identity (nearly) in its entirety. The subtype identity for cortical projection neurons comprises at least the following properties; i) expression of key molecular controls together with the absence of alternate controls that are specific for other neuronal subtypes, ii) subtype-appropriate morphology, iii) manifestation of specific

electrophysiological properties, and iv) the capacity to integrate into circuitry to form appropriate afferent and efferent connections *in vivo*.

Here, we systematically investigated whether ENVOF-directed neurons *in vitro* exhibit key molecular characteristics of endogenous CFuPN/SCPN neurons. First, we looked for the expression of CTIP2, a transcriptional control expressed by SCPN in the cortex, that regulates outgrowth and fasciculation of SCPN axons (Arlotta *et al.*, 2005). We found that at 7DPT, 50% of ENVOF-induced neurons express CTIP2 (n=5, 65-350 cells, average=180 cells) (Figure 4.10a-b), whereas none of control GFP-expressing cells express CTIP2 (n=2, ~200 cells) (data not shown). Furthermore, ENVOF-induced neurons express PCP4 (Purkinje cell protein 4), a calmodulin binding protein that is expressed by SCPN in the cortex (77% at 7 DPT, n=3, >100 cell) (Figure 4.10b-c).

Next, we investigated whether ENVOF-induced neurons simultaneously express molecular controls corresponding to callosal projection neurons, a feature of either immature differentiation or a mixed/hybrid identity, commonly observed with ES/iPS derived neurons (Sadegh and Macklis, 2014; Sances *et al.*, 2016). We found that ENVOF-induced neurons appropriately do not express Satb2 (n=3, 115-130 cells) (Figure 4.10b and 10e) or Cux1 (n=2), molecular controls that are exclusively expressed by callosal and other intra-cortical projection neurons.

Subcerebral projection neurons (SCPN) and corticothalamic projection neurons (CThPN) (together called corticofugal projection neurons (from the Latin, “traveling away from the cortex”)) are two developmentally closely-related cell populations. They are born sequentially during early corticogenesis, are located in deep cortical layers, and both send their axons away from the cortex through the internal capsule to their respective targets. Molecular programs that regulate their specification, post-mitotic differentiation and axon guidance are largely shared between the two populations. Differential dose and timing of these key controls, and combinatorial co-expression with other factors, delineates their differentiation. For example, both Fezf2 and Ctip2 are expressed at higher levels in SCPN, while at a comparatively lower level by CThPN, and both populations are severely affected in the absence of Fezf2 (Greig *et al.*, 2013). Therefore, we investigated whether ENVOF-induced neurons exhibit molecular controls that are enriched in CThPN.

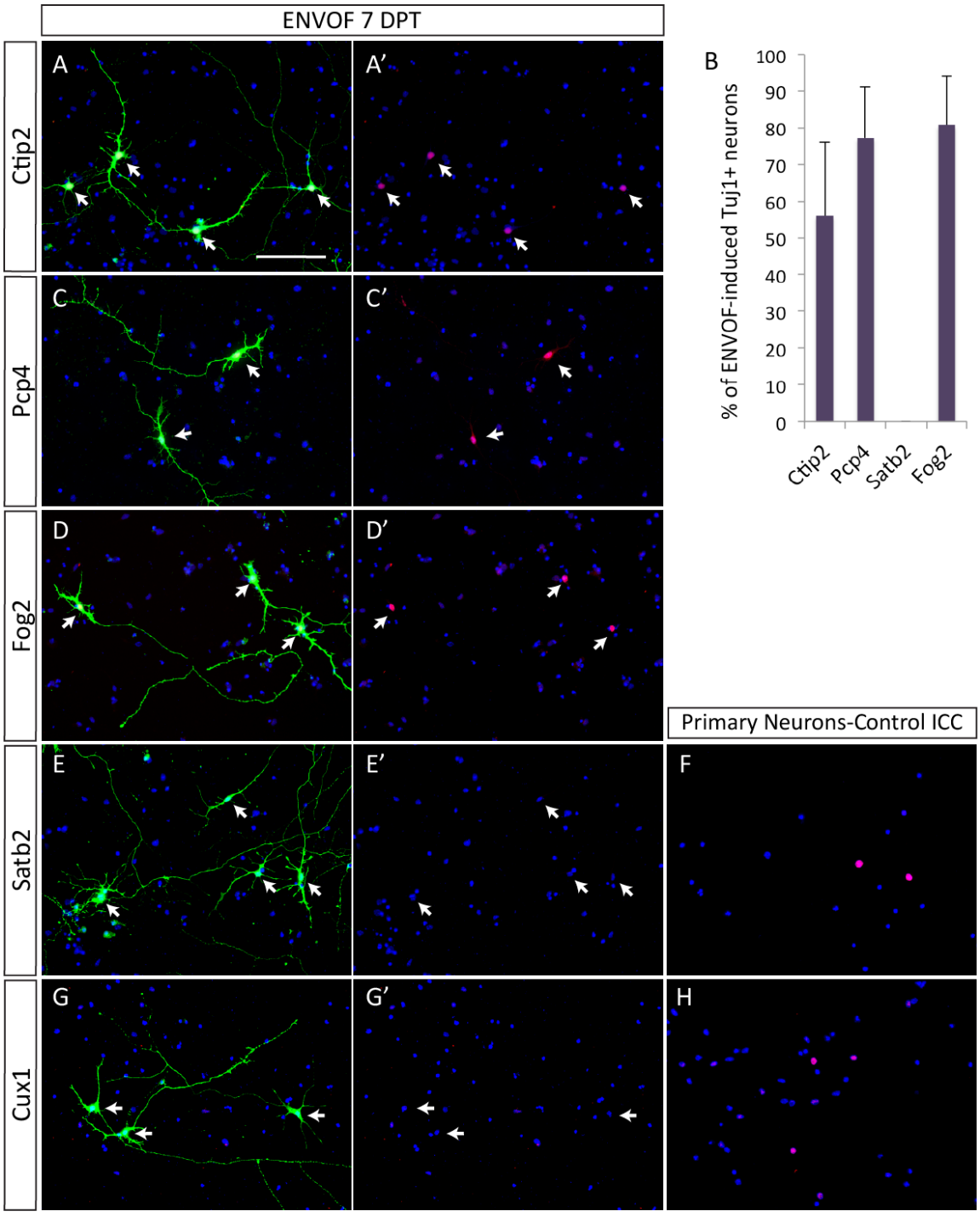

Figure 4.10. ENVOF-induced neurons exhibit molecular hallmarks of cortocfugal identity *in vitro*.

Details of Figure 4.10: ENVOF-induced neurons express the subcerebral projection neuron (SCPN) transcriptional controls Ctip2 (A), Pcp4 (B), and the corticothalamic projection neuron transcriptional control, Fog2 (D), but not callosal projection neuron

molecular controls Satb2 (E) and Cux1 (G). (B) Graph shows the quantification of percentage of ENVOF-induced Tuj1+ neurons that express Ctip2 (56% ±20%, n=5), Pcp4 (77% ±14%, n=3), Satb2 (0%, n=3) and Fog2 (81% ±13%, n=3). Cultured primary neurons were used as a positive control for immunocytochemistry staining for Satb2 and Cux1 (F, H). Scale bar: 100 μm. Error bars show standard deviations.

Intriguingly, we found that the great majority of ENVOF-directed neurons express Fog2 (~80% at 7 DPT, n=3, ~120 cells) (Figure 4.10b and 10d), a critical regulator of CThPN axonal targeting and diversity (Galazo et al. Neuron, in press). SCPN in cortical layer V in the postnatal motor cortex express high levels of Ctip2, and do not express Fog2 (though their co-expression might be differentially regulated across different cortical areas or earlier during neurogenesis). We also investigated the expression of Tbr1, a transcriptional control required for CThPN specification, and found that ENVOF-induced neurons do not express Tbr1 (data not shown). These data (together with absence of CPN controls) indicate that ENVOF induced neurons acquire broad CFuPN molecular identity; however, further refinement of SCPN versus CThPN identity is incomplete, indicating that additional layers of controls are needed for full refinement of their identity within the broad CFuPN population.

To further authenticate the specificity of CFuPN identity of ENVOF-induced neurons, we investigated the potential abnormal expression of molecules specific for distinct neuronal classes outside the cortex, across different regions of the CNS. We found that ENVOF-induced neurons appropriately do not express Darpp32 (expressed by striatal medium-sized spiny neurons, MSN) (Figure 4.11a vs. 11b); serotonin (5-hydroxytryptamine (5-HT), expressed by serotonergic neurons) (Figure 4.11c vs. 11d); TH (tyrosine hydroxylase, expressed by dopaminergic neurons) (Figure 4.11e vs. 11f), and Isl1 (Figure 4.11g vs. 11h), a transcriptional regulator that is required for a diverse set of cholinergic neurons across CNS, including spinal motor neurons. These results collectively indicate that ENVOF-induced neurons acquire the broad identity of cortical output neurons, but not other alternate neuronal fates.

Our initial analyses indicate that Ngn2 plays a central role in the activation of some of the deep layer molecular controls (data not shown), which is consistent with previous

reports (Mattar *et al.*, 2008; Gascon *et al.*, 2016). Understanding individual versus combinatorial roles of Ngn2 and Fezf2 for the activation of these molecules is immensely important for formulating precise combinations of factors for induction of neurons with more refined identity.

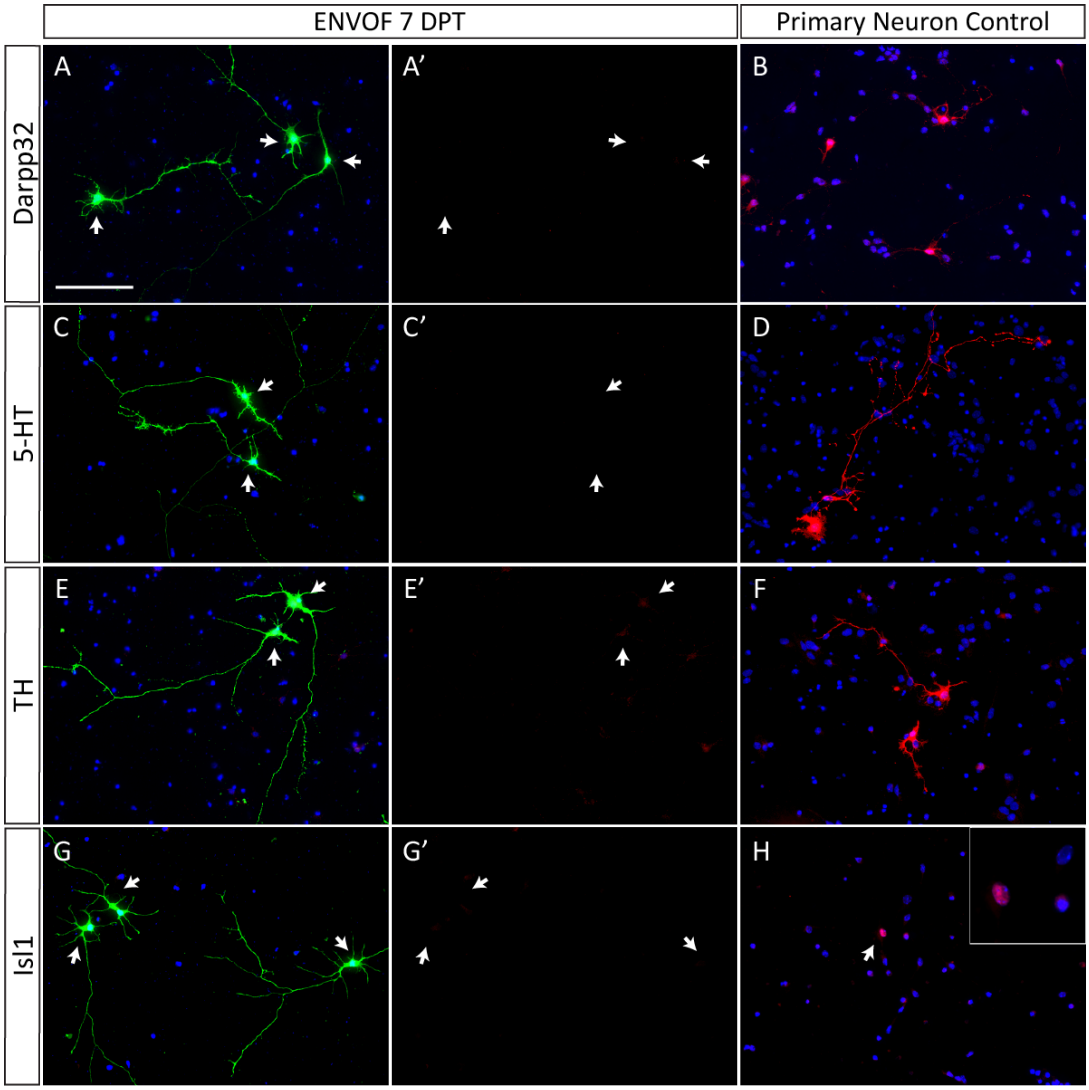

Figure 4.11. ENVOF-induced neurons appropriately do not co-exhibit multiple neuronal identities.

Details of Figure 4.11: Representative images of ENVOF-induced neurons with negative immunoreactivity for Darpp32 (A, A'), 5-HT (C, C'), TH (E, E') and Isl1 (G, G'). Cultured primary neurons from P2 wild-type mice were used as a positive control for immunocytochemistry staining (B, D, F, H). Scale bar: 100 μm.

Immunocytochemistry-based comprehensive molecular characterization provides important evidence that ENVOF-induced neurons are not generic neurons with mixed neuronal identity. To assess whether these molecular features are coupled with acquisition of electrophysiological characteristics of endogenous CFuPN/SCPN, currently we are performing whole-cell recordings of ENVOF-induced neurons, and comparing their electrophysiological features with cultured primary SCPN and CPN.

4.2.7. Temporal Control of Gene Expression for Refinement of Neuronal Maturation of CFuPN Derived from Cortical NG2-progenitors

Our analyses thus far reveal that ENVOF-induced neurons express a core set of molecules that are indicative of their true neuronal differentiation, and remarkably have intrinsic electrophysiological properties resembling those of primary neurons. Despite these, we noted that the density of (synapsin-positive) synaptic spines on presumptive dendritic structures of ENVOF-induced neurons at 14DPT was noticeably lower in comparison to primary neurons in co-culture experiments (data not shown). Also, as discussed in previous section, ENVOF-induced neurons co-express molecular controls normally expressed by multiple neuronal subtypes within the broad CFuPN population (i.e. Ctip2 and Fog2 co-expression).

A potential hindrance to the full functional and subtype specific maturation of ENVOF-induced neurons could be the constitutive expression of Ngn2, a transcription factor that is normally transiently expressed during the developmental generation of cortical glutamatergic neurons. It has also been reported that sustained expression of Ngn2 could be deleterious to differentiating neurons in the cortex (Cai *et al.*, 2000). To overcome these potential problems, we devised a strategy to restrict the expression of Ngn2 to a very short early time window during neuronal differentiation. Synthetic chemically modified RNA (modRNA), in which one or more nucleotides are replaced by modified nucleotides, represents a potential alternative to the plasmid-based transfection approach. Previous work in multiple systems has showed that modRNA mediates highly efficient, transient protein expression *in vitro* and *in vivo* without eliciting an innate immune response (Warren *et al.*, 2010; Chien *et al.*, 2015).

We adapted our transfection protocol to transfect cortical NG2-progenitors with modRNA at high efficiency (Figure 4.12a vs. 4.12b), and evaluated the time course of protein expression from modRNA using a modRNA encoding GFP reporter (Figure 4.12d-g). The expression of GFP protein from GFP modRNA appears to peak between 12-24 hrs post-transfection in cortical NG2 progenitors, and decline thereafter (Figure 4.12d-g). We compared the efficacy of the Ngn2 DNA construct and the Ngn2 modRNA in inducing neurogenesis from cortical NG2-progenitors, and found that a single dose of Ngn2 modRNA was capable of inducing neurons from cortical NG2-progenitors, albeit with a lower efficiency (Figure 4.12h-i) (n=3).

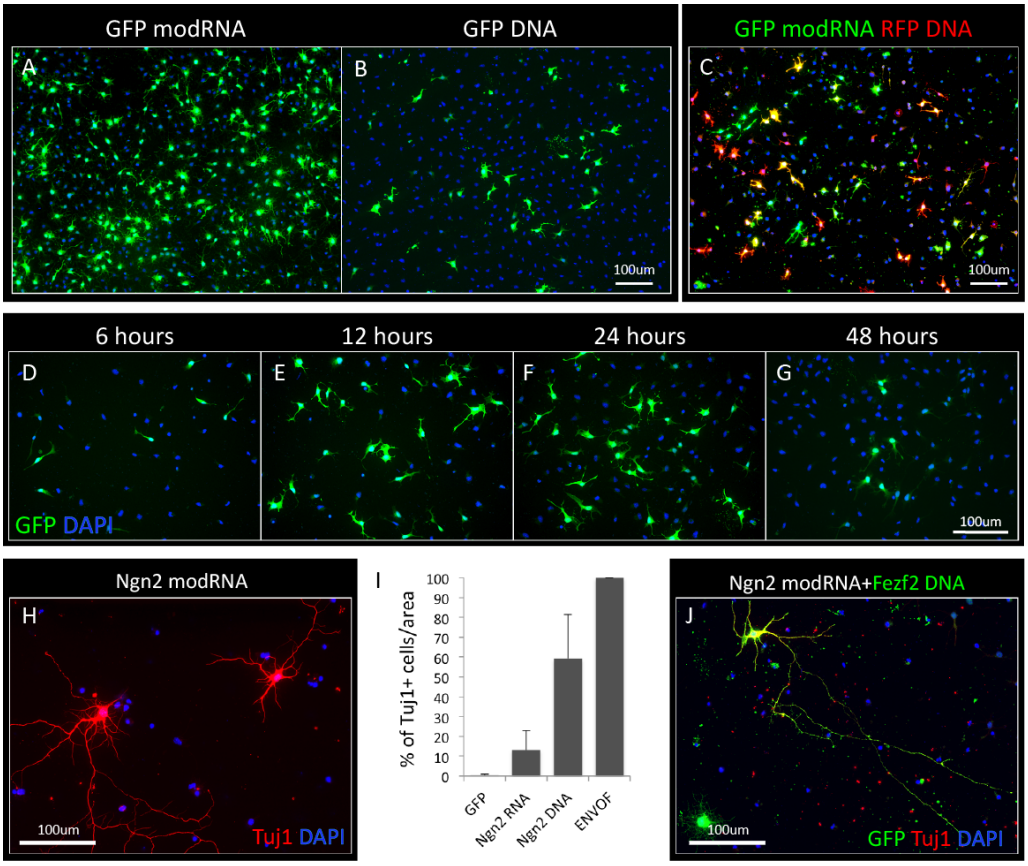

Figure 4.12. Synthetic modified RNA (modRNA) can induce neurons from NG2+ cortical progenitors.

Details of Figure 4.12: Cultured NG2+ cortical progenitors can be transfected with GFP modRNA at very high efficiencies (A), compared to a GFP DNA construct (B). (C) Co-transfection of GFP modRNA and a plasmid DNA construct encoding tdTomato reporter

shows substantial co-transfection efficiency. (D, G) Time course of gene expression from GFP modRNA transfected NG2+ progenitors suggests that expression of GFP begins by 6 hours-post-transfection (D), peaks between 12 to 24 hrs (E, F), and declines thereafter (G). A single pulse of Ngn2 modRNA can induce morphologically complex, Tuj1+ neurons from NG2+ cortical progenitors (H), albeit at lower efficiencies when compared to the Ngn2 DNA construct and ENVOF (I) (n=3). (I) Graph shows the percentage of Tuj1+ neuron-like cells in GFP control, Ngn2 modRNA and Ngn2 DNA construct-transfected cells relative to the number of Tuj1+ cells in ENVOF-transfected wells. (J) Co-transfection of Ngn2 modRNA and a Fezf2 DNA construct (also encodes GFP) induces Tuj1+ neurons from NG2- progenitors. Scale bars: 100 μm. Error bars show standard deviations.

Importantly, mature CFuPN/SCPN maintain Fezf2 expression, throughout development and adulthood, unlike transient expression of Ngn2 expression. To better mimic the endogenous expression patterns, we devised a co-transfection strategy by which we introduce Ngn2 as a modRNA and Fezf2 as a plasmid DNA construct under the control of a constitutively active CAG promoter. To test the feasibility of the DNA-RNA co-transfection approach, we used a two fluorescent reporter system, by which we co-transfected tdTomato reporter as a plasmid DNA and GFP as a modRNA (Figure 4.12c). We found that ~50% of fluorescent cells are co-transfected with both reporters (data not shown) (n=3). Co-transfection of plasmid DNA encoding Fezf2 together with Ngn2 modRNA resulted in a substantial number of neurons, morphologically similar to ENVOF-induced neurons (Figure 4.12j). We are currently evaluating and comparing the neuronal maturation, electrophysiological properties, as well as CFuPN subtype refinement of neurons induced by ENVOF, Ngn2 DNA plasmid, and those induced by the modRNA-based strategy.

4.3. Discussion

In this study, we demonstrate that developmental neuronal controls can direct the differentiation of NG2+ cortical progenitors into neurons with the molecular features of corticofugal projection neurons (CFuPN) *in vitro*. Fezf2, a molecular control over SCPN development, together with Neurogenin2 and VP16-Olig2 transcriptional regulators, is able to overcome the gliogenic differentiation program that is prominent in NG2 progenitors to generate neurons with a glutamatergic neuronal identity and the specific features of cortical

output neurons. Molecular, morphological, and electrophysiological analyses demonstrate that the newly generated neurons exhibit functional properties of mature neurons. Co-culturing NG2-progenitor-derived neurons together with primary cortical cells and astrocyte-conditioned medium further refines their maturation and differentiation. Our work provides compelling evidence that development-inspired directed differentiation is a viable strategy to generate defined neuronal subtypes for cellular repair of damaged neuronal circuitry.

4.3.1. Potential of NG2+ Cortical Progenitors for Cellular Repair of Neuronal Circuitry

NG2 progenitors constitute the largest progenitor population in the adult rodent brain, and are widely distributed. Progenitors lost due to differentiation or cell death are readily replenished by cell division and migration of neighboring progenitors (Hughes *et al.*, 2013). A substantial proportion (~30% in the adult, (Kessaris *et al.*, 2006)) of NG2-progenitors in the cortex share a common linage with cortical projection neurons, making them an ideal population for directed differentiation into cortical neurons. Though no work has directly investigated the cellular and molecular differences of NG2-expressing progenitors with distinct pallial and subpallial developmental origins, it is quite possible that they might retain certain epigenetic and molecular features of their origin. Of note, recent studies have discovered heterogeneity even within dorsal progenitors during development, with distinct fate potentials for callosal and cortical output neurons (Franco *et al.*, 2012). Therefore, it is tempting to postulate that cortical progenitors that originate from the dorsal proliferative zone would be more optimal targets for directed differentiation into cortical projection neurons because of their shared origin.

Several studies have documented that cell-of-origin-specific residual transcriptional, epigenetic and chromatin domain signatures persist during the derivation of iPSCs, especially during early passages (Polo *et al.*, 2010; Beagan *et al.*, 2016; Krijger *et al.*, 2016). Although similar studies have not been performed on fibroblast-derived neurons (iNs), or neurons derived from other cell types, it is conceivable that cell of origin-specific molecular signatures remain in iNs, and perhaps this could be a potential hindrance to full functional derivation of neuronal subtypes, and thus reliable modeling of the disease. Directed

differentiation of neurons from a related population of local progenitors could in principle result in fewer epigenetic blocks and therefore better functional differentiation. In line with this view, reprogramming of fibroblasts to neuronal lineage occurs at a much lower efficiency and over a longer time frame compared to reprogramming of cultured postnatal astrocytes (Ninkovic and Gotz, 2015). In this work, we found that upon ENVOF transfection, substantial numbers of NG2+ cortical progenitors acquire unipolar neuronal morphology by three days-post-transfection, demonstrating the significant plasticity and competence of NG2-progenitors to differentiate into neurons.

Several groups have demonstrated that, under certain culture conditions, cultured NG2+ progenitors (potentially a subset) can give rise to neurons *in vitro* (i.e. in the absence of molecular manipulation), suggesting that these cells are capable of lineage plasticity under appropriate conditions. However, though subsets have been reported to generate neurons in restricted regions of the brain *in vivo*, there is as yet no unequivocal evidence of physiological neurogenesis from these progenitors (Kondo and Raff, 2000; Belachew *et al.*, 2003; Aguirre *et al.*, 2004; Liu *et al.*, 2007a; Dimou *et al.*, 2008; Rivers *et al.*, 2008; Zhu *et al.*, 2008a; Guo *et al.*, 2009; Komitova *et al.*, 2009; Guo *et al.*, 2010; Kang *et al.*, 2010; Dewald *et al.*, 2011; Clarke *et al.*, 2012; Robins *et al.*, 2013). Nevertheless, the lineage plasticity displayed by at least a subset of these progenitors suggests that the epigenetic barrier for directed differentiation toward neuronal subtypes can potentially be overcome with fewer factors and extracellular cues.

4.3.2. Recent Progress in Targeting Endogenous Cells for Cellular Brain Repair

Prior studies have reported induced neurogenesis of glutamatergic or GABAergic neurons from cortical astrocytes and NG2 progenitors *in vitro*. The groups of Drs. Magdalena Götz and Benedikt Berninger have pioneered transcription factor-mediated reprogramming of brain glia into neurons. Using sustained expression of Neurog2 (Ngn2) or Dlx2, the researchers were able to direct the differentiation of cultured postnatal cortical astroglia into synapse forming glutamaterigic and GABAergic neurons, respectively (Buffo *et al.*, 2005; Heinrich *et al.*, 2010; Blum *et al.*, 2011; Heinrich *et al.*, 2011). Their studies also demonstrated that human and mouse pericytes could be reprogrammed similarly, expanding the cell types amenable to induced neuronal differentiation (Karow *et al.*, 2012).

Generation of immature neuron-like cells from astrocytes in the adult brain has been reported using a combination of Sox2 and extrinsic factors (Niu *et al.*, 2013; Su *et al.*, 2014; Niu *et al.*, 2015). Sox2 reprograms adult astrocytes into neural progenitor- or neuroblast-like cells, which then differentiate into neurons in the presence of growth factors or the HDAC inhibitor valproic acid, albeit with a very low efficiency. A combination of Ascl1/Brn2a/Myt1l (Torper *et al.*, 2013) in the striatum, or the single factor Ascl1 has also been reported to be sufficient to induce neurons from adult astrocytes in the midbrain (Liu *et al.*, 2015).

Several *in vivo* reprogramming and directed differentiation studies have reported induced neurogenesis from glial cells after injury to the brain. In a model of restricted injury and in an Alzheimers' disease mouse model, the single factor NeuroD1 induced neurogenesis from reactive astrocytes and NG2 progenitors (Guo *et al.*, 2014). Ngn2, together with exogenously supplied factors like Fgf2 and EGF, was shown to induce neurogenesis from a heterogeneous group of cells (including NG2-progenitors) after stab wound injury in the cortex and striatum (Grande *et al.*, 2013). The transcription factor combination of Sox2 and Ascl1 or the single factor Sox2 also can induce neurons from adult brain NG2 progenitors *in vivo* after stab wound injury (Heinrich *et al.*, 2014). These studies reported relatively low numbers of induced neurons potentially resulting from unfavorable conditions due to injury-induced inflammation (Abdul-Muneer *et al.*, 2015). Supporting this hypothesis, anti-oxidants and Bcl2 were shown to substantially improve Neurog2-mediated *in vivo* directed differentiation from astrocytes and NG2 progenitors after stab wound injury (Gascon *et al.*, 2016).

In contrast, a recent study reported generation of large numbers of neurons from a mix of activated microglia, NG2-progenitors, and reactive astrocytes transduced with pluripotency factors (Oct4, Sox2, Klf4, and c-Myc) after controlled cortical brain injury (Gao *et al.*, 2016). This could potentially be due to the fact that the reprogrammed glia transit through a pluripotent intermediate state enabling a significant expansion in progenitor numbers (Gao *et al.*, 2016). Of note, several reports indicate that directed differentiation using transcription factors (with the notable exception of Sox2) cause cell cycle exit and differentiation of progenitors. In agreement with these reports, we observed that both

ENVOF and Ngn2 cause NG2-progenitors to rapidly withdraw from the cell cycle and down-regulate their progenitor markers (Figure 4.5 and data not shown).

The above studies have focused on generating broad neuronal classes identified by their principal neurotransmitters such as glutamatergic neurons and GABAergic neurons. Repair of degenerated neuronal circuitry, though, requires further refinement of neuronal identity to the level of region-specific neuronal subtypes. This is essential to ensure that the newly generated neurons are able to synapse with appropriate partners, and avoid making aberrant connections. Our study is aimed toward this goal of going this critical and major step further from induction of neurons with generic features to generating subtype-specific neurons from endogenous progenitors.

4.3.3. Directed Differentiation of Neuronal Subtypes from Endogenous Cortical Progenitors

Recent work on cortical development has expanded our understanding of the molecular controls over neuronal subtype identity (Greig *et al.*, 2013). In addition to Ngn2 and VP16:Olig2 (to induce neurogenesis from NG2-progenitors), we selected Fezf2, a transcriptional control both necessary and sufficient to induce features of subcerebral projection neurons (SCPN). Our results indicate that ENVOF-induced NG2-progenitor derived neurons acquire basic features of cortical output neurons (SCPN belong to this broader class) with expression and appropriate localization of markers of neuronal maturation (MAP2, NF-M, NeuN, vGlut1, Synapsin1), a projection neuron-like morphology (a single long primary axon and an apical dendrite-like process), expression of molecular controls of CFuPN (e.g. CTIP2, Fog2 and PCP4), and importantly, exclusion of markers of alternate fates (Satb2, Cux1, GABA, TH, 5HT, Isl1 etc.).

ENVOF-induced neurons, however, have not acquired a completely refined SCPN-identity, since they express Fog2 (a marker of corticothalamic neurons that is not expressed by mature SCPN), and lack expression of some other markers of SCPN (e.g. Crym). Although unlikely, this might represent a relatively early stage of neuronal subtype identity acquisition, since early during normal development many molecular controls are expressed broadly, and their expression domains resolve over time to give rise to the subtype-restricted

patterns that are observed in the postnatal cortex (Azim *et al.*, 2009b). One possible reason for this could be that our neuronal induction medium lacks critical extrinsic factors needed for proper neuronal maturation and identity refinement of induced neurons. We have observed a similar but more severe stalling of developmental maturation of ES cell-derived cortical neurons under standard culture conditions (Sadegh and Macklis, 2014). Supporting our hypothesis, co-culturing ENVOF-induced neurons with primary cortical cells, and in the presence of astrocyte-conditioned medium, improves their survival and maturation (Figure 4.7).

One likely reason for not fully complete differentiation of ENVOF-induced neurons into SCPN could be the constitutive expression of Ngn2 (and VP16:Olig2). Ngn2 expression is dynamically regulated in neural progenitors (Shimojo *et al.*, 2011), and sustained expression of Ngn2 can cause cell death in developing neurons (Cai *et al.*, 2000). Beyond this, recent studies have also shown that, in addition to its well established role in activation of proneural genes, Ngn2 might activate some of the neuronal subtype specific genes, such as Fog2 and Ctip2 (Mattar *et al.*, 2008). Therefore, co-expression of Fog2 and Ctip2 by ENVOF-induced neurons might be due to constitutive Ngn2 expression. To overcome this issue, we apply a new synthetic modRNA-based approach to enable fine-tuning of both temporal expression and dose of Ngn2. Using a synthetically modified mRNA for Ngn2, we were able to induce neurogenesis from NG2+ cortical progenitors. We are investigating whether temporal and dose controlled expression of Ngn2 coupled with sustained expression of Fezf2 (Fezf2 is expressed constitutively by SCPN *in vivo*) will enable more refined differentiation of SCPN from NG2-progenitors.

In addition, we are investigating the capability of ENVOF-induced neurons for full functional integration by transplantation into the neonatal mouse cortex. Beyond expression of key molecular controls, exhibition of morphological and hodological features of CFuPN/SCPN *in vivo* is crucial for unequivocal assessment of cortical projection neuron identity. Future experiments will address if additional transcriptional regulators or extrinsic factors can further enable the generation of precise neuronal subtypes necessary for functional repair of damaged neuronal circuits.

5. MOLECULAR AND CELLULAR DEVELOPMENT OF CORTICOSTRIATAL PROJECTION NEURONS

5.1. Summary

As a first step toward the long-term goal of understanding the molecular mechanisms underlying Huntington's disease (HD) pathology, and of developing effective therapeutic treatment for HD, this work aims to investigate the development, connectivity, and diversity of corticostriatal projection neurons (CStrPN).

CStrPN are the major cortical efferent neurons connecting the cerebral cortex (the brain region responsible for cognition, integration of sensation, and control of precise voluntary movement) to the striatum of the basal ganglia. They are centrally involved in motor planning, execution, movement controls, learning, and cognitive functions (Pennartz *et al.*, 2009). There are two types of CStrPN (Figure 5.1). As the predominant type, intratelecephalic-type (IT or CStrPNi), send projections across the telencephalon to innervate contralateral striatum (and/or cortex) in order to convey information of sensory and motor planning. The alternative, pyramidal type (PT) CStrPNp are subcerebral-projecting corticospinal motor neurons (CSMN) or cortico-brainstem projecting neurons, extending axon collaterals to innervate the ipsilateral striatum (in addition to thalamus, subthalamic nucleus, midbrain, pontine nuclei and other brainstem areas), thus providing a "copy" of motor commands from the neocortex (Lei *et al.*, 2004; Reiner *et al.*, 2010; Shepherd, 2013).

In this study, we primarily focus on identifying and functionally investigating central molecular controls for the predominant intratelecephalic-type (IT-type) CStrPNi, because 1) they are responsible for complex motor programs and high-level cognitive functions in the mammalian neocortex; 2) they centrally degenerate in HD, a progressive neurodegenerative and developmental disorder selectively affecting the cortico-basal ganglia circuitry; and 3) CStrPNi possess anatomically and molecularly 'hybrid' characteristics of both callosal projection neurons (CPN) and corticofugal projection neurons (CFuPN), and thus, CStrPNi

loss in HD might specifically contribute to both deficits in cognitive executive functions (a trait of CPN degeneration), and in motor functions (a trait of CFuPN abnormality) (Montoya *et al.*, 2006; Walker, 2007).

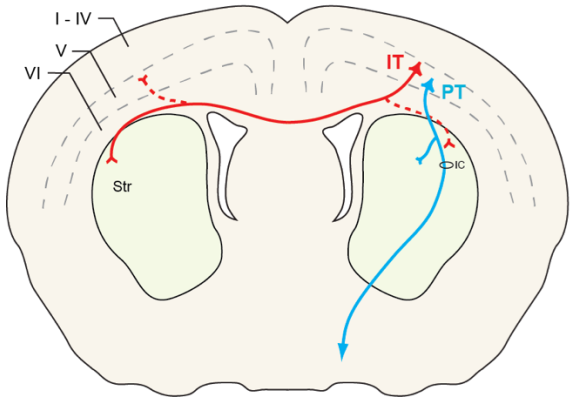

Figure 5.1. Categorization of corticostriatal projection neurons: Intra-telencephalic (IT, CStrPNi) and pyramidal type (PT, CStrPNp). CStrPNi extend primary axons to innervate contralateral striatum. CStrPNp consist of corticospinal and cortico-brainstem neurons.

Despite such significance, very little is known about the molecular basis of CStrPN development. Though there are anatomical and electrophysiological data investigating projection connectivity, topography, and synaptic connectivity at striatal terminal in rodents, dogs, and monkeys (Goldman-Rakic, 1981; McGeorge and Faull, 1987; Tanaka, 1987; Wilson, 1987; Flaherty and Graybiel, 1991), only recently, in a study from our laboratory, has important information on temporal and spatial development of CStrPNi been delineated (Sohur *et al.*, 2014). The technical difficulties in studying this important neuronal population, and functionally parcellating its role in diseased conditions echo with our hypothesis that subtle dysregulation in CStrPN development or trajectory might manifest as subtle abnormalities in corticostriatal connectivity, which might lead to subtle but specific synaptic and/or circuit dysfunction observed in HD.

The overall goal for this research is to identify key molecular controls and signatures for subtypes of CStrPN, which would serve as a solid step toward achieving a holistic view of the establishment of corticostriatal circuitry, and its potential dysgenesis in diseases.

5.2. Background and Significance

5.2.1. CStrPN are a Central Neuronal Population that Degenerates in Huntington's Disease

Many devastating neurodegenerative, developmental, and acquired central nervous system diseases and injuries primarily affect cortical projection neurons. In adult-onset HD, CStrPN are particularly vulnerable, and their degeneration critically contributes to the progressive deterioration of motor and cognitive functions, characterized by severe involuntary, choreic movements and dementia (Miller and Bezprozvanny, 2010). Besides the progressive loss of striatal medium-sized spiny neurons (MSN), the substantial degeneration of projection neurons in layers III, V, and VI (the majority of them are thought to be CStrPN) and the progressive disconnection between cortex and striatum (with thinning of corpus callosum, thus centrally implicating CStrPNi), have been demonstrated as central hallmarks of HD pathology (Figure 5.2) (de la Monte *et al.*, 1988; Cudkowicz and Kowall, 1990; Hedreen *et al.*, 1991; Heinsen *et al.*, 1994; Halliday *et al.*, 1998; Sapp *et al.*, 1999; Macdonald and Halliday, 2002; Rosas *et al.*, 2002; Selemon *et al.*, 2004; Cepeda *et al.*, 2007; Thu *et al.*, 2010; Hong *et al.*, 2012). Evidence from multiple HD animal models and preclinical human HD patients has suggested that abnormality and degeneration of cortical projection neurons might precede the onset of motor deficits and other symptoms, and that CStrPN dysfunction in HD might be primary rather than secondary to striatal degeneration (Rosas *et al.*, 2002; Cummings *et al.*, 2009; Miller *et al.*, 2011).

The wild-type huntingtin protein (HTT), encoded by the gene Htt, has been demonstrated to be essential for the normal neurogenesis, development, and survival in those brain areas most affected in HD. Knockdown of Htt expression in neuroepithelial cells of the neocortex causes aberrant cell migration, reduced proliferation, and increased apoptosis that is relatively specific to early neural development (Bhide *et al.*, 1996). This raises the possibility that a subtle loss of function on the part of the mutant protein, or a sequestering of wild-type HTT by the mutant form of HTT might contribute to HD pathogenesis (White *et al.*, 1997; Reiner *et al.*, 2003; Nguyen *et al.*, 2013). An HD knock-in mouse model has been shown to exhibit delayed acquisition of early striatal cytoarchitecture, and deregulated MSN subtype specification with aberrant expression of progressive markers of MSN

neurogenesis (Molero *et al.*, 2009). Neuronal changes in the striatum precede the onset of phenotype, whereas cortical changes, especially the accumulation of huntingtin protein in the nucleus and cytoplasm, and the appearance of dysmorphic dendrites, predicts the onset and severity of behavioral deficits (Laforet *et al.*, 2001).

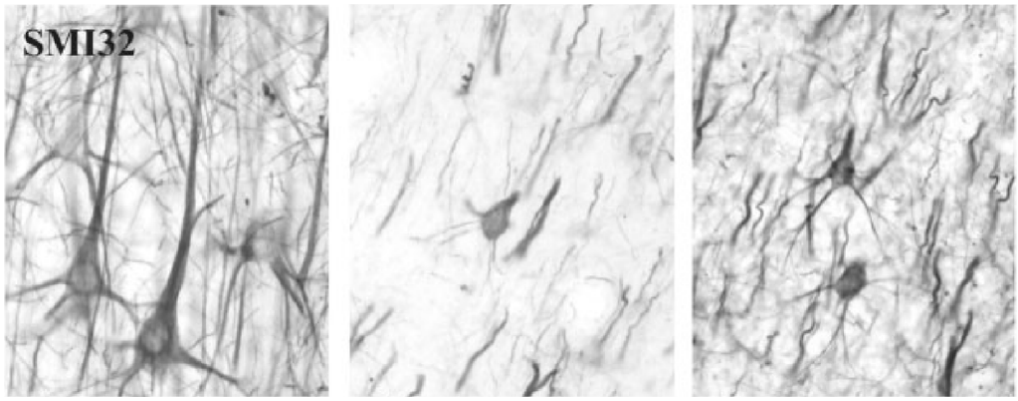

Figure 5.2. Photomicrographs of layer III pyramidal projection neurons (stained with SMI32) in the primary motor cortex of HD cases with predominantly motor (left), mixed motor-mood (center), and mainly mood (right) symptoms (Thu *et al.*, 2010).

Despite overwhelming attention and research efforts focusing on striatal MSN degeneration in HD, little is known about the complexity of CStrPNi that might underlie their selective vulnerability and death in HD, nor about the exact genes, molecules, and signals critical for their proper development, maintenance, function, and protection from degeneration. In addition, the subtype specificity of CStrPNi and CStrPNp involvement in HD has not been elucidated. Such profound lack of knowledge about CStrPN and their role in HD is a huge barrier to bringing advances to clinical diagnosis and molecular pathogenesis of HD. Identification of molecular controls and signature for CStrPNi development will provide a solid foundation for understanding root causes of this dual CStrPN and MSN neurodegeneration and pathophysiology in HD, which then could lead to directed searches for potential drugs and molecular therapies. Controlled recapitulation of developmental processes might ultimately enable large-scale production of CStrPN from pluripotent cells *in vitro* for high-throughput small molecule or drug screening. Similarly, knowledge of the mechanisms guiding CStrPN axons to project to correct targets, and to establish synaptic contacts to form functional cortico-basal ganglia circuitry might be used toward approaches to successful integration of transplanted CStrPN into recipient brains. An over-arching goal

of this research is to efficiently and productively move from discovery science to the development of new therapeutic approaches to prevent, delay, or reverse the currently untreatable HD degeneration.

5.2.2. CStrPNi is an Anatomically and Developmentally Hybrid Population with both CPN and CFuPN Characteristics

The hybrid anatomical connectivity of CStrPNi projecting first callosally, then subcortically, strongly suggests that they might also be a molecularly, and possibly evolutionarily, hybrid population of both CPN and CFuPN, and might utilize shared molecular and developmental programs with both broad projection neuron classes (Sohur *et al.*, 2014). There has been significant progress in identifying broader outlines of molecular programs for CFuPN and CPN development, in which neural progenitors and post-mitotic projection neurons express a sequential and combinatorial set of transcription factors to acquire subtype identity. Elucidating such mechanisms for CStrPNi development would greatly benefit from our existing knowledge of both CFuPN and CPN molecular controls.

Taken together, our research here 1) builds on new and existing knowledge on the birth and development of diverse classes of projection neurons in the neocortex, 2) aims to deliver findings that identify and delineate molecular controls over the development of this critically important CStrPNi population, and 3) provides a solid basis toward of future therapeutic treatments for HD.

5.3. Results and Discussion

5.3.1. Anatomic and Areal Distribution of CStrPNi

Spatiotemporal developmental characteristics of CStrPNi have recently been elucidated in a study from our laboratory. In this study, Sohur et al. utilize a retrograde labeling approach to label and systematically characterize the anatomical distribution of CStrPNi in the postnatal mouse brain (Sohur *et al.*, 2014). They demonstrated that CStrPNi reside primarily in layer V and sparsely in lower layer II/III, and are located predominantly in the motor and premotor cortices of the neocortex. Potentially through substantial pruning

and refinement of striatal axonal branches, the distribution of CStrPNi becomes sparse ventrolaterally in somatosensory, visual, and auditory cortices with age.

Both to substantiate these findings through different experimental approach, and to assess the areal distribution of cortical projection neurons that innervate the ipsilateral striatum (in contrast to contralateral striatum), we analyzed the anterograde tracing data in the Brain Connectivity Project conducted by the Allen Brain Institute (ABI). This online accessible database contains the tracing data of neurons (using recombinant rAAV2/1 virus expressing EGFP under the control of the human synapsin 1 promoter) from hundreds of different sites across the brain (Oh *et al.*, 2014). We analyzed all isocortex injections from this set of tracing data, including, but not limited to, primary and secondary motor, somatosensory, insular, visual, auditory, piriform, and entorhinal areas. We found that both motor and sensory cortical areas project substantially to innervate bilateral striata in varying degrees, but far caudal cortical areas (partially including visual and auditory cortices) showed only ipsilateral striatal innervation (Figure 5.3). These data independently confirm our published results on the areal distribution of CStrPNi (with projections to contralateral striatum) by retrograde labeling, and are in concert with previous studies on the anatomical topography of CStrPN (Wilson, 1987; Cavada and Goldman-Rakic, 1991; Mitchell and Macklis, 2005; Alloway *et al.*, 2006).

5.3.2. Temporal Development of CStrPNi

By dual BrdU birth-dating across different stages of cortical neurogenesis, and using retrograde analysis at P4, our lab previously demonstrated that CStrPNi are born predominantly between E12.5 and E14.5, along with populations of deep layer CPN and evolutionarily older CFuPN. In addition, anterograde and retrograde analyses for axon outgrowth and innervation of bilateral striata demonstrated that, around P0, pioneering CStrPNi axons cross the midline, but do not begin to innervate contralateral striatum till P2. Beginning at P3-P4, collaterals of CStrPNi reach bilateral striata simultaneously, which coincides with the time that contralateral cortex is innervated by CPN axons (Sheth *et al.*, 1998; Wang *et al.*, 2007a; Sohur *et al.*, 2014). We further confirmed these findings by analyzing coronal sections of postnatal mice brain where growing axonal terminals of developing CStrPN are labeled with GFP (from various *in utero* electroporation

experiments, conducted by us, and also by the other members of the Macklis Lab) (data not shown).

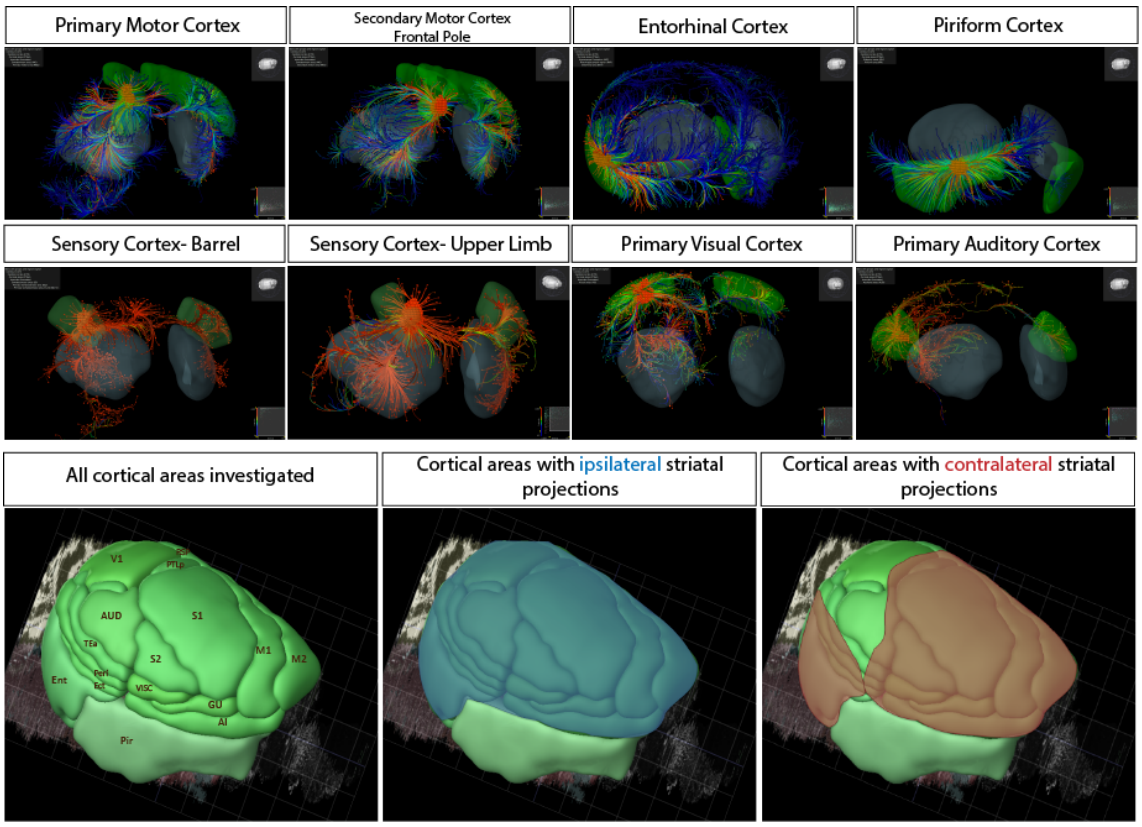

Figure 5.3. Cortical areas with ipsilateral and contralateral striatum projections. Snapshot representative images for investigation of areal distribution of CStrPNi and CStrPNp on Brain Explorer 2 software (The Connectivity Project, Allen Brain Institute).

To facilitate our analysis of the multiple "cross-axis" sets of comparative microarray data, and to elucidate the stage-specific molecular controls for CStrPNi, we summarized and divided the progression of CStrPNi development into four broad stages: 1) an early stage of CStrPNi identity determination, likely to start with expression of genes in early progenitors and post-mitotic neurons to establish and specify CStrPNi cell fate, and which might maintain some of this gene expression throughout maturity; 2) an axon outgrowth stage, with expression of genes that might regulate the extension and growth of axons out of neocortex (and in white matter tracts) before entering either ipsilateral or contralateral striatum; 3) a target innervation stage with expression of genes that might direct axons to exit from white matter tracts and regulate the branching of axon collaterals from main axons to innervate

targets such as contralateral and ipsilateral striatum; and 4) a stage of axon arborization in the striatum and of target cell recognition, with expression of genes that might affect the formation of terminal axon branches and development of functional synapses with striatal MSN (Figure 5.4).

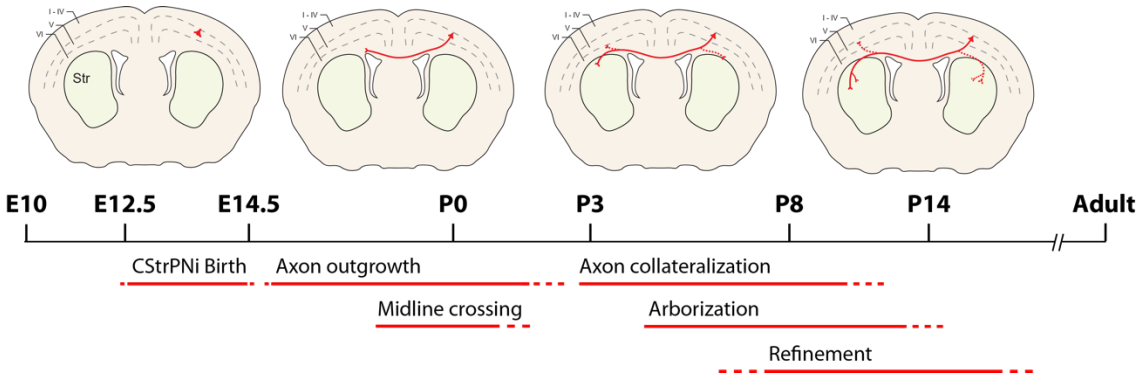

Figure 5.4. Timeline of CStrPNi development. CStrPNi are born ~E12.5-E14.5, and extend pioneering axons crossing the midline around P0. CStrPNi axons enter the contralateral striatum around P3, when a subset of CStrPNi project to the ipsilateral striatum.

5.3.3. Developmental Comparison of CStrPNi, CPN and CFuPN

The hybrid anatomical connectivity of CStrPNi, projecting first callosally between hemispheres, then subcortically to the striatum, clearly indicates that they share some developmental features with both CPN and CFuPN populations, and might incorporate molecular programs combinatorially from both projection neuron classes. At early stages of their development, during axonal outgrowth, CPN and CStrPNi (but not CFuPN) are potentially instructed by common molecular controls, because both extend their primary axons to the contralateral cortex (but not toward the internal capsule), and later form collaterals in the contralateral cortex. CFuPN axons enter to the striatum around E12, and pass through it by E15, while CStrPNi collaterals start entering the striatum around P3 (Sohur *et al.*, 2014). This indicates that CFuPN and CStrPNi possibly utilize different molecular programs than CFuPN in their entry to the striatum, but then follow similar instructions for axon collateralization and branching once within the striatum. This observation is further supported by our analysis of anterograde rAAV tracing data from ABI (Figure 5.5). While most CStrPNi axonal innervation in the striatum displays a fairly

discrete, defasciculated pattern of terminal organization, CFuPN axons (i.e. primarily PT-type CStrPNp) appear to fasciculate into discrete bundles, and could potentially share common molecular mechanisms for striatal innervation. Understanding the resemblance and differences of CStrPN with both CPN and CFuPN populations is crucial to interpret gene expression data, and formulate hypotheses regarding function of potential molecular controls.

5.3.4. Comparative Microarray Analysis and Progressive Filtering Toward Selection of Top Molecular Candidates for CStrPNi Development

The work by our laboratory and others has begun elucidating the outline of molecular programs for CFuPN and CPN development, in which neural progenitors and post-mitotic projection neurons express a sequential and combinatorial set of transcription factors to acquire subtype identity. Given the unique dual projecting, developmentally hybrid characteristics, understanding of the development of CStrPNi relies greatly on existing knowledge of both CFuPN and CPN development.

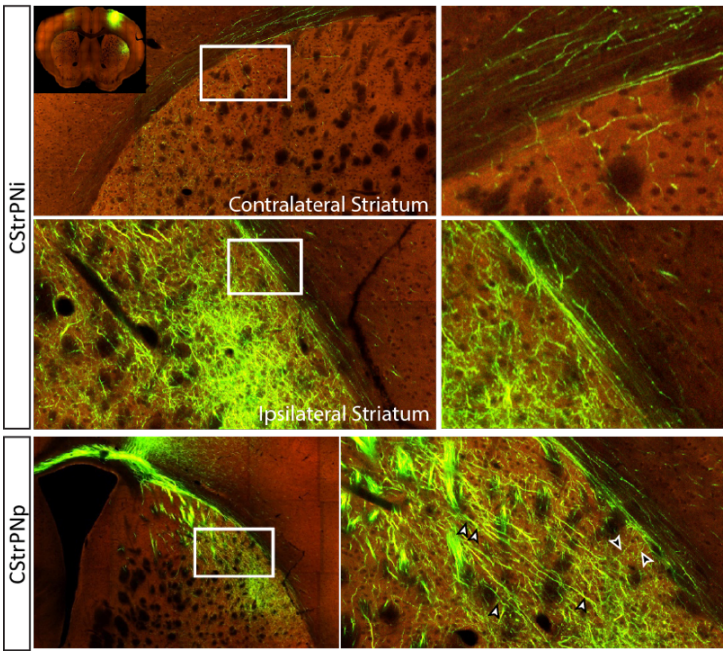

Figure 5.5. CStrPNi branches in striatum display defasciculated organization, whereas CStrPNp branches sprout from fasciculated discrete bundles (Allen Brain Institute, The Connectivity Project).

To identify the molecular controls that regulate CStrPN differentiation, former members of the Macklis Lab performed a microarray analysis of a differential gene expression between CStrPN and the comparator CPN at key developmental stages (P4, P8, P14) (Figure 5.6). They labeled CStrPN and CPN from their axonal terminals in the contralateral striatum and cortex, respectively, and isolated both populations via FACS-purification. They collected three biological replicate RNA samples, completed the Affymetrix GeneChip Mouse Genome Array analysis, and performed the data normalizations and validations as previously described (Arlotta *et al.*, 2005). The data were obtained with the same methods as prior work on CSMN, prior CPN analyses (Arlotta *et al.*, 2005, Molyneaux *et al.*, 2009), so those data can be directly compared and integrated.

We performed *in silico*, integrative analysis of these data sets to narrow down and identify candidate genes potentially involved in CStrPNi development. We have cross-referenced the CStrPN-CPN array data with separate CPN-CSMN-Corticotectal array data generated previously by our lab (Arlotta et al., 2005), to incorporate all available information on CPN and CFuPN development into current efforts of identifying CStrPNi molecular controls. While formulating filtering criteria to analyze the microarray data, we added biological sophistication to our array analysis to take into consideration the unique features of CStrPNi both as a neuronal population and regarding experimental limitations for microarray profiling as discussed below.

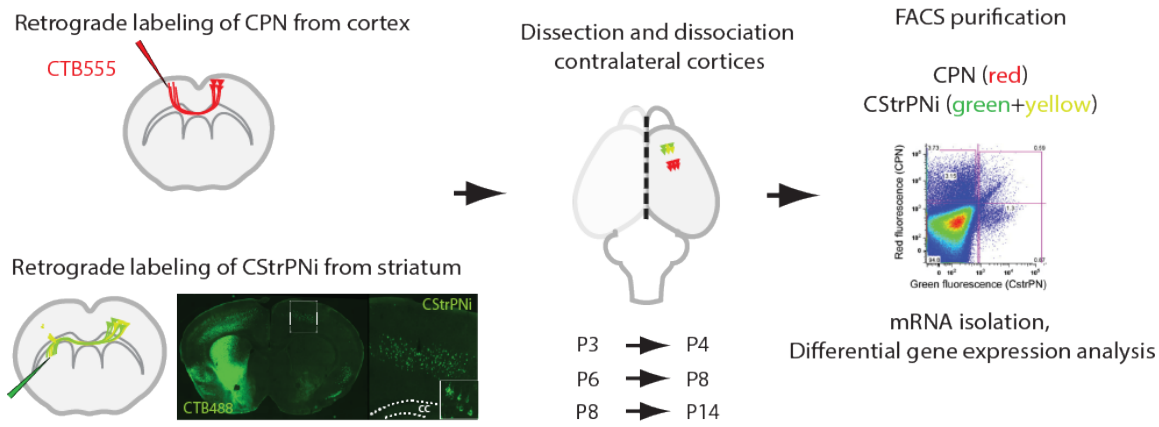

Figure 5.6. Experimental setup for comparative gene expression analysis for CStrPNi and CPN. CStrPN and CPN are retrogradely labeled, dissociated, and FACS-purified at key developmental stages, and comparative gene expression analysis is performed.

5.3.4.1. Biological Similarity of CStrPNi and CPN at Early Development. At early stages of development, CPN and CStrPNi are likely instructed by common molecular controls. Both extend their primary axons across the midline to the contralateral cortex (but not to the internal capsule) and form axon collaterals in the contralateral cortex. Previous evidence indicates that many CPN have transient collaterals innervating the striatum (Kadhim *et al.*, 1993), suggesting that signaling mechanisms that function in formation and entry of collaterals to the striatum are active in each cell population, but at varying doses. These data support our previous findings that, at early postnatal ages, CStrPNi display a more diffuse pattern of distribution in deep layers of neocortex along the rostra-caudal axis, but their distribution at caudal levels becomes gradually restricted to motor and premotor areas with age, suggesting that many eventually "pure" CPN had transient collaterals into the striatum. Thus, it is plausible to speculate that CPN and CStrPNi share common biology at early time points, which led us to enhance our candidate selection.

5.3.4.2. Expanding Candidate Gene Pool for Selection at Early Developmental Stage P4. CStrPNi are born predominantly between E12.5-E14.5. Their axons begin crossing the midline around E18-P0, and pioneering collaterals enter the striatum around P2-P3. Thus, the earliest time point to retrogradely label and isolate CStrPNi population is at P4. However, by P4, CStrPNi have already assumed their identity and began innervation to the striatum. Therefore, we hypothesized that, among those early-acting genes, there might be some that continue to function, and are specifically expressed at later stages as well.

5.3.4.3. Hybrid Nature of CStrPNi Affects Isolation for Comparative Microarray Analysis. For the comparative microarray analysis, CStrPNi and CPN populations are identified via retrograde labeling, and isolated via FACS-purification. Though this approach provides a pure population of CStrPNi, the labeled CPN population inevitably includes some CStrPNi, since cortical dye injections for CPN isolation potentially label some cortical axonal branches of CStrPNi. Thus, CPN-population consists of some CStrPNi, enabling modest enrichment; this was the best possible at the time. The proportion of CStrPNi in the isolated CPN population may vary over time and space, again leading to partial enrichment at early stages, P4 most strikingly.

5.3.4.4. Active Transport of RNA Molecules from the Soma for Local Translation. Potential cellular mechanisms involved in CStrPNi development include growth cone mediated axon guidance by extracellular cues, axon collateralization, and branching. Some molecules involved in growth cone functions are locally translated via active transport of mRNA molecules from the soma to growth cones (Wu *et al.*, 2005). In our microarray preparation, CStrPNi are retrogradely labeled, and only the somas are isolated for mRNA profiling. This could potentially affect the enrichment of CStrPNi-specific genes against CPN.

Our goal to refine and enhance the biological insight of candidate genes prior to functional analyses has led us to generate a candidate gene pool according to the following selection criteria: i) We have considered P-value <0.05 to be statistically meaningful because of the unique features of CStrPNi described above. 2) Because of the unique developmental connectivity of CStrPNi, we have decided to set the threshold to greater than 1.4-fold for P4, and greater than 1.5-fold for P8 and P14. Based on these selection criteria with the unique biological features of CStrPNi in consideration, we filtered our array data and obtained the following number of candidate genes to further refine, and from which to select highes-interest candidates: P4, 155; P8, 393; and P14, 507.

In the analysis of the microarray data, we aimed to identify candidate genes that might function at distinct, critical developmental stages of CStrPNi, including: identity acquisition, axon outgrowth, innervation of the striatum, branching within the striatum, synaptic connections with striatal MSN, maintenance, and survival. We considered two primary criteria and two complementary criteria for selecting candidate genes. Primary criteria included: 1) cellular and biological functions of the gene, and 2) the level of enrichment in CStrPNi and absolute expression in the microarray. Complementary criteria included: 3) spatial and temporal expression patterns in online databases (Allen Brain Atlas, GenSat.org, Eurexpress,org, GenePaint.org and BGEM) and from the published literature, and 4) temporal expression patterns in our published CSMN vs. CPN microarray analysis. We categorized genes that are functionally less interesting, but are highly enriched, as potential markers for CStrPNi, such as genes functioning in metabolic pathways (e.g. membrane transporters or enzymes involved in amino acid, glucose, lipid and nucleotide metabolism, ATP metabolism, lysosomal enzymes, pseudo-genes, genes encoded for mitochondrial proteins or ribosome biosynthesis, genes encoded for enzymes in major

signaling pathways, and DNA repair, etc). Based on these criteria and insight, we categorized the top CStrPNi candidate genes into three groups (Table 5.1): highly promising (Group A; Table 5.2), promising (Group B, as a secondary list), and less-promising.

5.3.5. Validation of Array Data

We performed in situ hybridization experiments for a select set of genes to verify the microarray data (Figure 5.7). We found that a substantial number of CStrPNi-enriched genes identified via microarray also show enhanced expression in cortical layer Va relative to other cortical layers. The spatial expression pattern of these genes matches the distribution of CStrPNi obtained by retrograde tracing (Sohur *et al.*, 2014), indicating that our multi-step, progressive filtering approach of array data was successful. Beyond this, we found that a number of genes are equally expressed in both layer Va and Vb (where CStrPNi and CStrPNp are located, respectively) (data not shown), further supporting our view that shared core biological processes might be operating in both CStrPNi and CStrPNp. Also, we found that a number of genes are enriched in layer Va in the adult mouse brain (data from Allen Brain Institute) (Figure 5.8). Once verified, these genes might be used as potential markers for CStrPNi.

5.3.6. Potential Experimental Approaches for Functional Analysis of Candidate Genes

To understand the function(s) of the selected top CStrPNi candidate genes, a next step might be to investigate the temporal, spatial, and cell type-specific expression of each gene, and characterize their CStrPNi-specific function via loss- and gain-of-function analyses. A wide range of already established experimental approaches are available for the functional investigation of candidate genes. In cases where conditional-null (floxed) mouse lines exist for a gene, a range of subtype-specific or cortex-specific Cre-driver lines have recently become available to selectively remove gene function (described below). Alternatively, Cre-recombinase expressing constructs can also be introduced to select subpopulations of CStrPNi at specific embryonic time points via *in utero* electroporation, or at postnatal stages via AAV-mediated transfection. This allows us to create a mosaic pattern in which mutant cells are scattered among control neurons, enabling more precise interpretation of observed phenotypes.

Table 5.1. Multi-step filtering process to identify the top CStrPNi candidate genes.

		P4	P8	P14	Total
Number of genes enriched in CStrPNi		155	393	507	1055
Step 1 (expression level and temporal course)		64	123	114	301
Step 2 (Functional relevance, cortical expression)		32	80	72	184
Step 3 (function, intensity of enrichment, expression level and pattern, comparative analysis between projection neuron subtypes)	Group A	5	6	6	17
	Group B	16	19	24	59
	Group C	11	55	43	109

Table 5.2. Top CStrPNi candidate genes (Class A) selected for further analysis.

Gene Symbol	Gene Name	Enrichment age
Igf2bp2	Insulin-like growth factor 2 mRNA binding protein 2	P4
Prdm16	PR domain containing 16	P4
Zic1	Zinc finger protein of the cerebellum 1	P4
Gfra2	Glial cell line derived neurotrophic factor family receptor alpha 2	P4
Rgs5	Regulator of G-protein signaling 5	P4
Cxcl12	Chemokine (C-X-C motif) ligand 12	P8
Aplnr	Apelin receptor	P8
Adcyap1r1	Adenylate cyclase activating polypeptide 1 receptor 1	P8
Sparc	Secreted acidic cysteine rich glycoprotein	P8
Apcdd1	Adenomatosis polyposis coli down-regulated 1	P8
Foxp1	Forkhead box P1	P8
Pbx1	Pre B-cell leukemia transcription factor 1	P14
Cd44	CD44 antigen	P14
Astn2	Astrotactin 2	P14
Itgb3	Integrin beta 3	P14
Chd6	Chromodomain helicase DNA binding protein 6	P14
S100a10	S100 calcium binding protein A10 (calpactin)	P14

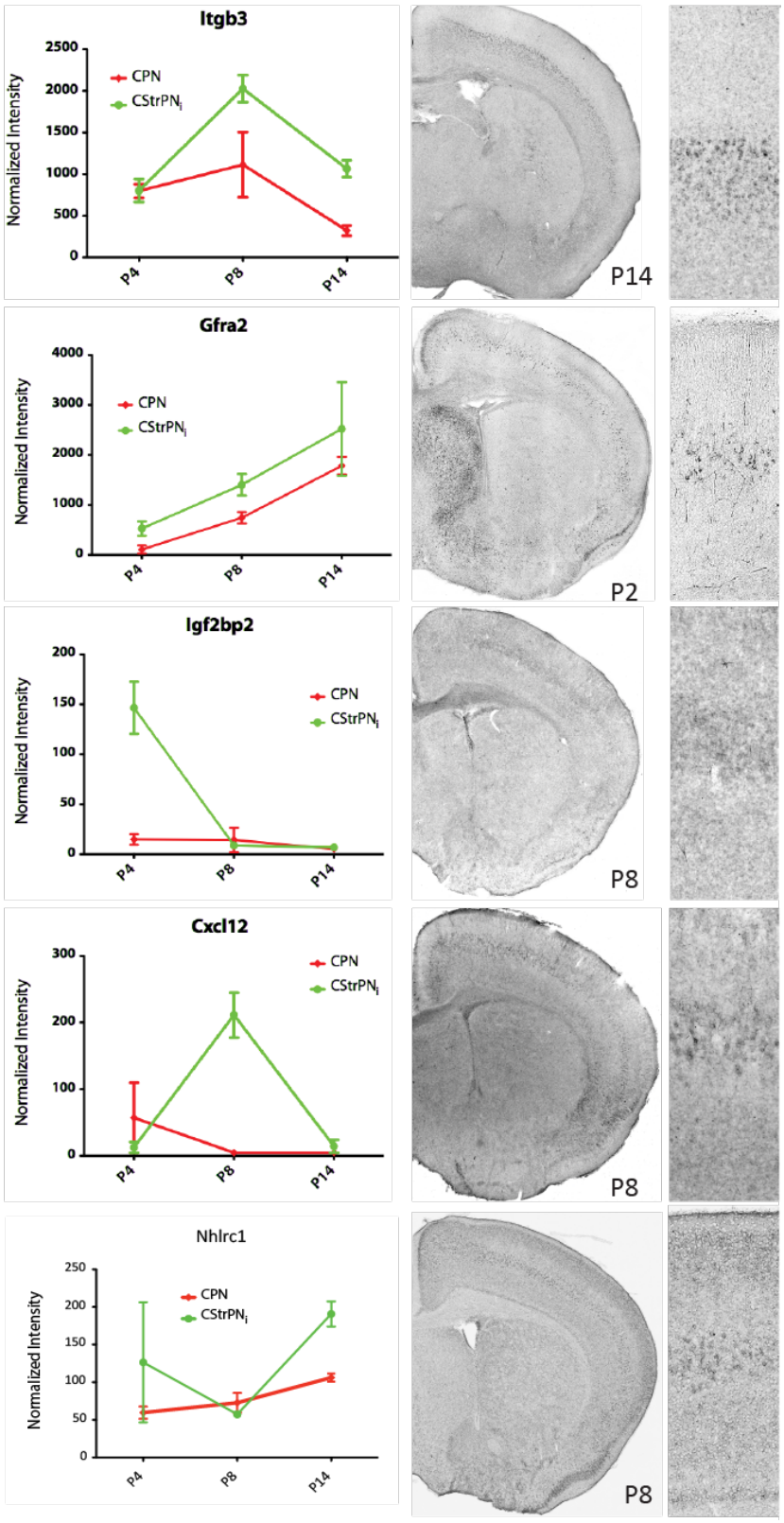

Figure 5.7. Temporal expression profiles of CStrPNi-enriched genes from microarray analyses, and *in situ* hybridization of cortical coronal sections showing layer V enrichment of CStrPNi genes during early postnatal stages.

Additionally, rescue experiments can be performed via delivery of over-expression constructs for a gene of interest on conditional- or global-knockout mice. If a conditional null mouse line is not available, shRNA knockdown constructs can be introduced via ultrasound-guided *in utero* electroporation, or by injection of AAV (adeno-associated virus) particles. In addition, mis-expression analyses (i.e. expression of a candidate gene in a different cell population or at a non-normal time) can be performed as complementary experiments to the loss-of-function experiments described above. This approach is especially useful to distinguish 'instructive' from 'permissive' roles of candidate genes.

5.3.7. Characterization of CStrPNi-specific Tlx3-BAC-Cre Mouse Line

We acquired a recently generated Tlx3.BAC-Cre driver line, a potential IT-type CStrPNi-specific transgenic mouse line expressing Cre recombinase under the control of the promoter of the T-cell leukemia homeobox 3 (Tlx3) gene. This line (generated by the Gene Expression Nervous System Atlas Project, GENSAT) can be used as a crucial tool for investigating the development and connectivity of CStrPNi. Immunostaining for the Cre protein, and labeling of axonal branches of Cre-expressing neurons (via a conditional GFP construct introduced as packaged AAV particles) suggested that this line is CStrPNi-specific (Gerfen *et al.*, 2013).

We characterized the Tlx3.Cre driver line at distinct embryonic and postnatal stages by crossing it to a conditional fluorescent reporter line (ROSA26-flox-STOP-flox-TdTomato). We investigated the specificity of Cre expression using both subtype-specific markers (Figure 5.9), and by retrograde labeling of CStrPNi and subcerebral projection neurons. We found that Cre expression starts around birth, specifically by neurons in layer Va (Figure 5.9). Cre activity is specific to CStrPNi (Figure 5.10a), and is completely absent in retrolabeled SCPN (Figure 5.10b). In line with this, we found that, while there are abundant projections in the striatum from reporter-positive neurons in these mice, there are no tdTomato+ axons in the internal capsule or along the rest of the corticospinal tract (data not shown).

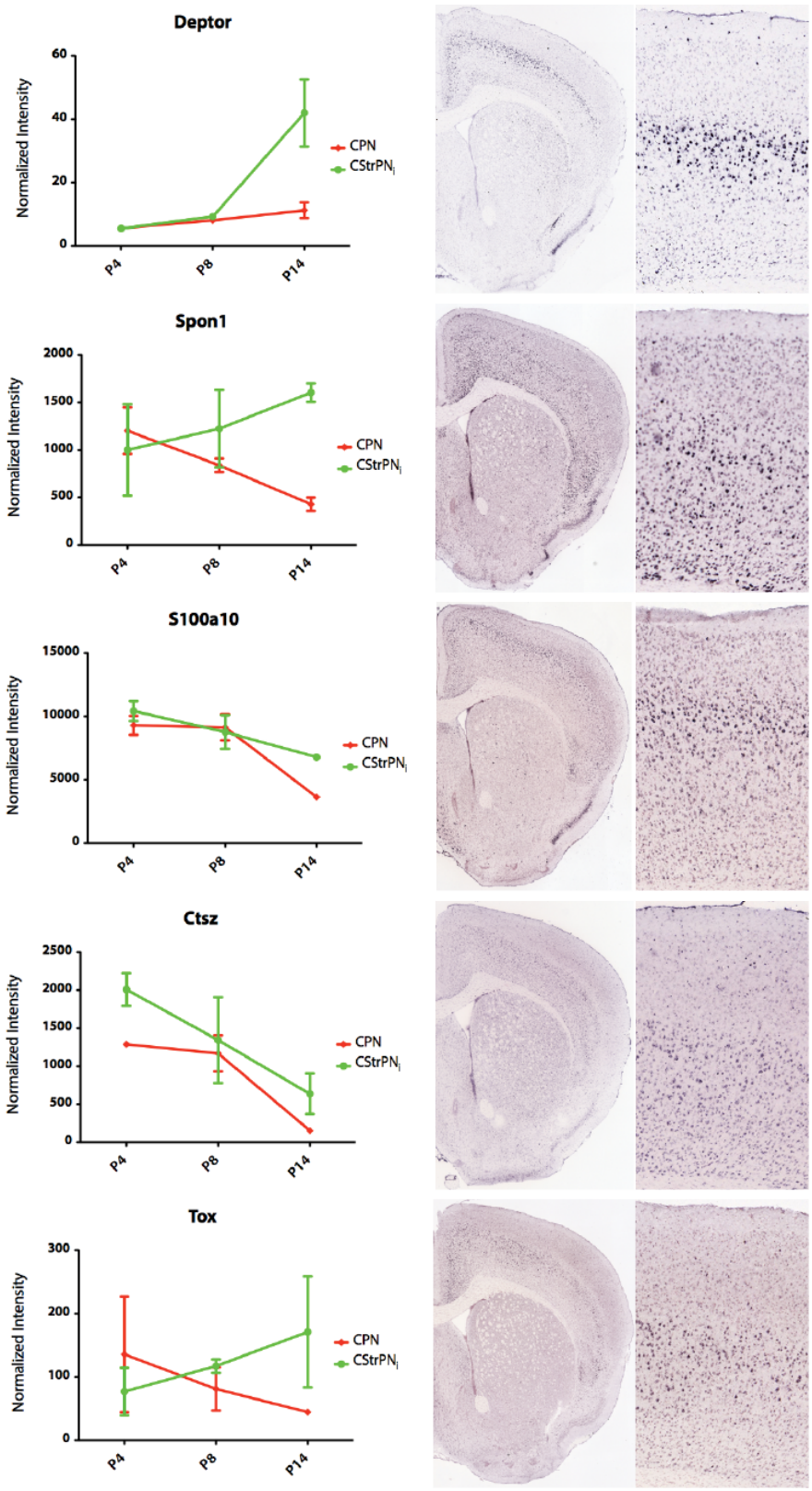

Figure 5.8. Temporal expression profiles of CStrPNi-enriched genes in the microarray analysis, and *in situ* hybridization in coronal sections of cortex showing layer V enrichment of CStrPNi genes in adult brain (Allen Brain Institue) (Lein *et al.*, 2007).

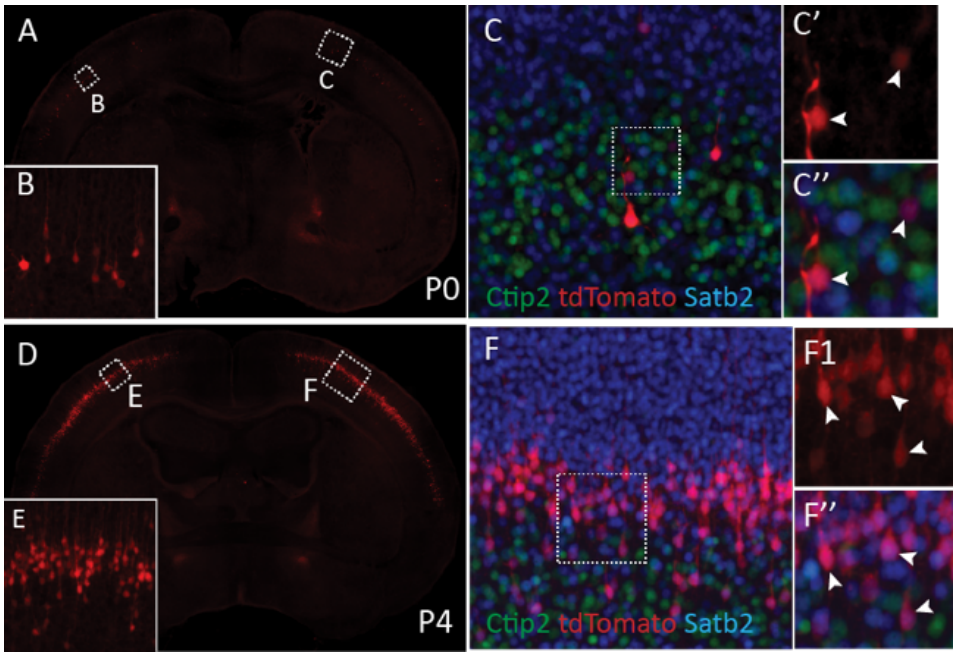

Figure 5.9. The Tlx3-BAC-Cre transgenic line displays layer Va CPN/CStrPNi, but not SCPN, molecular characteristics during development.

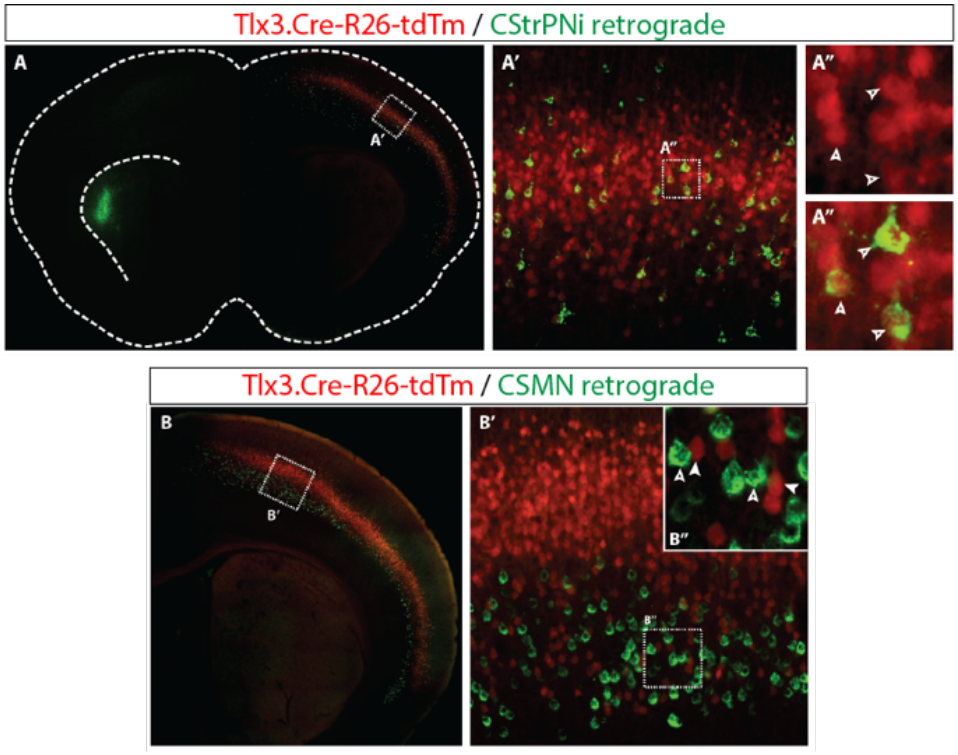

Figure 5.10. Tlx3-Cre-driven tdTomato expression co-localizes with CStrPNi (A), but not with CSMN (B).

Besides the Tlx3 driver, alternate Cre lines such as the Rbp4-BAC-Cre (layer V-specific; potentially covers both CStrPNi and CStrPNp subsets) and Emx1-IRES-Cre (expressed by progenitors of all projection neurons of the cortex) are valuable tools to knock out candidate genes in the broad corticostriatal population. Some of the top CStrPNi molecular candidates are also expressed in the striatum. In order to directly examine possible non-CStrPNi-autonomous, striatal MSN-specific effects on CStrPNi axon outgrowth and innervation, the Gsx2.Cre line can be used (Figure 5.11).

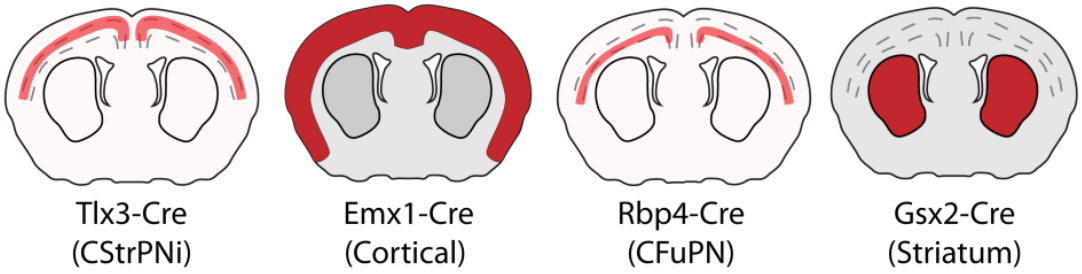

Figure 5.11. Potential Cre-driver lines that can be used in functional studies of CStrPNi.

5.3.8. Igf2bp2 as a Potential Molecular Control over CStrPNi Differentiation

Igf2bp2 (insulin-like growth factor-2 mRNA binding protein 2, also called IMP2) is the second-most enriched molecule in the CStrPNi population at P4 (~9.96 fold, p=0.0008). Igf2bp2, an RNA-binding protein, primarily localizes to the cytoplasm, and associates with cytoplasmic ribonucloprotein complexes, potentially regulating the cytoplasmic localization, turnover and translational control of target mRNAs (Bell *et al.*, 2013).

There are three members of the Igf2bp family in the mouse genome (Igf2bp1, Igf2bp2 and Igf2bp3), and they show high amino acid sequence conservation (73% identity between Igf2bp1 and Igf2bp3, 56% among the three members), suggesting that they potentially act in similar biological processes. Due to their high structural similarity, cross-reactivity of reagents (e.g. antibodies, *in situ* probes) between the three family members has made attributing a function to a particular family member difficult. While a systematic identification of transcripts that are targeted by Igf2bp proteins is lacking, *in silico* analyses suggests that they might regulate ~1000 target mRNAs. Of note, so far only Igf2bp1 has been shown to bind Igf2 growth factor mRNA. All family members are primarily expressed

during embryonic development (expression starting around E12.5 and declines toward birth) with relatively low or no expression in the adult tissues (Bell *et al.*, 2013).

They are implicated in a diverse range of developmental processes that primarily involve aspects of cellular migration, including cell polarization, adhesion, motility, progenitor differentiation, as well as tumor invasion. Intriguingly, Igf2bp family members can bind the transcript of CD44, a cell surface glycoprotein that is highly enriched in CStrPNi (~5-fold, $p<0.05$) in our microarray data. Binding of the CD44 transcript to Igf2bp might prevent its premature degradation, thereby regulating CD44-mediated invadopodia formation and cellular motility (Vikesaa *et al.*, 2006).

Igf2bps might regulate motility-related features in developing neurons, such as axonal growth cone guidance, along with other aspects of connectivity like dendritic morphology and synaptic properties (Eom *et al.*, 2003). In cultured hippocampal neurons, Igf2pb1 binds to beta-actin mRNA (ACTB), and this complex is dynamically transported along neurites. Remarkably, inhibition of Igf2bp1 binding to ACTB mRNA (via antisense oligonucleotides against 3-UTR of ACTB) impairs the directional responsiveness of growth cones to chemo-attractants *in vitro* (Zhang *et al.*, 2001; Huttelmaier *et al.*, 2005).

Based on the above data, I hypothesized that Igf2bp2 might be a potential regulator of axonal branching, enabling CStrPNi to innervate the contralateral cortex in addition to its targets in the bilateral striata. To explore this possibility, I performed mis-expression of Igf2bp2 in upper layer callosal projection neurons. During cortical neurogenesis, neural progenitors located in the dorsal ventricular zone initially give rise to deep layer neurons between embryonic day (E) 12.5 and E14.5, and later to upper layer neurons (primarily callosal projection neurons) between E15.5 and E17.5. I introduced Igf2bp2 over-expressing constructs (via *in utero* electroporation at E15.5) into the progenitors of upper layer CPN to investigate whether Igf2bp2 might induce CPN axons to ectopically branch and innervate the striatum. Although axonal branching of CPN was not affected by expression of Igf2bp2, the electroporated neurons were ectopically positioned in deep layers (Figure 5.12). This specific positioning of electroporated neurons in layer V suggests that this ectopic location is not the result of an arbitrary migration deficiency, and might indicate a potential role for Igf2bp2 in the positioning of CStrPNi. This suggests that Igf2bp2 might also function in

regulating additional aspects of CStrPNi specific differentiation. Supporting this hypothesis, the high-mobility group A (HMGA2) protein was recently shown to promote the expression of Igf2bp2 in early stage neocortical progenitors and in proliferative myoblasts, which in turn regulates the stability of target mRNAs, and mediates the timely differentiation of progenitors. To investigate the potential role of Igf2bp2 in CStrPNi development, I plan to interrogate its function through loss-of-function experiments via electroporation of either CRISPR/Cas9 or shRNA-constructs targeting Igf2bp2 *in utero*.

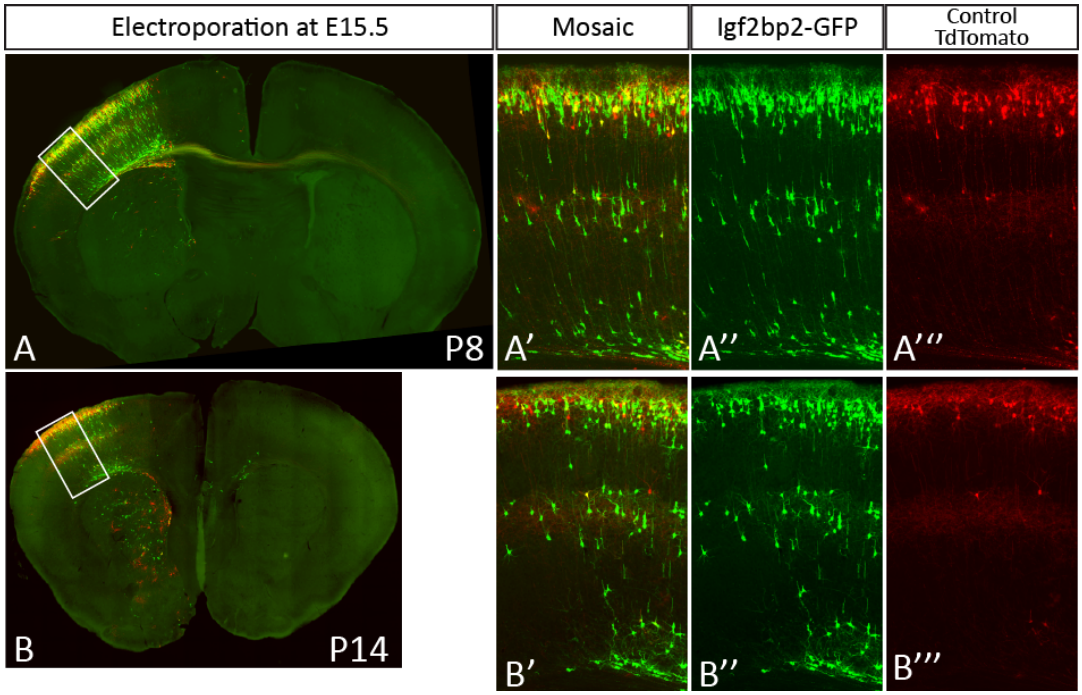

Figure 5.12. Igf2bp2 likely regulates cortical positioning and identity acquisition of CStrPNi. Control neurons (red) migrate normally and settle in their default destination, whereas a substantial number of Igf2bp2-expressing (GFP) neurons settle in layer V.

6. CONCLUSION

Many devastating neurodegenerative, developmental, and acquired central nervous system diseases and injuries primarily affect defined subsets of cortical projection neurons. Amyotrophic lateral sclerosis (ALS) is defined by degeneration of both corticospinal motor neurons (CSMN) and spinal motor neurons. CSMN degeneration also defines the "upper motor neuron" diseases primary lateral sclerosis and hereditary spastic paraplegia. Corticostriatal projection neuron degeneration accompanies striatal medium spiny neuron degeneration in Huntington's disease. Corticobasal degeneration involves degeneration of related projection neurons. Loss of motor function in spinal cord injury results centrally from damage to CSMN axons in the corticospinal tract.

Until recent years, functional, cellular brain repair has seemed impossible. However, there are now good reasons to believe that this may indeed be possible in the future. Strong natural precedents exist in which complex, behaviorally critical neural circuitry is normally repopulated and reconstructed throughout life in the mammalian olfactory bulb (the part of the brain dealing with smell) and dentate gyrus of the hippocampus (dealing with learning and memory), and in some high-level vocal learning and control networks of songbirds, but not in mammalian cerebral cortex under normal conditions. Work from our laboratory and then others indicates that therapeutic repair of complex neuronal circuitry might be possible, if we can precisely control the development of progenitors already in the brain or in the laboratory, and the environment for their growth, maturation, and function. Neuron replacement could make possible future therapies for neurodegenerative diseases.

In the first part of this thesis, I present work aimed to understand and refine how to manipulate endogenous neural progenitors using developmental controls, to enable directed population-specific neurogenesis and repair of complex neuronal circuitry, in particular of the neocortex and its output circuitry. We show that neural progenitors from the neocortex can be isolated with exceptional purity, and be expanded in culture without losing their endogenous features. We demonstrate that manipulation of a select set of central and complementary developmental molecular controls in cultured neocortical progenitors can direct the differentiation of cortical output neurons, a broad group of clinically relevant

neurons that includes CSMN. Newly generated neurons acquire morphological, molecular, and electrophysiological characteristics similar to their primary *in vivo* counterparts. Our work provides compelling evidence that development-inspired directed differentiation is a viable strategy to generate defined neuronal subtypes for cellular repair of damaged neuronal circuitry.

6.1. Future Prospects for ENVOF Directed Differentiation Approach

In our directed differentiation approach, we employ upstream central transcriptional regulators that are capable to activate downstream molecular pathways required for CSMN differentiation and glial fate suppression. Yet, our growing understanding of basic biology of cortical development, adult progenitors and mechanisms of cellular reprogramming will enable continuous refinement of directed CSMN neurogenesis approaches.

In light of recent data in the field, additional transcriptional regulators and epigenetic modifiers can be considered to further refine and improve the ENVOF approach. For example, NeuroD4/Math3 was identified as a cofactor of Ngn2 that synergizes with Ngn2 in specification of glutamatergic neurons (Mattar *et al.*, 2008). Future experiments investigating combinatorial effects of NeuroD4 and ENVOF might enable better induction of neurogenesis from NG2+ cortical progenitors. Additionally, expression of Ctip2 (acts in downstream of Fezf2 in CSMN axon outgrowth (Arlotta *et al.*, 2005; Chen *et al.*, 2008)) in addition to ENVOF can be investigated to further augment CSMN differentiation of newly generated neurons.

Also, in addition to VP16:Olig2 (acts as antagonizer of Olig2), additional and/or alternate approaches can be considered to ‘de-repress’ the broad neurogenic program in NG2+ cortical progenitors. For example, suppression of REST (RE1-silencing transcription factor, which normally acts as repressor of neuronal genes in non-neuronal cells by recruiting chromatin remodeling proteins) prevents oligodendrocyte differentiation by NG2+ progenitors; and initiates a neuronal gene expression program (Dewald *et al.*, 2011). This strategy offers the potential refinement and increase in efficiency of overcoming epigenetic blocks that may exist in progenitors, to enhance their competence for directed differentiation.

Likewise, recent *in vivo* and *in vitro* data has demonstrated that NG2+ progenitors respond to histone deacetylase inhibitors, and transform into multipotent precursors with the ability to differentiate into neurons (Liu *et al.*, 2007b; Lyssiotis *et al.*, 2007). Liu et al., observed neuronal differentiation of transplanted GFP+/NG2+ cells in rats treated with valproic acid. Thus, this approach can be extended to future *in vivo* experiments to enhance the differentiation of CSMN from NG2+ progenitors.

6.2. Transplantation of ENVOF-transfected Progenitors into Postnatal Mouse Brain

Our systematic characterization of subtype identity of ENVOF-induced neuronal cells in culture provides robust proof that these neurons gain CSMN-like identity (i.e. expression of key molecular factors, absence of non-CSMN key molecules, gain of appropriate morphology). However, definitive proof for subtype identity for a given neuron has to be tested *in vivo*. One potential approach would be transplantation of ENVOF-transfected cultured NG2+ progenitors back into early postnatal mouse brain, and assessment of morphological, hodological and electrophysiological features *in vivo*.

Additionally, previous work from Macklis Lab has shown that growth factors Igf1 and BDNF both support CSMN survival, and Igf1 specifically activates and enhances CSMN axon growth (Ozdinler and Macklis, 2006). Therefore, delivery of Igf1 (+/- BDNF) as exogenous peptides can be considered to enhance survival and axon outgrowth of transplanted CSMN to spinal cord. Direct infusion of Igf1 locally into motor cortex can be achieved via established methods such as Alzet minipump placement. Together, these experiments inform and refine approaches toward potential repair of CSMN circuitry in human ALS model mice described below.

6.3. Translation of Findings *in vivo*

While the work presented in this thesis is a significant step forward, there are fundamental challenges that remain to be tackled for neuronal replacement approaches to be therapeutically promising. To effectively replace the function of diseased neurons, newly generated neurons would need to not only exhibit subtype specific features *in vitro*, but also the capacity to integrate into *in vivo* neuronal circuitry to form functional afferent and

efferent connections. This would likely require new neurons to be in correct anatomical and laminar positions, to receive appropriate dendritic inputs, and to project their axons to correct target areas.

The fact that environmental cues required for guidance of axons to correct targets exist only during development, pose an important problem for long distance axonal growth in adult central nervous system. However, in brain areas such as the olfactory bulb, hippocampus and hypothalamus, constitutively generated and transplanted immature neurons or progenitors are capable of integrating into adult neuronal circuitry. Beyond, both clinical observations and animal experiments have shown that in CSMN circuitry, successful approaches to functional repair might require only a relatively small number of neurons. If long-distance axonal outgrowth can be at least somewhat specifically guided, potentially even partial restoration of diseased pathways and quite imprecise connections might be sufficient to restore the behavior.

6.4. Fezf2-null and ALS Model Mice for Induction of CSMN Neurogenesis *in vivo*

Our proof-of-concept experiments *in vitro* demonstrate the feasibility of achieving subtype–specific differentiation of cortical projection neurons from cortical progenitors. However, corroboration of these findings *in vivo* is immensely important for these efforts to be therapeutically relevant. Here, I describe two potential animal models that can be exploited for *in situ* induction of CSMN neurogenesis in future.

Human ALS is characterized by specific and progressive degeneration of both CSMN and spinal motor neurons. Ozdinler and Macklis Labs recently demonstrated that the transgenic mouse model of familial ALS (with G93A point mutation in human SOD1 gene) undergo specific and early degeneration of CSMN, beginning by P30, peaking in rate ~P45-P60, and continuing through P90+ (Ozdinler *et al.*, 2011). This work confirms hSOD1G93A mice as an accurate model of ALS, and suitable model for induction of CSMN neurogenesis *in vivo*.

The transcription factor Fezf2 is a critical regulator of CSMN specification during early development. In Fezf2-null mice, CSMN are never born, resulting in absence of CSMN

and the corticospinal tract. This "genetic deletion" model provides a clean background for generation of new CSMN, since any observed CSMN is unambiguously newly generated. Fezf2-null mice survive to adulthood, therefore one can rigorously investigate directed differentiation of NG2+ cortical progenitors into CSMN in adult ages.

6.5. Corticostriatal Projection Neurons: Development and Selective Vulnerability

In the second part of this thesis study, I present initial efforts toward understanding of development, connectivity, and diversity of corticostriatal projection neurons (CStrPN). Elucidation of molecular controls over CStrPN development would not only provide insight into their basic circuit development, and the molecular basis of their selective vulnerability for Huntington's Disease (HD) degeneration, but also might enable directed differentiation both *in vitro* for mechanistic modeling for disease and screening for small-molecule therapeutics, and toward cellular repair of corticostriatal circuitry in adult HD cortex.

It has been long speculated that the dysfunction and degeneration of cortical projection neurons are primary or at least coincident to the onset and progression of HD, and that the initiation of striatal MSN degeneration might not be striatum-intrinsic (Laforet *et al.*, 2001). Considerable evidence demonstrating that among the earliest manifestations of HD are emotional and cognitive disturbances further support this hypothesis (Petersen *et al.*, 2005). It is possible that CStrPN degeneration occurs coordinately with MSN degeneration as pathology of synaptically connected corticostriatal circuitry, causing alterations in both motor and cognitive functions (contrary to historic view of gross degeneration of the striatum). Since the central neuronal populations affected by HD are interconnected in a precise circuitry loop, understanding the role of the CStrPNi population as the possible first step in HD degeneration would contribute substantially to our understanding of HD; both molecular pathogenesis and routes to modeling and therapeutics. Our insight regarding the stage-specific molecular controls and markers for CStrPN will be immensely valuable to investigate whether mutant huntingtin protein causes specific and subtle dysgenesis in CStrPN projections, and/or specific vulnerability of both CStrPN and other cortical projection neuron subtypes.

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