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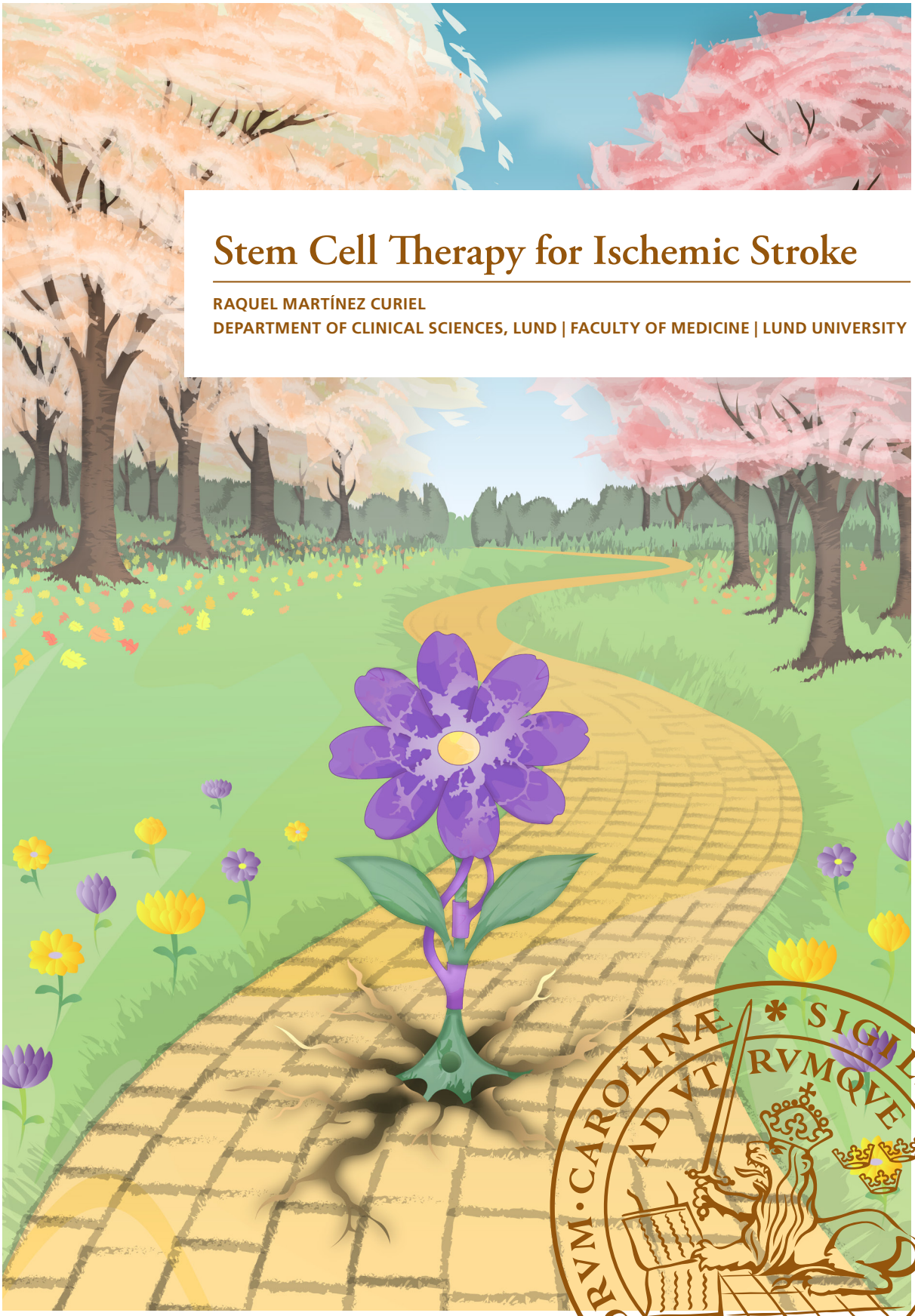

Stem Cell Therapy for Ischemic Stroke

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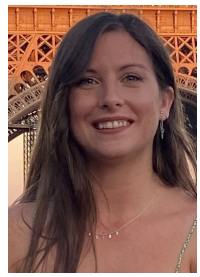

RAQUEL MARTINEZ CURIEL obtained her bachelor's degree in Biology from the Universidad Complutense of Madrid (Spain), where she became curious about biomedicine and a desire to contribute to research that could ultimately help patients in the future. She then pursued a master's degree in Advanced Therapies and Biotechnological Innovation at the Universidad Francisco de Vitoria in Madrid (Spain) and completed a year-long Erasmus internship with the Stem Cells and Restorative Neurology group at the Lund Stem Cell Center. During this time, she discovered her passion for neuroscience and stem cell therapies, inspiring her to pursue a PhD in this field. In the fall 2021, Raquel began her doctoral studies under the supervision of Professor Zaal Kokaia at Lund University. Her research focuses on developing novel stem cell-based therapeutic strategies for ischemic stroke and demyelinating diseases, aiming to restore neuronal networks.

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Stem Cell Therapy for Ischemic Stroke

Stem Cell Therapy for Ischemic Stroke

Raquel Martínez Curiel

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 7th of November at 13.00 in Segerfalksalen, Department of Clinical Sciences Lund, Sölvegatan 17, Lund

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Abstract:

Neurodegenerative diseases, such as ischemic stroke and multiple sclerosis, are characterized by progressive loss of neuronal populations, glial cells and demyelination, leading to irreversible cognitive and motor impairments. Despite the presence of endogenous reparative mechanisms, they are inefficient to promote substantial functional recovery and decline with age. Stem cell-based therapies have emerged as a promising strategy to replace lost cells and restore the damaged neural circuitry with the aim of functional improvement. Transplantation of human stem cell-derived oligodendrocytes (OLs) show strong potential for remyelination, improving axonal conduction and neuronal survival in demyelinating animal models. Parallel efforts focus on the differentiation of human pluripotent stem cells into cortical neurons to reconstruct damaged cortical networks. Advances in differentiation protocols, graft survival, synaptic integration and functional maturation have shown promising results. Nonetheless, significant challenges remain for clinical translation, since most evidence derive from animal models or *in vitro* settings that fail to recapitulate the human brain microenvironment.

In this thesis, stem-cell based therapies were developed and evaluated in various models to address the loss or damage of OLs and cortical neurons observed in neurodegenerative disorders. First, we wanted to improve current available models and bridge the gap between animal models to human brain environment. To do this organotypic slices of human adult cortical (hACtx) tissue were assessed as valuable 3D *ex vivo* model to evaluate the cell survival, differentiation, integration and electrophysiological properties of human stem cell-based therapies with human neuronal networks. Secondly, OLs were generated rapidly and efficiently from lt-NES cells using two different strategies: transcription factor programming for the sole generation of OLs or differentiation with small molecules, which produce OLs and cortical neurons. Both approaches proved to survive, differentiate into mature myelinating OLs and remyelinate both rodent and human axons following transplantation into either the stroke-lesioned rodent cortex or hACtx organotypic slices. Lastly, cortical neurons derived *in vitro* from human embryonic stem cells survived, differentiated and structurally integrated into the rodent neuronal networks after stroke injury.

Collectively these findings provide strong evidence supporting the therapeutic potential of human stem cell-derived products to promote clinical recovery in neurodegenerative disorders.

Key words:

Oligodendrocytes, cortical neurons, cell therapy, ischemic stroke, myelination, restoration

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Stem Cell Therapy For Ischemic Stroke

Raquel Martínez Curiel

LUND
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Cover image by Edoardo Sozzi, ilustrating stem cell-based therapies for neurodegeneration. In the background autumn symbolizes neurodegeneration, reflecting the decline and loss of neuronal function. Following the yellow brick road we walk towards spring, a season of renewal, representing regeneration and the potential for recovery. At the center of the path, a flower emerges, with its stem shaped as a neuron and its blossom formed by an oligodendrocytes. This flower breaking through the road symbolizes the promise of stem cell therapies to repair and restore damaged neural networks.

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MADE IN SWEDEN

To my family and the chosen one.

"*I'm through accepting limits, because someone says they're so,*
some things I cannot change, but 'til I try, I'll never know"
- Stephen Schwartz

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Lay summary

Neurodegenerative disorders, including multiple sclerosis (MS), stroke, Alzheimer's and Parkinson's disease, gradual or traumatic damage to the brain and spinal cord cells, causes problems with memory, movement, language and cognition. These conditions affect over 50 million people worldwide and this number is expected to double by 2050 as the population becomes older, highlighting the urgent need for new treatments.

Stroke is a leading cause of death and disability, with 1 in 4 adults over the age of 25 likely to experience it in their lifetime. Ischemic stroke occurs when the blood flow to a part of the brain is blocked, leading to the loss of neurons and supporting cells such as oligodendrocytes. Currently, there is no effective treatment to improve recovery after the initial hours. MS is a long-term disease where the protective coating around nerve fibers, called myelin, is damaged slowing down the nerve signals. While treatments can slow down its progression, there is no cure, and new cases in Europe are expected to rise to 2.5 per 100.000 people by 2050.

Stem cell-based therapies represent a new promising approach for the treatment of neurodegenerative diseases and currently they are being explored in clinical trials. The goal is to replace the brain cells that are lost due to disease, oligodendrocytes in MS and in the case of stroke, both cortical neurons and oligodendrocytes. However, studying these therapies in humans is extremely difficult due to the inaccessibility to living human brain tissue. Most of what we know today about stem cell-based transplantations comes from animal studies or experimental cell culture conditions, which fail to fully mimic the human brain environment. Even though there have been recent advances in cell culture techniques generating 3D "mini-brain" models from human stem cells, they still can't capture the complexity of the human brain.

The work in this thesis shows the generation of two main brain cell types using different starting stem cells: oligodendrocytes and cortical neurons. When transplanted into an animal model of stroke, these cells survive, become mature cortical neurons and myelinating oligodendrocytes which have the capacity to connect with the host damaged neuronal networks and myelinate injured neurons. Most importantly, to bridge the gap with animal models, in this thesis we use living healthy human brain tissue removed during surgery as human 3D model. This allows us to study how human stem cell-derived brain cells interact with human

neuronal networks providing a clearer picture of their potential as future therapies. In this human-to-human grafting situation, human stem-cell derived oligodendrocytes integrate into the host networks by interacting with human axons and producing new myelin around damaged fibers.

In summary, this thesis represents an important step towards future treatments in which stem cell-derived neural cells could replace or repair the brain in neurodegenerative disorders with the aim of promoting recovery.

Populärvetenskaplig sammanfattning

Neurodegenerativa sjukdomar, såsom multipel skleros (MS), stroke, Alzheimers och Parkinsons sjukdom samt gradvis eller traumatisk skada på hjärnans och ryggmärgens celler, leder till problem med minne, rörelseförmåga, språk och kognition. Dessa sjukdomar påverkar över 50 miljoner människor världen över, och antalet förväntas fördubblas till år 2050 i takt med att befolkningen blir äldre, vilket understryker det akuta behovet av nya behandlingar.

Stroke är en av de vanligaste orsakerna till död och funktionsnedsättning – en av fyra vuxna över 25 år beräknas få stroke under sin livstid. Vid ischemisk stroke blockeras blodflödet till en del av hjärnan, vilket leder till förlust av nervceller och stödjeceller som oligodendrocyter. I dag finns det ingen effektiv behandling som kan förbättra återhämtningen efter de första kritiska timmarna. MS är en kronisk sjukdom där det skyddande lagret runt nervtrådarna, myelinet, skadas, vilket bromsar nervsignalerna. Även om behandlingar kan bromsa sjukdomsförloppet finns inget botemedel, och antalet nya fall i Europa väntas stiga till 2.5 per 100 000 invånare fram till år 2050.

Stamcellsbaserade terapier är en ny lovande metod för behandling av neurodegenerativa sjukdomar och testas nu i kliniska studier. Målet är att ersätta de hjärnceller som gått förlorade – oligodendrocyter vid MS och kombinationen oligodendrocyter och kortikala nervceller vid stroke. Att studera dessa terapier i människor är dock mycket svårt på grund av svårigheten att få tillgång till human hjärnvävnad. Det mesta vi vet idag kommer från djurstudier eller experimentell cellodling, som inte fullt ut kan efterlikna den mänskliga hjärnans miljö. Trots framsteg inom cellodling, där forskare skapar 3D-”minihjärnor” från mänskliga stamceller, kan dessa modeller fortfarande inte återskapa hjärnans komplexitet.

I detta avhandlingsarbete har två viktiga hjärnceller framställts från olika typer av stamceller som startmaterial: oligodendrocyter och kortikala nervceller. När de transplanterades i en djurmodell för stroke överlevde cellerna, utvecklades till mogna kortikala nervceller och myelinbildande oligodendrocyter, och kunde koppla upp sig till skadade nervnätverk och återbilda myelin. För att överbrygga klyftan mellan djurförsök och mänskliga förhållanden användes levande frisk hjärnvävnad som tagits bort vid kirurgi som en mänsklig 3D-modell. Detta gör det möjligt att studera hur stamcellsderiverade hjärnceller samverkar med mänskliga nervnätverk och ger en tydligare bild av deras potential som framtida behandling. I detta

människa-till-människa-scenario integreras oligodendrocyterna i värdvävnaden genom att binda till mänskliga axoner och bilda nytt myelin runt skadade nervtrådar.

Sammanfattningsvis utgör detta avhandlingsarbete ett viktigt steg mot framtida behandlingar där stamcellsderiverade nervceller kan ersätta eller reparera hjärnan vid neurodegenerativa sjukdomar för att främja återhämtning.

Resumen en español

Las enfermedades neurodegenerativas, como la esclerosis múltiple (EM), ictus, Alzheimer y Parkinson, dañan de forma gradual o repentina las células del cerebro y la médula espinal, causando problemas de memoria, movimiento, lenguaje y otras funciones cognitivas. Estas enfermedades afectan a más de 50 millones de personas en todo el mundo, y se espera que esta cifra se duplique para 2050 con el envejecimiento de la población, lo que resalta la necesidad urgente de nuevos tratamientos.

El ictus es una de las principales causas de muerte y discapacidad: 1 de cada 4 adultos mayores de 25 años sufrirá uno a lo largo de su vida. En el ictus isquémico, un coagulo bloquea el flujo sanguíneo hacia una parte del cerebro, provocando la pérdida de neuronas y células de soporte como los oligodendrocitos (células encargadas de producir mielina, la capa protectora de las fibras nerviosas). Actualmente, no existe un tratamiento eficaz que favorezca la recuperación pasadas las primeras horas tras el ictus. Por otro lado, la EM es una enfermedad crónica en la que se daña la mielina ralentizando la transmisión de señales nerviosas. Aunque existen tratamientos que frenan su progresión, no hay cura, y se estima que para 2050 habrá 2,5 nuevos casos por cada 100.000 habitantes en Europa.

Las terapias basadas en células madre ofrecen una alternativa prometedora para tratar las enfermedades neurodegenerativas y actualmente, se están evaluando en ensayos clínicos. Su objetivo es reemplazar las células cerebrales perdidas: oligodendrocitos en la EM y, en el caso del ictus, tanto neuronas corticales como oligodendrocitos. Sin embargo, estudiar estas terapias en humanos es muy difícil, dado que no se puede acceder fácilmente a tejido cerebral vivo. Gran parte del conocimiento actual proviene de estudios en animales o de cultivos celulares experimentales, que no reproducen fielmente el entorno del cerebro humano. Incluso los más recientes avances, que incluyen la creación de “mini-cerebros” en 3D a partir de células madre humanas, aún no reflejan toda la complejidad del cerebro humano.

En esta tesis, se muestra cómo se pueden generar dos tipos principales de células cerebrales - oligodendrocitos y neuronas corticales - a partir de distintas células madre humanas y evaluamos su potencial terapéutico. Tras el trasplante en un modelo animal de ictus, estas células sobreviven, maduran y se integran en las redes neuronales dañadas, formando neuronas funcionales y oligodendrocitos capaces de

mielinizar las fibras nerviosas lesionadas. Lo más innovador para acortar la distancia entre los estudios en animales y la investigación del cerebro humano, utilizamos tejido cerebral humano sano obtenido durante cirugías como un modelo 3D. Esto permite observar directamente cómo las células cerebrales derivadas de células madre humanas interactúan con redes neuronales humanas, y brinda una visión más clara de su potencial terapéutico. En este escenario de injerto "humano a humano", los oligodendrocitos derivados de células madre humanas se integran en los circuitos cerebrales, interactúan con axones humanos, y generan nueva mielina alrededor de las fibras dañadas.

En conjunto, esta tesis representa un paso importante hacia futuros tratamientos en los que las células cerebrales derivadas de células madre humanas podrían contribuir a reparar el cerebro, y promover la recuperación en enfermedades neurodegenerativas.

Original articles

Paper I

Organotypic cultures of adult human cortex as an *ex vivo* model for human stem cell transplantation and validation
Palma-Tortosa, S., **Martínez-Curiel, R**., Aretio-Medina, C., Avaliani, N., Kokaia, Z.
J. Vis. Exp.; (190), e64234. 2022. DOI:10.3791/64234-v

Paper II

Oligodendrocytes in human induced pluripotent stem cell-derived cortical grafts remyelinate adult rat and human cortical neurons
Martínez-Curiel, R., Jansson, L., Tsupykov, O., Avaliani, N., Aretio-Medina, C., Hidalgo, I., Monni, E., Bengzon, J., Skibo, G., Lindvall, O., Kokaia, Z., Palma-Tortosa, S.
Stem Cell Reports (2023), Vol. 18:1643-1656. DOI: 10.1016/j.stemcr.2023.04.010

Paper III

Human cortical neurons rapidly generated by embryonic stem cell programming integrate into the stroke-injured rat cortex
Martínez-Curiel, R., Hajy, M., Tsupykov, O., Jansson, L., Avaliani, N., Tampé, J., Monni, E., Skibo, G., Lindvall, O., Palma-Tortosa, S., Kokaia, Z.
Stem Cells (2025), Accepted Manuscript. DOI: 10.1093/stmcls/sxaf049

Paper IV

Rapid and efficient generation of human oligodendrocytes myelinating adult human cortical neurons
Martínez-Curiel, R., Rincón-Cerrada, P., Al Khani., A.M., Tsupykov, O., Martín-Hernandez, D., Monni, E., Savchenko, K., R.Rodriguez, L., Kidnapillai, S., Bruzelius, A., Hidalgo, I., Canals, I., Bengzon, J., Skibo, G.,Ottosson, D.R., Falk, A., Ahlenius, A., Lindvall, O., Kokaia, Z., Palma-Tortosa, S.
Manuscript

Publications outside of the thesis

Aging and ischemic stroke: mechanisms, trends and strategies for improved outcomes
Kokaia, Z. and **Martínez-Curiel, R**.
Aging and longevity, 6(2), 152-158. 2025. DOI: 10.47855/jal9020-2025-2-7

Abstract

Neurodegenerative diseases, such as ischemic stroke and multiple sclerosis, are characterized by progressive loss of neuronal populations, glial cells and demyelination, leading to irreversible cognitive and motor impairments. Despite the presence of endogenous reparative mechanisms, they are inefficient to promote substantial functional recovery and decline with age. Stem cell-based therapies have emerged as a promising strategy to replace lost cells and restore the damaged neural circuitry with the aim of functional improvement. Transplantation of human stem cell-derived oligodendrocytes (OLs) show strong potential for remyelination, improving axonal conduction and neuronal survival in demyelinating animal models. Parallel efforts focus on the differentiation of human pluripotent stem cells into cortical neurons to reconstruct damaged cortical networks. Advances in differentiation protocols, graft survival, synaptic integration and functional maturation have shown promising results. Nonetheless, significant challenges remain for clinical translation, since most evidence derive from animal models or *in vitro* settings that fail to recapitulate the human brain microenvironment.

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Collectively these findings provide strong evidence supporting the therapeutic potential of human stem cell-derived products to promote clinical recovery in neurodegenerative disorders.

Abbreviations

aCSF	Artificial cerebrospinal fluid
APs	Action Potentials
BBB	Blood Brain Barrier
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenetic Protein 4
BSA	Bovine serum albumin
CC	Corpus callosum
CNS	Central nervous system
Cy	Cyclopamine
D	Day
DCX	Doublecortin
dMCAO	Distal middle cerebral artery occlusion
DMTs	Disease modifying therapies
dox	Doxycycline
EBs	Embryoid bodies
EGF	Epidermal growth factor
FA	Formaldehyde
GFP	Green fluorescent protein
hACtx	Human adult cortical
hES	Human embryonic stem
hES-iNs	Human embryonic induced neurons
hiOLs	Human induced oligodendrocytes
hiPS	Human induced pluripotent
hPS	Human pluripotent

HSCs	Hematopoietic stem cells
Hygro	Hygromycin
iNs	Induced neurons
KPBS	Potassium Phosphate Buffered Solution
MBP	Myelin basic protein
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
MSCs	Mesenchymal stem cells
NDS	Normal donkey serum
NeuN	Mature neuronal nuclei
NPCs	Neural progenitor cells
NSCs	Neural stem cells
OLs	Oligodendrocytes
OPCs	Oligodendrocyte precursor cells
PDGFR	Platelet derived growth factor receptor
PFA	Parafolmaldehyde
PPMS	Primary progressive multiple sclerosis
Puro	Puromycin
RA	Retinoid Acid
RMP	Resting membrane potential
RNA	Ribonucleic Acid
RRMS	Relapsing-remitting multiple sclerosis
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SPMS	Secondary progressive multiple sclerosis
TKPBS	Triton potassium phosphate buffered solution
Wnt3a	Wingless-type MMTV integration site family member 3[a]

Introduction

Neurodegenerative disorders

Neurodegenerative disorders are characterized by the deterioration and death of neural cells in the brain or spinal cord, leading to functional and cognitive impairments [1]. They can be caused by ischemic or traumatic injuries, as well as genetic predisposition or accumulation of certain proteins. Such disorders can be categorized as acute or chronic depending on their rate of progression and cause. While chronic diseases develop slowly and often have a genetic or age component, acute diseases occur suddenly and progress quickly due to injury, infection, or ischemia. Ischemic stroke serves as an example of an acute neurodegenerative disorder, while multiple sclerosis (MS) represents a chronic neurodegenerative disorder [2-5].

Ischemic stroke

Stroke in numbers

Stroke is defined by the World Health Organization as the "*rapid development of clinical symptoms indicative of a local or widespread impairment of brain function, with symptoms persisting for 24 hours or more, leading to death with no apparent cause other than vascular disease*" [6]. In 2021, stroke was the second leading cause of death and third leading cause of adult disability-adjusted life-years and mortality worldwide [7]. Yearly, this condition affects over 13.7 million people and one in four people above the age of 25 will have a stroke in their lifetime [8]. It is estimated that by 2050, deaths and costs due to stroke will climb by more than 50% due to population aging [9].

Depending on the nature, stroke can be classified as haemorrhagic or ischemic. Haemorrhagic stroke is caused by the rupture of a blood vessel. Ischemic stroke, on the other hand, is the most frequent type, accounting for over 60%, and is caused by the obstruction of a cerebral artery due to a thromboembolism (Figure 1) [10, 11]. The lack of nutrients and oxygen to a specific area of the brain induces cell death, leading to sensory, motor and cognitive impairments. More than half of ischemic stroke patients suffer significant residual deficits [12]. It has been shown that 60% of acute ischemic strokes show impairments in the somatosensory cortex and such

impairment leads to major disability compared to striatal damage after stroke in humans [13, 14].

Pathology of acute ischemic stroke

Ischemic stroke is a dynamic process that progresses through different phases with characteristic processes: hyperacute (first 24 hours), acute (24 hours to 1 week), subacute (1-4 weeks) and chronic phase (more than 4 weeks). The hyperacute phase is characterized by the breakdown of the brain-blood barrier (BBB) and massive cell death. During the next week, the BBB continues to rupture with the infiltration of immune cells to the brain, activation of microglia and astrocytes, which leads to inflammation [15]. In the subacute phase, there is a delayed immune response and the start of recovery processes like angiogenesis [16]. Afterwards, the chronic phase is characterized by the reparative mechanisms such as neural plasticity and scar formation [17].

In an ischemic lesion, tissue affected by the loss of blood flow can be divided into two regions: the stroke core and peri-infarct. The ischemic core is the region closest to the affected blood vessel where neurons, astrocytes, oligodendrocytes (OLs) and other cell types die and the damage is irreversible [18]. The peri-infarct area, which surrounds the core, has reduced blood flow that leads to functionally impaired but viable tissue, as ion channels are maintained, which could be potentially salvageable [19]. It is important to highlight that ischemic lesions often lead to secondary degeneration in remote brain regions as connectivity is affected and may worsen long-term recovery [20, 21].

In addition to neuronal loss, ischemia also leads to axonal demyelination and the death of OLs, the myelinating cells of the central nervous system (CNS), as they are highly vulnerable in the acute phase. The brain attempts to compensate through two mechanisms: recruitment of surviving resident OLs for the production of new myelin [22] or an increase in endogenous oligodendrogenesis [23]. However, these reparative responses are limited, as most newly generated OLs fail to differentiate and remyelinate [24, 25]. This regenerative capacity is further diminished in elderly individuals [25].

Treatments

The primary aid in clinical practice for acute ischemic stroke is the mechanical removal of the thrombus, which is more invasive, or dissolution of the clot by administration of tissue plasminogen activator (tPA), which aims to restore blood flow in the affected brain areas. The treatment window for these interventions is very short after onset, within 4.5 hours for tPA and 24 hours for thrombectomy, resulting in only 10% of stroke patients being eligible for this treatment (Figure 1) [26, 27]. Post-stroke recovery is a complex process, as some degree of spontaneous recovery occurs, from modest improvement to almost complete restoration. It involves plastic changes in surviving neurons, release of growth and anti-

inflammatory factors, as well as generation of new neural cells from endogenous stem cells [28, 29]. Following the acute phase, physical rehabilitation remains the primary treatment shown to improve the patient's condition [30]. Although these mechanisms are promising, their efficacy diminishes with age, and currently, there are no effective therapeutic strategies to enhance functional recovery during the chronic phase after injury.

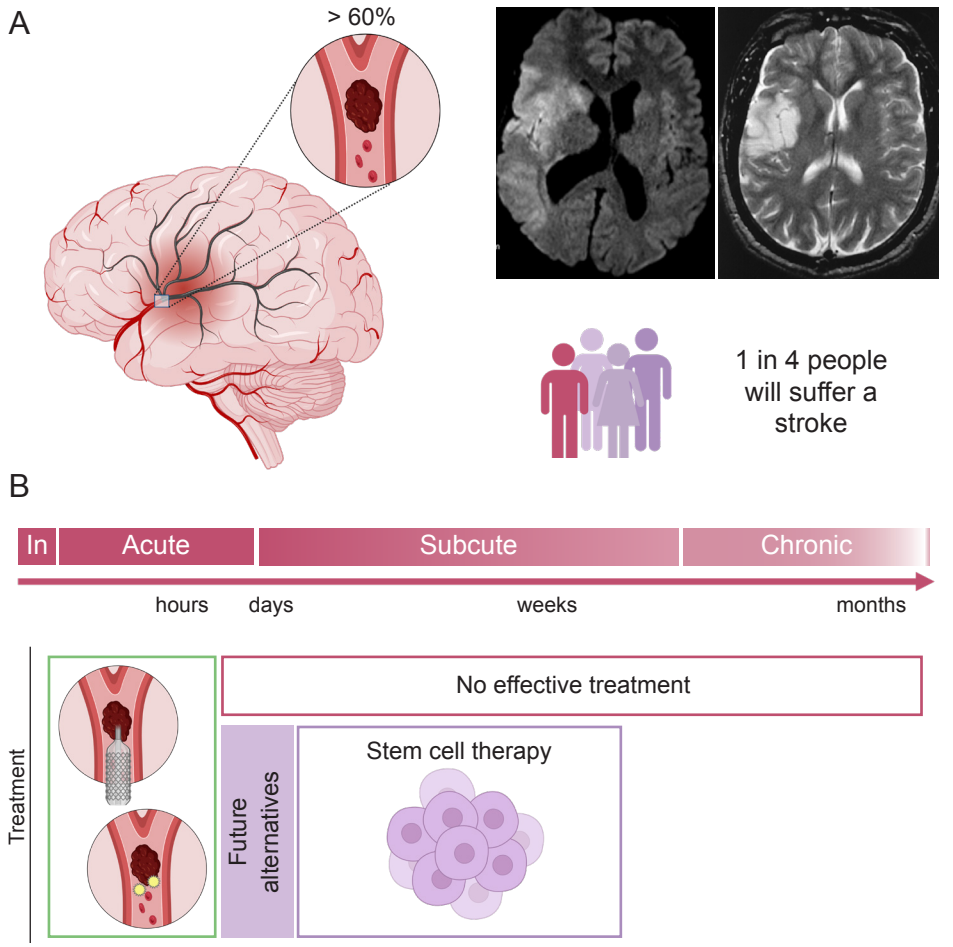

Figure 1. Ischemic stroke: statistics, phases and treatment
A. Ischemic stroke occurs when blood flow to a part of the brain is blocked by a bloodclot and accounts for over 60% of all strokes. Right: representative MRI images of an ischemic stroke which shows area of ischemia as bright signal. Predictions show that 1 in 4 people over the age of 25 will suffer a stroke in their lifetime. **B**. Current and emerging treatments for ischemic stroke. To date, the treatment window for stroke is restricted to the first 24 hours (acute phase), during which blood clots can be removed enzymaticaly (tPA) or mechanically. Beyond this period, no effective treatments exist to enhance functional recovery. Stem cell-based therapies offer a promising strategy to extend the therapeutic window into the subacute phase for patients who fall out of the 24 hours period. In, insult. MRI images from [31, 32]. Figure made with BioRender.

Demyelinating disorders and multiple sclerosis

Demyelinating diseases are characterized by damage to myelin sheaths wrapping the axons of the central and peripheral nervous system, or to the cells that produce myelin, OLs. This damage results in axonal degeneration, which leads to neurological deficits [33]. Demyelination is the defining feature of these diseases, while the underlying mechanisms and lesion patterns vary, they share some similarities. It is important to note that myelin breakdown is not exclusively the result of cellular dysfunction, as it also occurs naturally as part of normal myelin turnover, where old myelin is renewed by new myelin [34]. Nonetheless, the progression of demyelination is associated with impaired OL maturation and migration, deficits in myelin sheath renewal, dysregulated myelin turnover and inflammation [35, 36].

Focusing on the CNS, they can be classified as either primary or secondary based on the underlying cause of the myelin damage. The primary demyelinating diseases, such as MS, are often immune-mediated. In MS, the myelin is targeted and damaged by autoimmune or genetic mechanisms. While in secondary demyelinating diseases, myelin loss is associated with neuronal or axonal degeneration and can be associated with ischemic events like stroke [37, 38].

Multiple Sclerosis in numbers and types

MS is a chronic, inflammatory, demyelinating and neurodegenerative disease of the CNS and one of the most common causes of non-traumatic disability in younger adults (18-40 years) [39]. Over the last decades, the incidence of MS has risen and globally, around 2.8 million people are affected [40].

The clinical manifestations and progression of MS are heterogeneous. Based on the progression of the disease, MS can be classified into relapsing-remitting (RRMS), secondary progressive (SPMS) and primary progressive (PPMS). The majority of the patients, around 85%, present the RRMS phenotype, which is characterized by periods of acute neurological symptoms (relapses) followed by partial or full clinical recovery (remissions) [41]. Over time, as the disease advances, neurological deficits become permanent without remission periods and there is a steady progression of the disease. This is known as SPMS, which in 15-20% of patients starts as RRMS and transitions to SPMS. At diagnosis, a minority of patients (10-15%), have a slow progression of disability from the first manifestation of the disease and are classified as PPMS [42].

Pathology of Multiple Sclerosis

MS is initiated by autoreactive T lymphocytes that produce antigens against the CNS myelin proteins. The activation of T cells is triggered by genetic or environmental factors, such as infection. Activated immune cells cross the BBB and migrate into the brain parenchyma. Locally, T cells are reactivated, local microglia

cells become reactive and initiate an inflammatory cascade. This response causes OL death, demyelination, and astrogliosis, which results in axonal degeneration leading to neuronal loss that extends beyond focal lesions.

MS lesions or plaques are the defining feature of the disease and correspond to regions of inflammation and demyelination. Inflammation damages OLs, leading to demyelination and consequently axonal degeneration. In order to remyelinate damaged axons, oligodendrocyte precursor cells (OPCs) are recruited to the lesion site, where they differentiate into mature myelinating cells or resident surviving OLs contribute to form new myelin [22]. Remyelination can occur in MS [43, 44], giving rise to the shadow plaques and is suggested to be by resident mature OLs, as no new OLs are observed [45]. However, the extent of remyelination is heterogeneous in MS patients. In later stages of the disease, inflammation impairs OPC migration and differentiation, limiting remyelination, while at earlier stages and in younger patients, remyelination and greater OPCs recruitment are observed [46-48]. Regarding resident surviving OLs, they have limited capacity for remyelination [48, 49], as although they extend new processes, they rarely generate new myelin internodes [50] or if they remyelinate, the myelin is mistargeted to the cell body [48].

Treatments

Currently, MS remains incurable, however, over the last decades, disease-modifying therapies have been developed for RRMS. The main effect of these therapies is primarily suppressing the immune response by reducing the migration of immune cells to the CNS or depleting specific types of immune cells. The main first-line treatments for MS are approved disease-modifying therapies such as interferon β and glatiramer acetate. Unfortunately, treatments used for RRMS cannot prevent aggravation in those with PPMS or SPMS [51]. Two recent therapies, siponimod and ocrelizumab, have shown benefits for progressive forms of MS as they modulate inflammatory cell migration and their depletion, respectively [52, 53]. However, all currently approved disease-modifying therapies are primarily anti-inflammatory/immunomodulatory, with only limited inhibitory effect on neurodegeneration or disease progression, and none have been shown to promote repair or remyelination. This highlights the urgent need for therapies that support remyelination and neural repair to improve disability. There are novel clinical trials investigating strategies fostering neuroprotection (NCT03161028) or remyelination (clemastine, NCT02040298) [54].

Stem cell therapies for neurodegenerative disorders

Neurodegenerative diseases are characterized by physical and cognitive impairments due to the loss or degeneration of neurons. Current treatments for these

diseases focus on alleviating symptoms, which can be transient and partially effective without substantial functional improvement. As the CNS has limited capacity for self-repair, alternative strategies, such as cell therapies, are highly warranted. Over the last decades, stem cell-based approaches have gained attention as a promise for brain repair. Cell therapy aims to replace, repair, or modulate the brain microenvironment to restore lost function.

In the late 80s and the beginning of the 90s, there was a breakthrough in the cell therapy field. Groundbreaking clinical studies on the transplantation of fetal midbrain tissue in Parkinson's disease patients provided proof-of-principle that neuronal replacement is feasible in the adult human brain and can induce long-lasting improvements [55-57]. Grafted fetal midbrain dopaminergic precursors were able to survive for many years in the human adult brain, mature into the correct subtype of dopaminergic neurons, integrate into the host circuitry and improve motor function [57, 58]. Although these studies were revolutionary, limitations such as tissue availability, quality and ethical concerns surrounding the use of primary fetal tissue have shifted the field towards the use of stem cell-based therapies. The advantage of stem cells is that they can be produced using standardized protocols, expanded *in vitro* and undergo rigorous quality controls.

Stem cell sources for cell therapy

Stem cells used for transplantation can be obtained from different sources (Figure 2) that differ in terms of availability and ethical issues.

Pluripotent stem cells

In 1998, human embryonic stem (hES) cells were isolated for the first time, which have the stemness and can differentiate into a variety of specific neural subtypes relevant for cell therapy [59]. One of the ground-breaking discoveries regarding this field has been the generation of human induced pluripotent stem (hiPS) cells from human adult somatic cells, more specifically human adult fibroblasts, by overexpression of a minimal combination of four transcription factors: Oct4, Sox2, Klf4 and cMyc; bringing them back to the developmental stage of pluripotency state [60]. Further studies showed that hiPS cells were equivalent to hES cells, as they could form teratomas and give rise to cells of the three germ layers [61]. More importantly, iPS cells can be derived from patients' fibroblasts or blood cells avoiding the need for long immunosuppressive treatments, risk of graft rejection and the ethical concerns related to the use of hES cells [62, 63]. As mentioned previously, hiPS cells offer the possibility to produce patient-specific cells, which expands the potential for disease modelling as these cells recapitulate the disease in the dish [64]. hiPS cells are able to differentiate into any neural cell, similar to hES cells, such as dopaminergic neurons [65, 66], cortical neurons [67], OLs [68, 69], astrocytes [70] and interneurons [71].

The use of both cell sources for cell therapy presents advantages and limitations. The use of hES cells raises ethical concerns due to their origin, whereas hiPS cells bypass such issues. By contrast, the generation of patient-specific hiPS cells is time-consuming, requires rigorous quality controls, and involves high costs. Nonetheless, both cell sources are freely available on demand and can be easily expanded in culture. However, hES and hiPS cells carry a risk of tumorigenicity, which remains a critical concern for their therapeutic application. At the moment, there are active clinical trials for Parkinson's disease running in three different locations using different stem cell sources. Two of them are using hES cells-derived neural progenitors [72, 73], while the other one is using hiPS cells-derived neural progenitors, which are human leukocyte antigen (HLA) matched to patients [74].

Human long-term neuroepithelial-like stem (lt-NES) cells

As an alternative to hPS cells, long-term self-renewing neuroepithelial-like stem (lt-NES) cells have been generated from both hES and hiPS cells. These cells constitute a stable source with the capacity for self-renewal, long-term expandability and neuronal and glial differentiation [75, 76]. When compared to human fetal neural stem cells, which express radial glia markers, lt-NES cells present neuroepithelial and neural rosette markers representing an early stage of the CNS development [76].

Lt-NES cells have the ability to differentiate into functional neurons, astrocytes and OLs [76, 77]. When exposed to region-specific morphogens, such as those of ventral midbrain, lt-NES cells can differentiate into defined neuronal phenotypes [76]. In order to produce a specific neuronal population, Tornero and collaborators developed a protocol to generate neuronal progenitors with a cortical identity [78]. Cortically primed lt-NES cells expressed markers of upper and deep cortical neurons, such as SATB2, TBR1 and CTIP2. Most importantly, cortically fated lt-NES cells differentiated to functional neurons which form synaptic connections after 8 weeks of differentiation establishing a neuronal network [78, 79].

In contrast to hES and hiPS cells, an important feature of lt-NES cells is the absence of tumorigenicity. When non-fated lt-NES cells are transplanted into the damaged rodent brain, no teratomas were observed, as they are multipotent, giving rise to only neural cells [80]. These characteristics make lt-NES cells a great candidate as a stem cell source for transplantation.

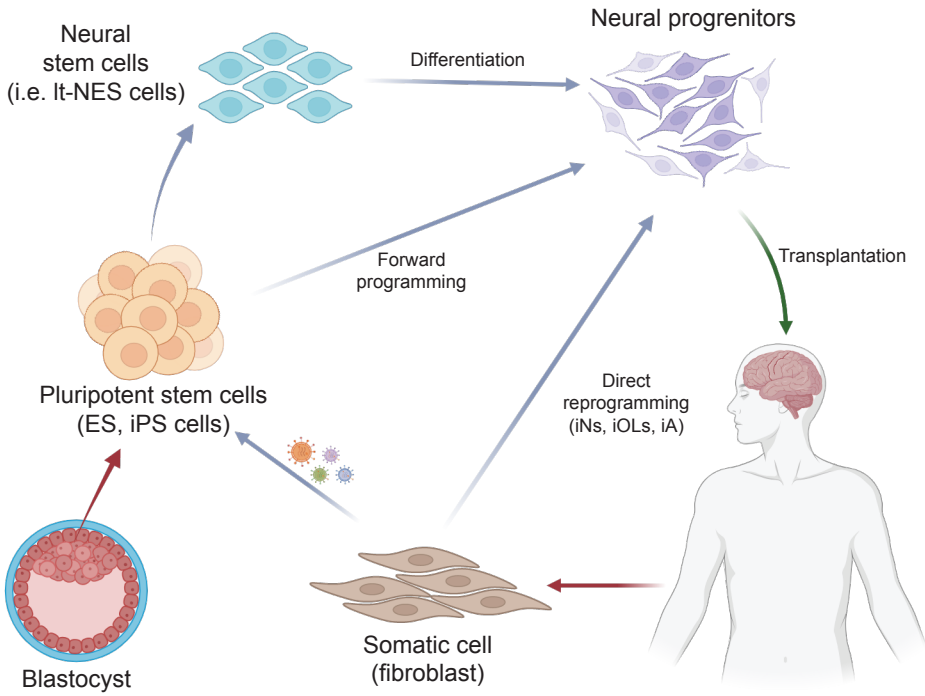

Figure 2. Stem cell sources for cell therapy
Neural progenitors can be obtain from different cell sources: differentiated from pluripotent stem cells (hES and hiPS cells) passing through a neural stem cell state, forwarded programmed from pluripotent stem cells by overexpression of different transgenes or directly reprogrammed from somatic cells to induced neurons (iNs), oligodendrocytes (iOLs) or astrocytes (iAs). Figure made with BioRender.

Direct reprogramming of adult somatic cells

Another important landmark in the field is the direct reprogramming of adult somatic cells from one germ-layer to another. This strategy bypasses the pluripotent state, thereby decreasing teratoma risk and accelerating the differentiation process. In 2010, Vierbuchen and colleagues successfully reprogrammed mouse fibroblasts, which are mesodermal lineage, into functional induced neurons (iNs) of ectodermal origin [81]. This was replicated in human fibroblasts, which also converted into iNs [82]. Moreover, other neural cell types, including functional astrocytes and OLs, can be generated through direct reprogramming of fibroblasts from young, adult and old-aged humans [83, 84]. However, there are many concerns regarding this method, such as low conversion rate and limited cell yield.

Stem cell-derived cortical neurons for neurodegenerative disorders

Generation of cortical neurons

Two main strategies have been used for the generation of cortical neurons from hES and hiPS cells. First, through small molecules that recapitulate cortical development with an initial neural induction via dual SMAD and WNT inhibition [85-87]. These protocols have shown that the differentiation of hES cells into cortical neurons follows the temporal order observed in the cortex development where neurons expressing deep-layer markers, such as TBR1, appear first followed by upper cortical layer neurons, expressing markers such as BRN2 and SABT2 [85, 87]. However, the proportion of layer-specific cortical neurons varies between protocols, which suggests that external molecular cues are required for the generation of proper proportions of different layer subtypes [88]. It has also been shown that after 8 weeks of cortical priming, hiPS cell-derived lt-NES cells give rise to neurons expressing markers of both upper and deep cortical layers [78, 79]. Secondly, by the forced overexpression of transcription factors involved in neuronal specification. hES cell-derived neurons (hES-iNs) programmed by the overexpression of the transcription factor neurogenin-2 (NGN2) generate functional excitatory neurons which exhibit neuronal morphology, gene expression and are able to establish synaptic connections [89-92]. However, hES-iNs seemed to be primed to become cortical neurons as their genetic profile shows the co-expression of all cortical markers resembling fetal human cortical neurons [92].

Transplantation of hPS cell-derived neural progenitor cells (NPCs) or neuronal cortical progenitors in neurodegenerative diseases

An important aspect when evaluating the therapeutic potential of stem cell-derived cortical progenitors is their capacity to mature, integrate into the host neuronal circuitries and form proper projection patterns following transplantation in models of neurodegenerative diseases, such as ischemic stroke and MS.

hPS cell-derived cortical progenitors, generated via neural induction protocols, are able to integrate and connect with the correct brain regions after transplantation in the rodent newborn and adult cortex [87, 93, 94]. Most importantly, when transplanted into the adult rodent injured cortex, hPS cell-derived cortical neurons express cortical layer markers, reestablish damaged axonal pathways and integrate into the host circuitry [95]. hES cell-derived cortical progenitors and cortically primed hiPS cell-derived lt-NES cells after transplantation in the stroke-damaged rat cortex, give rise to mature functional neurons that extend axonal projections to both hemispheres that improve neurological deficits [78, 96]. These grafted neurons, derived from hiPS cell-derived lt-NES cells, receive afferent connections from a normal pattern of different brain areas, including synaptic inputs from the thalamus, which are activated by sensory stimuli, demonstrating that the host brain regulates the activity of grafted neurons [97]. Furthermore, grafted neurons send axonal

projections to both hemispheres, which establish excitatory synapses with host cortical neurons providing the integration of efferent projections from graft to host. Most importantly, grafted lt-NES cells-derived cortical neurons reversed the sensorimotor deficits in stroke-injured rodent brains [98].

Recently, the emergence of new technologies and advances in cell culture has led to the development of 3D brain organoids from hiPS cells and exploring their therapeutic potential to repair the damaged brain. Organoid protocols can be unguided, which relies on hPS cells' self-patterning properties, or guided, where factors are incorporated to direct the formation of specific brain regions, like the cortex [99]. Transplantation of cortical organoids generated from hES cells and hiPS cells, into the adult visual cortex and the peri-infarct area of a stroke model, has shown neuronal differentiation, axonal projection, integration into the host neuronal circuitry and responded to stimuli 2-6 months after transplantation [100, 101]. Most importantly, sensorimotor impairment was reversed after transplantation [101].

Aside from cell replacement, transplantation of hPS cell-derived neural progenitor cells (NPCs) can modulate the surrounding environment through different mechanisms after brain injury, a phenomenon known as the bystander effect. Previous studies have shown that after transplantation of hiPS cell-derived neural cells, such as astroglia and NPCs, in the injured brain promoted oligodendrogenesis and remyelination [102-105]. Llorente and colleagues showed increased oligodendrogenesis and, therefore, promote remyelination, which enhanced motor recovery after transplantation of hiPS cell-derived astroglia in a model of white matter stroke [103]. In a demyelination and MS model (experimental autoimmune encephalomyelitis, EAE), transplantation of hES cell-derived NPCs reduced demyelination, promoted remyelination and limited neuroinflammation. None of the grafted cells differentiated into OLs, which suggests that NPC transplantation activated endogenous OPCs [105, 106]. Similarly, transplantation of mouse iPS cell-derived NPCs in an EAE model promoted the survival and differentiation of endogenous OPCs and mature OLs [104].

Stem cell-derived oligodendrocytes for demyelinating diseases

Generation of OLs

OLs can be derived from both hES and hiPS cells through different techniques that are based on OL development. By the addition of small molecules for OL specification (i.e, dual SMAD inhibition combined with retinoids, sonic hedgehog agonist, platelet-derived growth factor receptor alpha (PDGFRa) and basic fibroblast growth factor (bFGF)) for OL specification, previously published protocols [68, 107-112] have shown OPC differentiation times of 50-100 days and up to 140 days for mature myelinating OLs with a low yield of $O4^+$ cells.

Nonetheless, these mature OLs were able to myelinate neurons *in vitro* [68, 107, 110, 111].

Due to the time-consuming protocols based on small molecules, the use of programming strategies via overexpression of transcription factors involved in OL specification has arisen. Mainly, SOX10, OLIG2 and NKX6.2 are used depending on the starting cell source [113, 114]. In 2017, Ehrlich and collaborators showed that after 28 days of transduction of hiPS cells- NPCs with the previously mentioned transcription factors, 70% of the cells expressed O4 and by day 35, around 30% of the cells expressed the myelin basic protein (MBP). These mature OLs are able to ensheathe neuronal processes from iPS cell-derived neurons *in vitro* [113]. In line with this, Garcia-León and colleagues showed that the sole overexpression of SOX10 in hiPS cell-derived NPCs gave rise to 50% of O4 positive cells in around 20 days and the generated OLs can remyelinate neurons from organotypic slices of myelin-deficient rodents. It must be highlighted that hiPS cell-derived NPCs expressed OLIG2 [114]. Reprogramming of fibroblasts to OLs is a feasible protocol. The use of the 3 previously mentioned transcription factors gave rise to 20% O4-positive cells after 16 days [83]. Apart from the programming, most of the protocols use a cocktail of maturation factors such as PDGFRa and triiodothyronine (T3) [83, 113].

Transplantation of hPS cell-derived OLs in demyelinating disorders

Demyelination models, such as cuprizone-induced demyelination, and MS-like models, such as EAE, have been used to assess the capacity of hPS cell-derived OLs to migrate, differentiate and myelinate denuded axons.

After transplantation in demyelinated neonatal rodent models, such as congenitally hypomyelinated, OPCs generated from hPS cells with small molecules migrate, differentiate into mature OLs, which remyelinate the denuded axons as compact graft-derived myelin was observed [68, 107, 110]. On top of structural integration, transplantation of hiPS cell-derived OPCs improved survival and reduced mortality in a model of hypomyelination (shiverer mouse) [68]. hPS cell-derived $O4^+$ cells, generated through overexpression of OLIG2, SOX10 and NKX2.6, dispersed widely in the host brain and myelinate host axons in the developing CNS after transplantation in the corpus callosum of shiverer newborn mice [113].

Neonatally transplanted hPS cell-derived OPCs have been shown to survive and differentiate into myelinating mature OLs. However, to consider OLs as a viable option for stem cell therapy, it is essential to determine whether hPS cell-derived OLs can efficiently remyelinate the demyelinated adult brain. In a model of focal demyelination by lysophosphatidylcholine, transplanted human OLs were capable of producing normal compact myelin sheaths within the adult demyelinated spinal cord [113]. Similarly, human glial progenitor cells dispersed through the host forebrain and differentiated into mature myelinating OLs that ensheathed denuded

host axons following transplantation in adult rodent models of demyelination, as cuprizone-induced demyelination [115]. Functionally, transplantation of hES cell-derived OPCs has been shown to promote complete recovery of cognitive function as well as motor recovery in a model of radiation-induced demyelination [111]. Comparable improvements in hindlimb motor function have also been observed in spinal cord injury models after transplantation of hPS cell-derived OPCs [110].

OPCs derived from hiPS cells have also been tested in MS-like models. In 2016, Thiruvalluvan and colleagues showed that after transplantation of hiPS cell-derived OPCs into the cortex of a non-human primate model of EAE, grafted cells migrated towards MS-like lesions and differentiated into mature OLs, myelinating denuded axons [116]. It must be highlighted that OPCs differentiated from hiPS cells had a higher speed and efficiency of myelination than those generated from hES cells [68].

Understanding the environment into which cells are transplanted is crucial, as it may contain inhibitory cues that impair migration or differentiation. Recently, CRISPR-edited hES cell-derived OPCs were engineered to overcome the inhibitory environment characteristic of MS. When transplanted into a rodent model of chronic MS, edited OPCs successfully migrated towards the lesions, differentiated into mature OLs, and contributed to the remyelination of the chronic lesions [117].

Another valuable tool to understand the role of OLs in disease, specifically MS, is the accessibility to patient-derived iPS cells. hiPS cell-OLs have been successfully generated from MS patients with diverse clinical subtypes [107, 118-120]. While some studies show that MS-derived OPCs were able to mature into myelinating OLs and form dense compact myelin resembling normal myelin in demyelinated rodent brains [107, 120], others indicate that their migration capacity is impaired *in vitro* [119] and some adopt an immune-like phenotype, resulting in decreased OL formation and overall, failed remyelination [118].

Few studies have investigated the effect of the transplantation of hPS cell-derived OPCs/OLs in ischemic stroke models. Transplantation of rodent OPCs following transient middle cerebral artery occlusion has been shown to enhance endogenous oligodendrogenesis, promote neurite outgrowth as well as synapse formation, preserve the BBB integrity, reduce inflammation and ameliorate neurological damage after ischemic stroke [121, 122].

Clinical trials with stem cells for stroke/MS

Clinical trials for stroke

Up to date, most of the clinical trials performed in ischemic stroke involve the intravenous transplantation of autologous or allogenic mesenchymal stem cells (MSCs) from different sources like adipose tissue or bone marrow. These types of transplantations do not aim for cell replacement. Instead, the modest improvements

observed in patients have been attributed to the so-called bystander effect, as MSCs release trophic factors which lead to changes in the plasticity and modulate inflammation [123-126]. These trials demonstrated the safety and feasibility of intravenous transplantation of MSCs in stroke patients. Similarly, intracranial transplantation of MSCs have shown safety, feasibility and significant neurological improvements [127, 128].

With the aim of cell replacement, in 2017 Zhang and collaborators transplanted fetal human neural stem cells (hNSCs) intracerebrally, which improved neurological deficits in stroke patients and, through magnetic resonance imaging, showed new neural tissue formation in the lesion cavity [129]. Similarly, the PISCES clinical trial showed that hNSCs can be safely delivered into the brain by stereotactic intracerebral injection and patients who had residual upper limb movement before treatment showed functional improvements [130]. Among the stem cell-based therapies explored, hNSCs hold great promise for the functional recovery of stroke patients.

Even if shown to be safe, the efficacy remains inconsistent between clinical trials. As previously mentioned, some studies report functional improvements, while others didn't reach significant differences between treatment and control groups [131, 132]. In some cases, results were mixed as in some measures there were no differences and in others there was [124].

Clinical trials for MS

Based on preclinical results, clinical trials for the treatment of MS have been focused mostly on hematopoietic stem cells (HSCs) and MSCs due to their immunomodulatory effects. Autologous transplantation of HSCs in most RRMS patients reduced the relapse rates and lesion activity. Nonetheless, there is variability between individuals [133, 134]. Similar outcomes were reached after intravenous or intrathecal transplantation of MSCs in RRMS and PMS patients with a reduction in relapse time, new lesions and lesion volume, as well as neuroprotective effects shown by decreased levels of neurofilament light chain and CXCL13, biomarkers for MS [135-138].

Although these results show promise in the MS treatment, clinical trials targeting remyelination and replacement of the damaged OLs are needed. To date, two clinical trials have used human fetal NSCs (hfNSCs). Leone and collaborators showed that SPMS patients after intracerebral transplantation of hfNSCs maintained stability with no progression of the disability or worsening of lesions/biomarkers [139]. Preliminary results of a second clinical trial using hfNPCs showed their safety and feasibility after transplantation in the spinal cord of PMS patients. It also exhibited less brain atrophy and increased anti-inflammatory and neuroprotective molecules in cerebrospinal fluid [140]. These results show great promise in the therapeutic use of hPS cell-derived NSCs for the treatment of MS.

Bridge between preclinical and clinical studies

An important consideration in the clinical translation of stem cell-based therapies is whether these cells can survive and, most importantly, integrate into the adult human brain circuitries. Clinical trials are based on results from preclinical studies carried out in immunodeficient animal models (xenotransplantation), which misses the interaction between human cells and can be a reason why many clinical trials fail [141, 142]. Although rodent and human brains have similarities, differences in gene expression, synaptic composition and plasticity, as well as connectivity architecture influence how grafted neurons form connections and organize into functional networks, contributing to the translational gap [143-145]. In preclinical studies, usually younger and healthier animal models do not capture the complexity of the disorder, which typically affects older individuals with comorbidities. This creates a mismatch between preclinical findings and clinical settings [146].

With the aim to bridge preclinical studies and clinical trials, the use of human brain organotypic slices obtained from neurosurgical resections is a relevant *ex vivo* 3D model to assess the functional and morphological integration of human stem cell-based therapies into a human neuronal network. This ex *vivo* system preserves the synaptic connectivity, 3D cytoarchitecture, spatial distribution, as well as the microenvironment [147-150]. In most cases, human organotypic cultured slices from the hippocampus are used [151, 152]. However, in diseases such as stroke or MS, where the cerebral cortex is affected, there is a clear need for a human 3D cortical platform to evaluate the therapeutic potential of different treatments. Human adult cortical (hACtx) organotypic slices have previously been used in the literature for disease modelling [149, 153, 154]. In the context of cell replacement, transplantation of hES-iNs into hACtx tissue led to the differentiation into mature cortical neurons that integrated into the host neuronal circuitry [92]. Similarly, hPS cell-derived lt-NES cells become functional, mature, layer-specific cortical neurons that stablish both efferent and afferent connections with the host human neurons after grafting [79]. These findings support the feasibility of using this *ex vivo* model for stem cell-derived cortical neuron transplantation.

Aims

The main aim of the thesis was to test different potential stem cell-based therapies for acute and chronic neurodegenerative disorders and their capacity for integration into rodent and human neuronal circuitries.

Specific objectives

I. Structural and functional characterization of hACtx tissue organotypic cultures as a candidate for *ex vivo* validation of stem cell-based therapies (**Paper I**).

II. Assess the generation of OLs from hiPS cell-derived lt-NES cells following cortical priming and their role in axonal remyelination after intracortical transplantation in a rat model of ischemic stroke and into healthy human adult 3D organotypic cultures (**Paper II**).

III. Characterize neurons generated from ES cells prior to transplantation and study their maturation and integration into the stroke-injured rodent cerebral cortex after grafting (**Paper III**).

IV. Develop a rapid and efficient protocol for the generation of myelinating OLs and evaluate their ability to myelinate human adult neuronal axons (**Paper IV**).

Key experimental methods

This section provides an overview of the main methods used in this thesis. For additional details and description of all the methodology used, please refer to the Materials and Methods of the relevant papers (see *Appendix*).

Cells and Tissue Cultures

hES cells culture and expansion

In **Paper III**, hES cells H1 (WAO1) from WiCell Research Institute (WiCell, WI) were used. hES cells were cultured in feeder-free conditions on Matrigel-coated (Corning) 6-well plates using mTeSR 1 medium (STEMCELL Technologies, UK) that was changed every day. After reaching 80% confluence, cells were dissociated with Accutase (Thermo Fisher Scientific) and re-plated on plates in mTeSR1 medium supplemented with ROCK Inhibitor (10 μM, Y27632, Selleckchem) to enhance cell survival for the first 24 hours.

Differentiation of hES cells into induced neurons (hES-iNs)

To start programming, on day -2 H1 cells were passaged using Accutase and $5x10^5$ cells were replated on Matrigel-coated 6-well plates in mTeSR1 media with ROCK inhibitor. 24 hours after seeding, the medium was replaced with fresh mTeSR1 and hES cells were transduced with TetO-Ngn2-T2A-Puro and FUW-M2rtTA (teT-controlled transactivator #20342, Addgene) lentiviruses (MOI 2). One day after transduction (day 0), mTeSR1 media was replaced with an induction medium (Dulbecco's modified Eagle medium/F12 [DMEM/F12] supplemented with N2 (1:100, Gibco) and B27 (1:50, Gibco), where doxycycline (dox) (2.5 μg/mL, Sigma-Aldrich) was added on day 0 and kept until the end of the experiment to continuously induce NGN2 expression. From days 1 to 7, puromycin (Puro, 1.25 μg/mL, Thermo Fisher Scientific) selection was applied. Cells were collected on day 6 for RT-qPCR and on day 7 for transplantation. Cells subjected to immunostainings or electrophysiological recordings were transferred to coverslips on day 6 and analyzed 24 hours later (Figure 3).

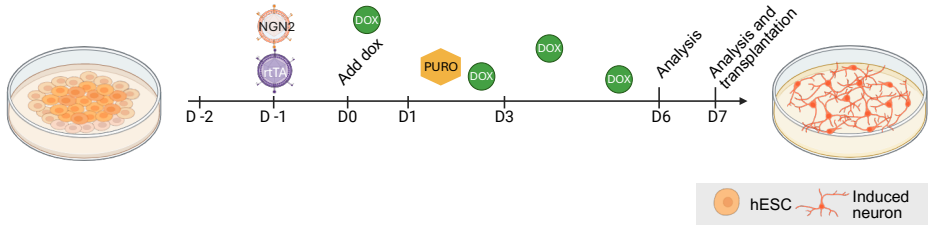

Figure 3. Timeline for the generation of induced neurons from hES cells
Schematic illustrating the timeline of the protocol used for the generation of hES-iNs by the overexpression of *Ngn2* using the doxycycline-inducible Tet-On system. D, day; DOX, doxycycline; PURO, puromycin. Figure made with BioRender.

Derivation of human iPS cell-derived lt-NES cells

Human dermal fibroblasts from healthy adult donors were subjected to Sendai virus transduction with the reprogramming factors Oct4, Sox2, KLF4, and c-MYC (CytoTune iPS 2.0 Sendai Reprogramming kit, Invitrogen). Colonies were picked to establish iPS cell lines using mTeSR medium. For neural induction, iPS cells were split and colonies were gently resuspended in embryoid body (EB) medium (DMEM/F12, 10% Knockout serum replacement, 2-Mercaptoethanol [1:1000], nonessential amino acids [NMEAA] [1:100], Glutamine [1:100]) with ROCK Inhibitor (1:1000), 3 µM Dorsomorphin (Sigma-Aldrich) and 10 µM SB431542 (Sigma-Aldrich). On day 5, EBs were collected and plated on poly-L-ornithine/ laminin-coated plates in an EB medium with 3 µM Dorsomorphin and 10 µM SB431542. On day 6, the media was changed to N2 medium (DMEM-F12 [without Hepes + Glutamine], N2 [1:100], Glucose [1.6 g/L]) supplemented with 1 µM Dorsomorphin and 10 ng/mL bFGF. Neural rosettes were carefully picked, six days later, and grown in suspension in an N2 medium with 20 ng/mL bFGF. On day 14, neural rosette spheroids were collected, and the small clumps obtained were grown in adhesion on poly-L-ornithine/laminin-coated dishes in the presence of 10 ng/mL bFGF, 10 ng/mL epidermal growth factor (EGF) (both from Peprotech) and B27 (1:1000).

The lt-NES cell lines were routinely cultured and expanded on 0.1 mg/mL poly-L-ornithine and 10 mg/mL laminin (Merk and Thermo Fisher Scientific, respectively)-coated plates into the same media supplemented with bFGF, EGF, and B27 and dissociated every second to the third day, when over 100% confluence is reached. Trypsin-EDTA (Sigma) at a concentration of 0.025% is used for dissociation, trypsin inhibitor (0.5 mg/mL; Thermo Fisher Scientific) to stop the enzymatic reaction and cells are resuspended in N2 medium supplemented with growth factors and B27.

Cortical priming of human lt-NES cells to cortical neurons and OLs

Differentiation of lt-NES cells to OLs and neurons with a cortical phenotype was performed as described previously [78, 79].

In brief, growth factors (EGF, bFGF) and B27 were omitted from the culture media and lt-NES cells were cultured at low density in a differentiation-defined medium containing DMEM/F12 with glutamine (Gibco) supplemented with N2 (1:100), NMEAA (0.1mM, Gibco), sodium pyruvate (1mM, Gibco), bovine serum albumin (BSA, 500mg/ml, Gibco) and 2-mercaptoethanol (0.1mM), in the presence of bone morphogenetic protein 4 (BMP4, 10 ng/mL, R&D Systems), wingless-type MMTV integration site family, member 3A (Wnt3A, 10 ng/mL, R&D Systems), and cyclopamine (Cy, 1 µM, Calbiochem) for 7 days. On day 7, neural progenitors were then dissociated using trypsin and plated on poly-L-ornithine/laminin-coated glass coverslips in a 1:1 proportion of N2 medium/Brain Phys Medium (STEMCELL Technologies) supplemented with B27 without retinoic acid (RA) (1:50, Gibco). Media was changed every 4 days to BrainPhys Medium supplemented with B27(-RA) until day 21 of differentiation (Figure 4).

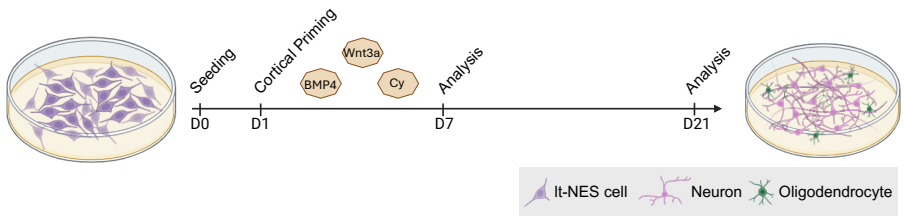

Figure 4. Differentiation of human lt-NES cells via cortical priming protocol
Schematic ilustrating the timeline protocol used for the generation of cortical neurons and OLs from lt-NES cells via cortical priming protocll. D, day; BMP4, bone morphogeneitc protein 4; Wnt3a, wingless-type MMTV integration site family member 3A; Cy, cyclopamine. Figure made with BioRender.

Human lt-NES cells programming into induced OLs (hiOLs)

Lt-NES cells were passaged on day -3 using trypsin and cells were replated on a poly-L-ornithine/laminin T25 flask in N2 medium supplemented with bFGF, EGF and B27. 24 hours later (day -2), media was supplemented with growth factors and B27. On day -1, lt-NES cells were transduced with three lentiviral vectors: teT-ON-Sox10-Puro, teT-ON-Olig2-Hygro and FUW-M2rtTA, on fresh N2 media supplemented with growth factors and B27. The expression of both transcription factors, SOX10 and OLIG2, was initiated at day 0 by administration of 2.5 µg/mL dox and kept until day 10. On day 1, N2 medium was replaced with glial differentiation medium (GM) composed of DMEM-F12, N2 supplement (1:200), B27(-RA) (1:100) and 1% Glutamax. Antibiotic selection was carried out on days

1 and 3 for puromycin (0.25 µg/mL), and from days 1 to 5 for hygromycin (hygro, 0.2 mg/mL, Thermo Fisher Scientific). In addition, for the optimization of the protocol, in some culture conditions the media was supplemented with smoothened agonist (SAG) (1 µM, Selleckchem), ascorbic acid (AA) (200 µM, Sigma) , insulin-like growth factor 1 (IGF-1) (10 ng/mL, Peprotech), neurotrophin-3 (NT3) (10 ng/mL, Peprotech), PDGF (10 ng/mL, Peprotech) and triiodothyronine (T3) (20 ng/mL, Sigma-Aldrich). On day 7, cells were detached for characterization and destined to either flow cytometry analysis, co-cultures with induced neurons, or further differentiation until day 10 using immunocytochemistry (Figure 5).

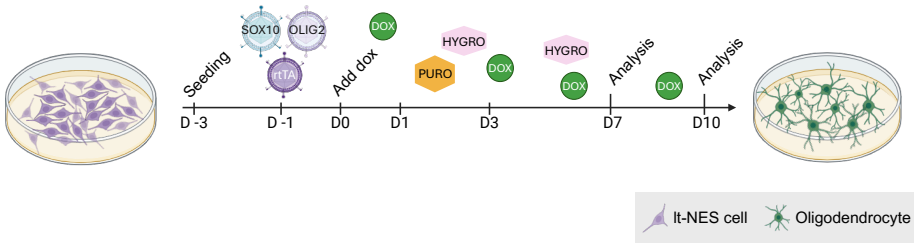

Figure 5. Outline for the generation of a pure population of OLs from human lt-NES cells
Schematic ilustrating the timeline of the protocol used for the generation of hiOLS from lt-NES cells by the overexpression of two transgenes *Olig2* and *Sox10* using the doxycycline-inducible Tet-On system. D, day; DOX, doxycycline; PURO, puromycin; HYGRO, hygromycin. Figure made with BioRender.

Co-culture of iNs with hiOLs

The generation of mature excitatory neurons from hES cells and human lt-NES cells was performed as previously described in the section "*Differentiation of hES cells into induced neurons*". Human inhibitory neurons were generated from ES cells with a cocktail of factors [155]. For the establishment of neuron-OL co-cultures, hiOLs programmed for 7 days were plated on top of coverslips containing either excitatory or inhibitory mature neurons (ratio 10.000 OLs/100.000 neurons) in the presence of induction media (DMEM/F12 with N2 and B27). Dox was daily added to the media until day 9 and every other day from day 9 to day 14. Co-cultures were either fixed after 7 days (14 days of programming for both cell types) for characterization via immunocytochemistry or subjected to live cell imaging from 12 to 14 days for posterior recording analysis and then fixed at day 14 of co-culture (day 21 of programming).

Human adult cortical (hACtx) tissue

Healthy human neocortical tissue was obtained with informed consent by resection of a big piece of the middle temporal gyrus from patients undergoing elective

surgery for temporal lobe epilepsy (n = 3, for **Paper II;** n=1 for **Paper IV**) according to guidelines approved by the Regional Ethical Committee, Lund (Dnr. 2021-07006-01).

The surgically resected tissue was instantly kept in ice-cold modified human artificial cerebrospinal fluid (mhACSF) and quickly assessed for the best cutting orientation and glued to the slicing stage within the chamber of the Vibratome (Leica VT1200S) filled with ice-cold mhACSF continuously bubbled with 95% O2 and 5% CO2. Slices were cut at 300 µm thickness, 0.05 mm/s speed and 1.7mm amplitude, after which they were transferred to cold rinsing media containing HBSS, HEPES (4,76 g/L), Glucose (2 mg/mL, Sigma) and Penicillin/Streptomicin (500 U/mL). Slices were fixed with 4% paraformaldehyde (PFA) for acute characterization of the tissue (**Paper II, III**) or transferred to cell culture inserts containing Alvetex scaffold membranes (Reinnervate) (**Paper II**) or Millicell membranes (PICM0RG50, Millipore) (**Paper IV**) in 6 well plates filled with hACtx media (Brain Phys medium [without phenol red] supplemented with B27 [1:50], glutamax [1:200, Gibco] and gentamycin [50 mg/mL, Gibco], Antibiotic/Antimycotic [100x, Gibco], BDNF [50 ng/mL, Peprotech] and NT3 [50 ng/mL, Peprotech]), and incubated in 5% CO2 at +37ºC. The media was changed once a week for Alvetex membranes and every second day for the Millicell.

Transplantation into hACtx organotypic slices

Human organotypic cortical slices were maintained in culture for 4 or 7 days before transplantation of GFP^+ hiOLs (**Paper IV**) or GFP^+ lt-NES cells (**Papers I, II**), respectively, was performed. Briefly, both types of cells were detached at day 7 of differentiation and resuspended to a concentration of 100.00 cells/µL in pure cold Matrigel. After part of the medium was removed from the top of the insert, the cell suspension was collected into a cold glass capillary and injected as small drops, stabbing the semi-dry slice at various sites. After Matrigel was solidified, additional medium was carefully added to immerse the tissue. Media was changed once a week (**Paper I,II**) or every other day (**Paper IV**). Dox was added until day 14 of programming (1 week after transplantation) for **Paper IV**. Tissue was fixed 4 weeks post-transplantation for graft characterization via immuno-histochemistry and immuno-electron microscopy (iEM).

Non-transplanted slices maintained for 2-4 weeks in culture were used for characterization by immunohistochemistry and electrophysiological recordings of the neural populations in cultured tissue.

Viral vectors

Different lentiviral vectors were used in the experiments described in **Papers II-IV** (Figure 6). The lentiviral constructs integrate into the cell genome, allowing stable gene expression and inheritance through cell division. The Tet-On inducible system was used to deliver specific transcription factors. In **Paper III**, *Ngn2* was introduced into hES cells, while in **Paper IV,** *Olig2* and *Sox10*, along with *GFP*, were introduced into lt-NES cells.

The lt-NES cell-derived progenitors used for transplantation onto *ex vivo* human tissue in **Papers I, II, IV** were transduced with a lentiviral vector carrying GFP either under a constitutive promoter (pKG-GFP) or a TetOn promoter.

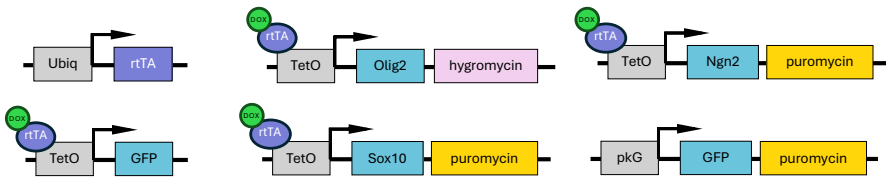

Figure 6. Lentiviral particles used in this thesis
Construct maps of the lentivirus constituting the Tet-On system: FUW-M2rtTA (Addgene #20342), TetO-GFP, TetO-Olig2-Hygromycin, TetO-Sox10-Puro and TetO-Ngn2-T2A-Puro; as well a lentiviral particle with a constitutive promoter pKG-GFP-Puro.

Live cell analysis

Live cell imaging of hiOLs and iNs co-culture

The GFP^{+}hiOLs and lt-NES-iNs were transferred to the incubation chamber of a Zeiss LSM780 laser scanning confocal microscope. The cultures were maintained and imaged for 2 days at +32ºC in 5% CO2, with fresh medium added daily. Fluorescence and brightfield time-lapse images were acquired every 30 min using a 20X dry (air) objective. Time-lapse single-plane images were collected to monitor the samples over time. The 488 nm argon laser was used to detect GFP expression in GFP^{+} cells, with the signal collected using a Gallium Arsenide Phosphide (GaAsP) detector. The same 488 nm argon laser was employed for brightfield imaging of non-labeled neuronal cells, with the transmitted light captured using a Transmitted Photomultiplier Tube (T-PMT). Differential Interference Contrast (DIC) imaging was utilized to enhance the contrast of the transmitted light images,

facilitating detailed visualization of the sample's structural features. The Zeiss LSM780 was operated using Zen Black software version 2012. Time-lapse videos and individual frames extracted from these videos, where GFP^+ hiOLs had migrated towards neuronal cells and overlapped with their axonal structures, were analyzed. Particular attention was paid to detecting the overlap of the GFP signal with axonal structures, as this phenomenon strongly indicates interactions between OLs and neurons and possibly early myelination events.

Electrophysiology *in vitro*

Electrophysiological recordings were done with HEKA double patch clamp EPC10 amplifier (HEKA Elektronik, Lambrecht, Germany) and sampled at 10 kHz, using PatchMaster for data acquisition. For whole-cell patch-clamp recordings, coverslips were transferred to the recording chamber and constantly perfused with carbonated artificial cerebrospinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 26 NaHCO3, 1.25 NaH2PO4, and 11 glucose (pH ~7.4, osmolarity ~305 mOsm). Patch pipette was filled with an internal solution containing (in mM): Kgluconate 122.5, KCl 17.5, NaCl 8, KOH-HEPES 10, KOH-EGTA 0.2, MgATP 2, and Na3GTP 0.3 (pH ~7.2, 295 mOsm). The average pipette tip resistance was ~4-5 MW, and recordings were done at +32ºC. Pipette current was corrected online before gigaseal formation. In contrast, fast capacitive currents were compensated for during the cell-attached configuration.

Resting membrane potential (RMP) was measured in current clamp mode at 0 pA immediately after establishing the whole-cell configuration. Input resistance (Ri) was calculated from a 5 mV pulse and monitored throughout the experiment. The ability to generate an action potential (AP) was determined by 500 ms square depolarizing current step injections at RMP, with 10 pA increments and ramp injection of 1 s depolarizing current, which was also used to determine the action potential threshold. AP amplitude was measured from threshold to peak, the half AP amplitude width was defined as the time between the rising and decaying phase of the AP measured at half the amplitude of the AP, and the afterhyperpolarization (AHP) amplitude was determined as the difference between the AHP peak and the AP threshold. Whole-cell sodium and potassium currents were observed in voltage-clamp mode at a holding potential of −70 mV, and 200 ms voltage steps were delivered in 10 mV increments.

Flow Cytometry Analysis

For **Papers II, IV**, lt-NES cells or hiOLs cultures were harvested on day 7 and washed before antibody incubation. Anti-O4 APC-conjugated antibody (Miltenyi, no. 130-119-155) was diluted 1:200 in FACS buffer (PBS + 2% fetal bovine serum + 2 nM ethylenediaminetetraacetic acid). Cells were incubated for 45 min at +4ºC

in darkness and washed with FACS buffer by centrifugation. Finally, cell pellets were incubated with viability marker propidium iodide (PI, Life Technologies), diluted 1:1000 in FACS buffer for at least 5 min before acquisition. The flow cytometry analysis was performed on a LSR II flow cytometer (Becton Dickinson) or BD LSR Fortessa flow cytometer. The data was analyzed using BD FACSDiva 9.0 software (BD Biosciences) and FlowJo v.10.8 Software (BD Life Sciences). As for the gating strategy, viable single cells were selected from the total events by size, complexity, and PI negativity.

Ex vivo electrophysiology

For whole-cell patch-clamp recordings, hACtx organotypic slices were transferred to a recording chamber and were constantly perfused with carbogenated human artificial cerebrospinal fluid (hACSF, in mM: 129 NaCl, 21 NaHCO3, 10 glucose, 3 KCl, 1.25 NaH2PO4, 2 MgSO4 and 1.6 CaCl2, pH ~7.4) during the recordings. Recordings were performed with a HEKA EPC10 amplifier using PatchMaster software for data acquisition. The internal pipette solution contained (in mM): 122.5 K-gluconate, 12.5 KCl, 10 HEPES, 2.0 Na2ATP, 0.3 Na2-GTP, and 8.0 NaCl. Biocytin (1-3 mg/mL, Biotium) was dissolved in the pipette solution for *post-hoc* identification of recorded cells.

To study the ability to generate AP and its characteristics, either a current ramp of 0-300 pA, or 10 pA current steps were applied at RMP in the current clamp configuration. In voltage clamp mode, sodium and potassium currents were evoked by a series of 10 mV steps ranging from -70 mV to +40 mV. RMP was measured in current clamp mode immediately after establishing the whole-cell configuration. Ri was calculated from a 10 mV pulse and monitored throughout the experiment. Data were analyzed offline with FitMaster and IgorPro software.

Animals and surgical procedures

Animals were housed in individually ventilated cages under standard temperature and humidity conditions and a 12-hour light/dark cycle with free access to food and water. All procedures were conducted in accordance with European Union Directive 2010/ 63/EU and approved by the ethical committee for the use of laboratory animals at Lund University and the Swedish Department of Agriculture (Dnr. 5.8.18-07222/2021 M68-16). Details on experimental animals from each paper are detailed in Table 1. Information on the number of animals and experimental groups is in the papers.

Table 1. Summary of practicalities regarding experimental animals.
Information on the specie, strain, age of surgery, gender , number of animals used and company.

Paper	Specie	Strain	Age	Sex	N	Company
II	Rat	NIH-Foxn1 RNU	8 weeks	Male	18	Charles River
III	Rat	NIH-Foxn1 RNU	8 weeks	Male	12	Charles River

Focal cortical ischemic injury was induced in the somatosensory cortex by distal middle cerebral artery occlusion (dMCAO), as described previously [80, 156] with some modifications. Animals were anesthetized with isoflurane (3.0% induction; 1.5% maintenance) mixed with air, exposing the temporal bone. A craniotomy of 3 mm was made, the *dura mater* was carefully opened, and the cortical branch of the middle cerebral artery was ligated permanently by suture, cauterized, and cut. Both common carotid arteries were isolated and ligated for 30 min. After releasing the common carotid arteries, surgical wounds were closed, and rats were injected with 1.5 mL Ringer's solution.

Stroke analysis was carried out by magnetic resonance imaging (MRI) 24 hours after experimental ischemia using the Biospec Avance III, 9.4T (BrukerBioSciences Corporation, Ettlingen, Germany). Animals with no ischemic lesion are excluded from the studies.

Intracortical transplantation of GFP^{+}lt-NES cell-derived progenitors and hES-iNs was performed stereotactically 48 h after dMCAO as described previously [78, 98]. Briefly, on the day of surgery, cortically primed lt-NES cells on their third day of differentiation (**Paper I, II**) or programmed hES-iNs on day 7 (**Paper III**) were resuspended to a final concentration of 100.000 cells/μL in cytocon buffer. A volume of 1 μl was injected in two sites for **Paper I, II** at the following coordinates (from bregma and brain surface): anterior/posterior: +1.5 mm; medial/lateral: +1.5 mm; dorsal/ventral: −2.0 mm; and anterior/posterior: +0.5 mm; medial/lateral: +1.5 mm; dorsal/ventral: −2.5 mm. On **Paper III**, 1 μL was injected in three sites at the following coordinates: anterior/posterior: +1.5 mm; medial/lateral: +2.0 mm; dorsal/ventral: −2.0 mm; and anterior/posterior: +0.5 mm; medial/lateral: +1.5 mm; dorsal/ventral: −2.0 mm and anterior/posterior: +0.5 mm; medial/lateral: +2.5 mm; dorsal/ventral: −2.5 mm.

Cells and tissue fixation

All different cell cultures for immunocytochemistry and electrophysiology were plated on glass coverslips of a 24-well plate. On the day of fixation, cells were washed with PBS +/+, then fixed with 4% PFA (Sigma) for 10-20 mins at room temperature and finally washed 3 times with potassium phosphate buffered saline

(KPBS). In contrast, for electron microscopy (EM), coverslips were fixed with 2% formaldehyde (FA) and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4).

Regarding animals, for histology, rats were sacrificed by intraperitoneal injection of sodium pentobarbital. Following the loss of reflexes, animals are transcardially perfused with 0.1 M PBS and then with ice-cold 4% PFA. After perfusion, brains were extracted and post-fixed for 24 hours with PFA at +4ºC. Then, the brains are transferred to 20% sucrose for at least 24 hours at +4ºC, until the brains sink. Brains were cut on a microtome into 30 µm-thick coronal sections and stored in glycerol-based antifreeze solution at -20ºC until use. For iDISCO, animals were perfused with 2% PFA and brains were post-fixed for one hour, then kept in PBS at +4ºC shortly before the protocol started. iEM, rats were transcardially perfused with 0.1 M PBS followed by ice-cold 2% FA, containing 0.2% glutaraldehyde, in 0.1 M PBS, pH 7.4. Brains were removed, post-fixed for 1 h and washed in 0.1 M PBS.

Human organotypic cultured slices, on the day of fixation, were removed carefully from inserts by submersion of the slice in PBS +/+ and transferred to a 24-well plate for fixation with 4% PFA for at least 4 hours at +4ºC. Finally washed 3 times with KPBS until use. For *ex vivo* iEM, tissue was fixed with 2% PFA + 0.2% glutaraldehyde, in 0.1 M PBS for 1 hour.

Stainings

Immunocytochemistry

Cells were permeabilized with 0.025% Triton X-100 in 0.1 M KPBS and blocked with 5% of normal donkey serum (NDS) (Merk Millipore) for 45 min at room temperature. Afterwards, primary antibodies diluted in blocking solution were applied overnight at +4ºC, followed by 3 rinses with KPBS. Fluorophore-conjugated secondary antibodies (1:500, Jackson Laboratories) diluted in blocking solution were applied for 2 h at room temperature. Subsequently, cells were rinsed 3 times with KPBS and nuclei were stained for 10 mins with Hoechst (Molecular Probes or Jackson Laboratories) or 4′,6-diamidino-2-phenylindole (DAPI) diluted 1:1000 in KPBS at room temperature. Stained glass coverslips were mounted on slides with Dabco (Merk) mounting media. The list of primary antibodies can be found in Table 2.

For the stainings requiring antigen retrieval, before permeabilization, it is recommended to stain the nuclei, after which an incubation with sodium citrate, pH 6.0, Tween 0.05% at +65ºC was done for 30 min, followed by 3 washes with KPBS.

Immunohistochemistry

Stored rat brain sections were rinsed 3 times with KPBS and incubated in a blocking solution for 1 h (0.25 Triton X-100 in 0.1 M KPBS [TKPBS] with 10% NDS). The rest of the procedure follows that outlined above for cell cultures.

For staining of hACtx organotypic cultured or acute slices, tissue was incubated overnight at + 4ºC in permeabilization solution (0.02% BSA, 1% Triton X in PBS). Next, slices were incubated overnight at +4ºC in blocking solution (KPBS, 0.2% Triton X-100, 1% BSA, Sodium azide [NaN3] [1:10000] and 10% NDS). Primary antibodies were diluted in blocking solution and incubated for 48 h at +4ºC. Secondary antibodies, also diluted in blocking solution, were applied for 48 h at +4ºC. Slices were washed 3 times with KPBS and incubated in Hoechst/DAPI for 2 h at room temperature. Finally, mounted with Dabco mounting media after rinsing with deionized water.

Some stainings required antigen retrieval before the permeabilization step. Rat sections, or human organotypic cultures were incubated with sodium citrate pH 6.0, Tween 0.05%, for 30 min (for sections) or 2 h (for organotypic cultures) at +65 ºC.

Imaging and quantifications

Overview images of rat brain slices for different markers were taken using a Virtual Slide Scanning System (VS-120-S6-W, Olympus, Germany). Fluorescent staining images were acquired using a confocal microscope (LSM 780, Zeiss, Germany).

In vitro quantifications were performed through 10 µm thick Z-stack images, which were taken with a 20x objective. A total of 10 images separated 1000 µm were taken per coverslip and/or condition. Maximum intensity projection images were analyzed using ImageJ software. The percentage of positive cells for the different markers was quantified as follows: marker$^+$cells / total DAPI$^+$ cells. Cell counting was performed independently by three blinded researchers, with the final result representing the average value.

For the morphological analysis of the hiOLs (**Paper IV**), a classification was established based on morphological complexity according to the number of processes using the cytoplasmic staining O4: unbranched cells were classified as "non-ramified"; cells with less than 5 ramifications were termed "mid-ramified" and, cells with more than 5 ramifications were classified as "highly ramified".

The volume of infarction was measured in sections stained with a mature neuronal marker (NeuN). The intact area was determined by cells in both the ipsilateral and contralateral hemispheres, marked out, and measured using ImageJ software. The infarcted area was determined by subtracting the non-lesioned (NeuN-stained) area in the damaged hemisphere from the corresponding location in the contralateral hemisphere. The lesion volume was calculated by multiplying the infarcted area by

the thickness and spacing between sections (300 μm). The graft area was determined using sections stained with STEM101. The immature and mature regions within the graft were determined by outlining areas positive for DCX and NeuN, respectively.

On **Paper II**, CNPase and OLIG2 quantification in the core of the transplant in rat slices was performed in 10 mm thick 40x images and positive cells were counted by sampling different areas (($CNPase^+$-$STEM101^+$)/total $STEM101^+$ and ($OLIG2^+$-$STEM101^+$)/total $STEM101^+$).

Quantification of $OLIG2^+$ cells in the corpus callosum in rat slices was performed in 20x confocal images (10 μm thick z stack). For analysis of myelination, confocal images (5 μm thick z stack) were taken in the corpus callosum (40x), peri-infarct area (20x), and striatum (20x). For analysis of the dorsal lateral striatum, starting 700 μm lateral to the dorsal part of the lateral ventricle, two 20x images (x = 700 and 1,400 μm) were taken. To measure the thickness of the corpus callosum, confocal images were taken with a 20x Zoom 0.6. All quantifications were performed on maximum-intensity projection images using ImageJ software by blinded researchers.

In **Paper III**, co-expression was assessed via confocal microscopy by observing the overlap of the two chosen markers within the same plane and area. Co-expression of layer-specific cortical markers (BRN2, CTIP2) with the human nuclear marker (STEM101) was performed using 63x confocal images.

Evaluation of areas reached by hES-iNs-derived fibers was performed using a Virtual Slide Scanning System (VS-120-S6-W, Olympus, Germany). Fiber density was assessed semi-quantitatively in 10-μm thick maximum intensity projection confocal images captured with a 63x objective. Three to five images were analyzed for each area.

Quantification of GFP^+ $OLIG2^+$ cells in the human organotypic slices was carried out using 20x confocal images in areas where transplantation was observed, and the number of double-positive cells was counted through a z-stack by sampling different single planes.

iDISCO

iDISCO tissue clearing was performed according to Renier and collaborators with some modifications (details in **Paper III**)[157]. Primary antibodies mouse anti-STEM101 and mouse anti-STEM121, were added to identify human nucleus and processes, followed by incubation with the secondary antibody donkey anti-mouse Cy5. Clearing was performed using dibenzyl ether prior to sample imaging. Tissue-cleared samples were imaged in a sagittal orientation using a sCMOS-5.5-CL3 camera equipped light sheet microscope (Ultramicroscope II, LaVision Biotec, Germany) with a 2×/0.5 objective lens (MVPLAPO 2×) with a 6-mm working

distance dipping cap. All imaging used the Imspector-Pro219 software and scanned continuously with a step size of 10 μm at 3.2× magnification (7988 × 9472 pixels). Post-imaging visualization utilized the Arivis Vision 4D v.2.12.3 software.

Table 2. List of primary antibodies used for immunofluorescence.

Antigen	Host	*in vitro*	*in vivo*	*ex vivo*	Notes	Company	Catalog No
AiF1 (Iba1)	Goat	-	-	1:100		Bio-rad	AHP2024
BIII-Tubulin	Mouse	1:500	-	-		Sigma	T8660
BRN2	Goat	1:400	1:400	-	AR	Santa Cruz	SC-629
CC1	Mouse	1:200	1:200	1:200	AR	Abcam	ab16794
CNPase	Rabbit	-	1:500	1:500		Abcam	ab227218
CTIP2	Rabbit	1:100	1:200	-	AR	Merck	HPA049117
DCX	Goat	1:400	1:400	-		Santa Cruz	SC-8060
GFP	Chicken	1:1000	1:1000	1:1000		Millipore	AB18901
KGA	Rabbit	1:1000	-	-		Abcam	ab9343
Ki67	Rabbit	1:500	1:250	-		Abcam	ab16667
MAP2	Chicken	1:1000	1:1000	1:1000		Abcam	ab5392
MBP	Mouse	1:1000	1:500	1:1000	AR	Biolegend	808401
NANOG	Rabbit	1:150	-	-		Abcam	ab21624
NeuN	Rabbit	1:500	1:500	1:1000		Abcam	ab104225
NeuroFilament	Chicken	1:1000	-	-		Abcam	ab4680
Neurofilament Axonal Pan	Mouse	1:5000	-	-		Biolegend	SMI-312-R/837904
Neurofilament Neuronal Pan	Mouse	1:1000	-	-		Biolegend	SMI-311-R/837801
NG2	Rabbit	-	1:100	-		Millipore	AB5320
O4	Mouse	1:200	-	-	IgM	R&D	MAB1326
OLIG2	Rabbit	1:500	1:500	1:500	AR	Abcam	ab109186
PLP	Rabbit	1:200	-	-		abcam	ab105784
SATB2	Mouse	1:100	-	-	AR	Abcam	ab51502
SATB2	Rabbit	-	1:200	-	AR	Abcam	ab34735
SOX10	Goat	1:200	-	1:500	AR	Santa Cruz	SC-17343
SOX2	Rabbit	1:200	1:200	-		Millipore	Ab5603
STEM101	Mouse	-	1:500	-		Takara	AB-101
STEM121	Mouse	-	1:500	-		Takara	AB-121
STEM123	Mouse	-	1:2000	1:2000		Takara	AB-123
TBR1	Rabbit	-	1:300	-		Gifted	
Tmem119	Rabbit	-	-	1:200		Abcam	ab18533

Electron microscopy (EM)

For *in vitro* EM, lt-NES cell cultures (n = 8 wells) were frozen and cut into ultrathin sections with a diamond knife. Ultrathin sections were examined and photographed using a transmission electron microscope FEI Tecnai Bio-Twin 120 kv.

ImmunoEM (iEM)

For iEM in rat tissue, frontal 100 μm sections of the whole brain were cut on a Vibratome VT1000A (Leica, Germany). In **Paper II**, the sections were cryoprotected, freeze-thawed in liquid nitrogen, and incubated overnight in primary goat anti-GFP antibody (1:500, Novus Biologicals) at +4ºC. The tissue was then incubated at room temperature for 2 h with biotinylated rabbit anti-goat secondary antibody (1:200, DakoCytomation), and avidin-biotin-peroxidase complex (ABC) (Vector Laboratories), followed by 3,3′- diaminobenzidine tetrachloride (DAB) and 0.015% hydrogen peroxide. Following the DAB reaction, sections were processed for iEM.

DAB-immunostained sections of rat brain tissue and human organotypic cultures were postfixed in 1% osmium tetroxide in 0.1 M PBS, dehydrated in a graded series of ethanol and propylene oxide, and flat-embedded in Epon. Ultrathin sections were cut with a diamond knife. For post-embedding immunogold labeling of GFP or STEM121, ultrathin sections were incubated overnight in primary goat anti-GFP antibody (1:500, Novus Biologicals) or mouse anti-STEM121 antibody (1:500, Takara Bio Europe) at +4ºC. A secondary antibody (donkey anti-rabbit IgG conjugated to 12 nm colloidal gold; Jackson Laboratories) or (goat anti-mouse IgG conjugated to 15 nm colloidal gold, EMS) diluted 1:20 in 0.1% BSA in PBS was added for 1.5 h, then washed with PBS. Sections were then fixed with 2% glutaraldehyde, then washed with PBS, followed by dH2O. Sections were stained with uranyl acetate and lead citrate. Ultrathin sections were examined and photographed using a transmission electron microscope JEM-100CX (JEOL, Japan).

In **Paper IV**, for pre-embedding immunogold labeling, human adult cortical tissue samples were blocked and permeabilized in PBS containing 2% NDS and 0.05% Triton X-100 for 30 min. They were then incubated overnight at +4°C with a primary goat anti-GFP antibody (1:150, Abcam) diluted in PBS with 2% NDS and 0.05% Triton X-100, followed by PBS washes. A secondary Nanogold®-Fab' rabbit anti-goat antibody (1.4 nm gold-conjugated, 1:200, Nanoprobes) was applied for 2 hours, and samples were washed again. Fixation was performed with 2% glutaraldehyde in PBS for 30 min, followed by washing with deionized water. Silver enhancement was carried out using the HQ kit (Nanoprobes) under darkroom

conditions for 8 min. After enhancement, samples were washed with deionized water and post-fixed in 0.2% osmium tetroxide in 0.1 M phosphate buffer for 30 min on ice. They were then stained with 0.25% uranyl acetate in 0.1 N acetate buffer for 1 hour. Dehydration was performed through a graded ethanol series, followed by infiltration with ethanol/epoxy resin mixtures. Finally, samples were embedded in epoxy resin and polymerized at +60°C for two days. Ultrathin sections were cut, stained with lead citrate, and examined using a JEM-100CX transmission electron microscope (JEOL, Japan).

RNA extraction and RT-qPCR

RT-qPCR was performed on RNA extracted from: lt-NES cells at different time points of differentiation (D0, D4, D8, D12, and D15) and hES-iNs at 6 DIV. RNA extraction was performed with the RNeasy Mini Kit (QIAGEN) following the protocol described by the manufacturer. After extraction, RNA purity and concentration of samples were determined using a NanoDrop spectrophotometer (ND-1000). RNA (1 µg) was used for cDNA synthesis with qScript cDNA SuperMix (QuantaBio). TaqMan probes (Thermo Fisher Scientific, Table 3) were used, and RT-qPCR was run in triplicate samples on an iQ5 real-time cycler (Bio-Rad) for **Paper II** or QuantStudio™ 1 Real-Time PCR System (ThermoFisher Scientific, Sweden) for **Paper III**, with GAPDH as the housekeeping gene.

Table 3. Summary of probes used in RT-qPCR

Target	Expression	Assay ID
OLIG2	Oligodendrocyte linage	HS00300164_s1
MBP	Mature oligodendrocytes	HS00921945_m1
DCX	Neuroblasts	HS00167057_m1
GAPDH	Housekeeping gene	HS02786624_g1
NANOG	Pluripotency	Hs02387400_g1 HS00300164_s1
BIIITUB	Pan-neuronal	Hs00964963_g1
MAP2	Pan-neuronal	Hs00258900_g1
POU3F2	Upper-layer neurons	Hs00271595_s1
TBR1	Deep-layer neurons	Hs00232429_m1

Statistical analysis

Statistical analysis was performed using Prism 9 or 10.1.1 software (GraphPad, Dotmatics). An unpaired t-test was used when data were normally distributed, whereas a Mann-Whitney U test was used when data did not pass the normality test.

When different independent groups were compared, a one-way ANOVA plus Tukey's multiple comparison test was performed. A two-way ANOVA followed by Sidak's multiple comparison post hoc test was used to compare multiple groups. Significance was set at $p < 0.05$. Data are expressed as mean ± SEM (**Papers I-III**) or mean ± SD (**Paper IV**).

Summary of key results

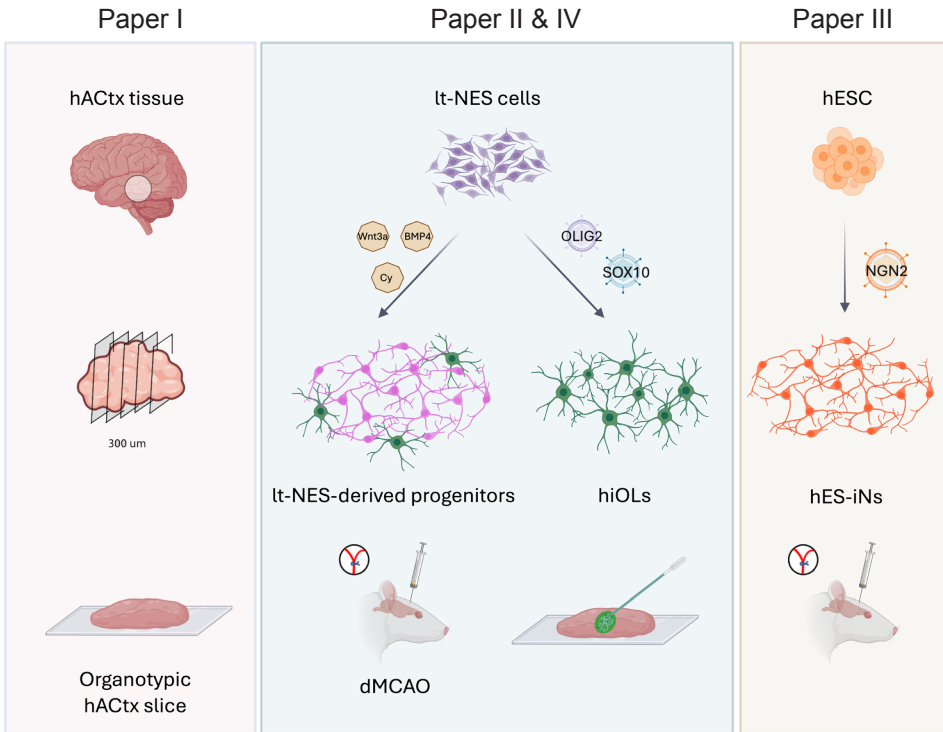

Figure 7. Overview of studies and experimental designs
This section summarizes the key findings from the articles included in this thesis, all aimed at restoring the injured brain. In Paper I, organotypic slices of hACtx tissue are validated as a platform to test potential stem cell-based therapies. Papers II and III explored the potential of cortical neurons and OLs derived from human lt-NES cells, and cortical neurons derived from human ES cells for transplantation into the stroke-injured brain. Finally, Paper IV describes the rapid generation in vitro of a pure population of OLs followed by their transplantation in a human-to-human setting to evaluate their therapeutic value. hACtx, human adult cortical; lt-NES, long-term neuroepithelial stem; hiOLs, human induced oligodendrocytes; hESC, human embryonic stem cells; hES-iNs, human embryonic induced neurons; dMCAO, distal middle cerebral artery oclussion. Figure made with BioRender.

Generation of functional cortical neurons and myelinating OLs from human lt-NES cells using a cortical priming protocol (Paper II)

In our previous study, we obtained some evidence for the presence not only of neurons but also of myelin-forming OLs in grafts, 6 months after intracerebral transplantation of cortically primed human lt-NES cells in a rat stroke model [98]. To assess whether lt-NES cells can form functional OLs in addition to neurons, we first differentiated them *in vitro* for 21 days using our cortical priming protocol, using the following small molecules: BMP4, Wnt3a and Cy [78]. We characterized the generated cells by protein and gene expression.

We found that after 8 days, lt-NES cells started expressing immature neuronal markers like DCX and OL markers, OLIG2 (pan-OL marker) and MBP (myelin basic protein), both at protein and gene level (Figure 8A). By day 21 of differentiation, 5% of the cells were OLIG2 positive and 3% expressed CC1, a marker of mature OLs. About 1.5% of the lt-NES cells expressed O4, a marker of immature and mature OLs (Figure 8B). Importantly, MBP expression was found near the axonal marker Neurofilament, suggesting that OLs in the culture may contribute to axonal myelination.

In order to provide further evidence that lt-NES cells-derived OLs have the ability to myelinate human axons in the culture system, we used EM. After 21 days, in addition to neurons [79], we observed a cell population exhibiting the ultrastructural features of mature OLs, i.e., irregularly shaped dark nucleus with clumped chromatin near the inner nuclear membrane. The cytoplasm was electron-dense and contained short cisternae of rough endoplasmic reticulum with short mitochondria. Importantly, the lt-NES cell-derived OLs formed loose myelin sheaths around axons, which could be the initial stage of myelination (Figure 8C,D). These findings indicated that cortically fated lt-NES cells form myelinating OLs in 21 days.

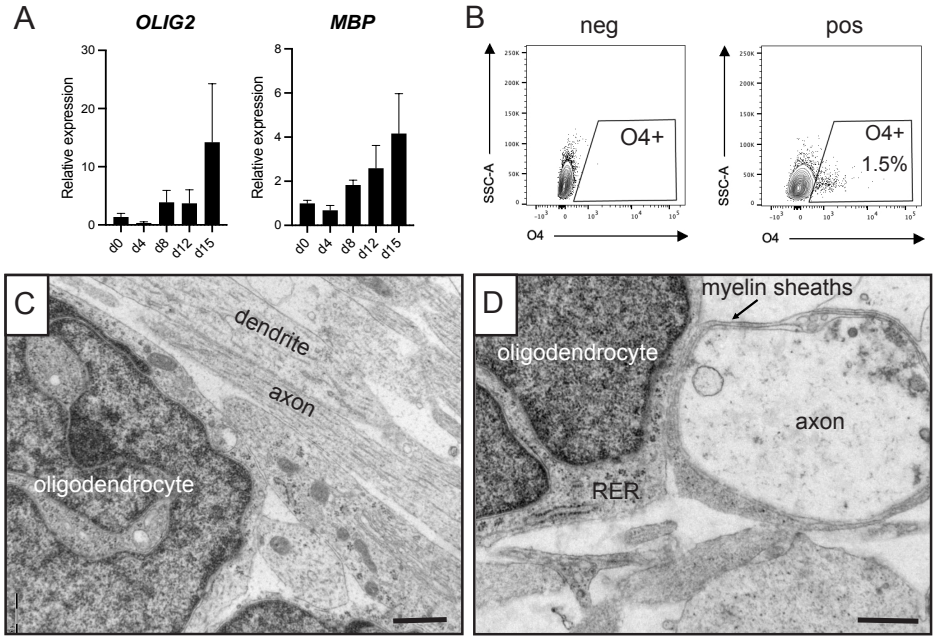

Figure 8. Human lt-NES cells cortically primed give rise to myelinating oligodendrocytes (OLs) and mature neurons after 21 days of *in vitro* differentiation
A. Flow cytometry analysis of $O4^+$ cells after 21 days of differentiation. **B**. Temporal analysis of OLIG2 and MBP gene expression by RT-qPCR. **C**. Mature lt-NES cell-derived OL and neuronal proceses shown by EM. **D**. lt-NES cell-derived axon wrapped by myelin generated by lt-NES cell-derived OL. Scale bars, 500 nm. Data shown as mean ± SEM.

Generation of functional cortical neurons from hES cells through transcription factor programming (Paper III)

We previously found that a 2-week induction of NGN2 expression in hES cells gives rise to mature neurons expressing the neuronal marker MAP2 and with morphology similar to adult human cortical neurons [92]. To explore the potential use of hES-iNs for transplantation with the aim of restoring neuronal circuitries, we first performed a detailed characterization of the differentiation stage of the cells at the time point chosen for transplantation (7 days).

85% of the hES-iNs were positive for DCX and 63% expressed the mature neuronal marker NeuN at day 7 of programming (Figure 9A). Specifically, 24% of the cells expressed only DCX, 61% co-expressed both DCX and NeuN, and 2% solely expressed NeuN. Importantly, we found no expression of pluripotency markers and just less than 1% were Sox2 positive, suggesting the presence of a small population

of neural stem cells. Taken together, our data provide evidence that NGN2 programming of hES cells gives rise to neurons at different stages of differentiation: immature neurons ($DCX^+/NeuN^-$), intermediate stage ($DCX^+/NeuN^+$), and a small population of mature neurons ($DCX^-/NeuN^+$).

Regarding their phenotype, 95% of the generated hES-iNs expressed the excitatory neuronal marker, KGA. They also expressed markers characteristic of both upper cortical layers, such as BRN2 and SATB2, and deep layers, e.g., CTIP2. Interestingly, and in line with our previous results [92], around 94% of the cells co-expressed upper and deep cortical layer markers (Figure 9B,C).

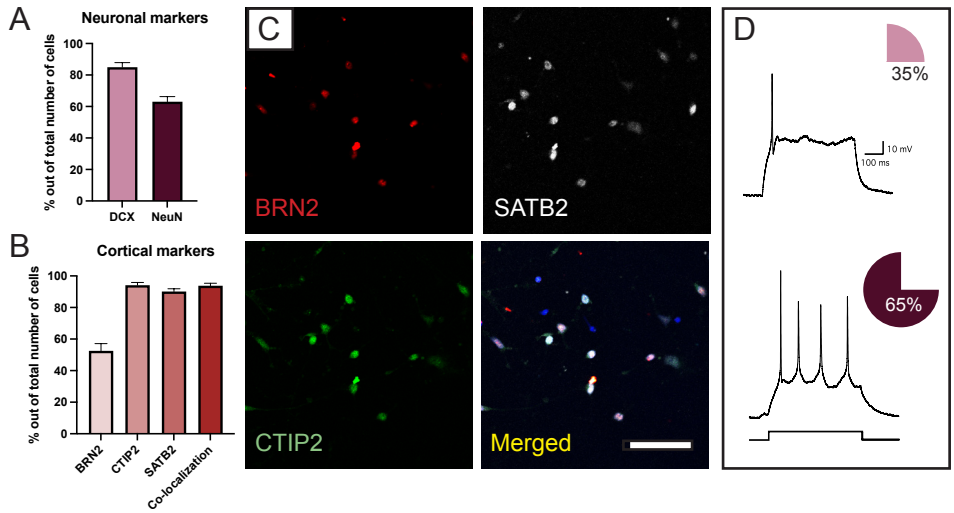

Figure 9. hES-iNs generated by NGN2 overexpression gives rise to immature and mature neurons following 7 days of programming *in vitro*

A, Quantifications of the expression of neuronal progenitor marker DCX and mature neuronal marker NeuN. **B-C**, Quantifications and representative confocal images of upper and deep cortical layer markers BRN2, CTIP2, SATB2 and colocalization of this markers. **D**, Whole-cell patch-clamp recordings of hES-iNs at day 7. Representative voltage traces illustrates the ability of the cells to generate single (upper trace) or multiple (lower trace) action potentials (APs) in response to a 20 pA current step. Pie charts indicate the ratio of cells exhibiting single versus multiple APs upon depolarization. Scale bar, 50 µm. Data is represented as mean ± SEM.

We then performed whole-patch clamp recordings to assess whether hES-iNs were functional at 7 days. hES-iNs exhibit all basic properties of functional neurons, i.e., the ability to fire APs and presence of both fast inward sodium and sustained outward potassium currents. 65% of the cells were able to generate multiple APs upon step or ramp current injection, while 35% only fired a single AP (Figure 9D). These observations indicate the occurrence of more and less mature neuronal populations simultaneously. We also observed spontaneous AP firing at resting membrane potential in 40% of those with multiple APs. No spontaneous synaptic

activity was detected in any of the recorded cells at this time point. Taken together, our electrophysiological analysis suggests that hES-iNs have acquired certain neuronal characteristics, they are still young and not yet synaptically integrated after 7 days of programming.

Generation of myelinating OLs from human lt-NES cells with transcription factor programming (Paper IV)

As a continuation of the inspiring results obtained in Paper II, to improve the yield of OLs with the aim of generating a pure source of myelinating cells from human lt-NES cells, we tested different induction protocols. To induce OL lineage, we overexpressed OLIG2 and SOX10, transcription factors involved in OL specification [113], using a tet-ON system combined with antibiotic selection (Tet-ON-Olig2-hygro and Tet-ON-Sox10-puro). We first overexpressed OLIG2, as undifferentiated lt-NES cells express SOX10 [158]. After 7 days of induction, the induced cells displayed neuronal morphology and expressed neuronal markers such as DCX, Tuj1 and MAP2. Tuj1 was expressed by 97% of the cells, while less than 1% expressed O4. On the other hand, overexpression of only SOX10 gave rise to 42% of Tuj1-positive cells and 30% of O4-positive cells. Our results show that the sole expression of OLIG2 or SOX10 is not sufficient for efficient OL specification.

We then combined the overexpression of OLIG2 and SOX10 for 7 and 10 days. We used a fixed concentration of OLIG2 lentivirus and tested two different concentrations of SOX10, corresponding to MOIs of 0.001 and 0.0025. We also cultured hiOLs in the presence or absence of a cocktail of maturation factors (SAG, PDGF, AA, T3, NT3 and IGF-1), extensively used in the literature for OL production [68, 113].

After 7 days of induction, we confirmed the expression of OLIG2 and SOX10 in all culture conditions and we could observe that 80% of the human lt-NES cells expressed O4 without differences in percentage between the different induction protocols (Figure 10A). Similarly, by day 10, the percentage of $O4^+$ cells remained at 80%. Taking advantage of the cytoplasmic nature of the O4 staining, we assessed the morphological diversity of OLs in our culture and classified them into three subtypes: *non-ramified*, *mid-ramified*, and *highly ramified* (for criteria, see *Methods*) (Figure 10B). At day 10 of programming, 3% of OLs exhibited highly ramified morphology except for the condition with high expression of SOX10 and the presence of maturation factors, where the percentage reached around 8%. To evaluate OL maturity, we performed MBP staining (Figure 10C) and observed a pattern consistent with our morphological analysis, where MBP was slightly increased in conditions with high SOX10 and added factors. It must be highlighted that all MBP^+ cells displayed a highly ramified morphology. Other myelin-

associated proteins, such as PLP, and mature OL marker CC1 were found across all four culture conditions, suggesting that part of the generated OLs might be mature at this time-point.

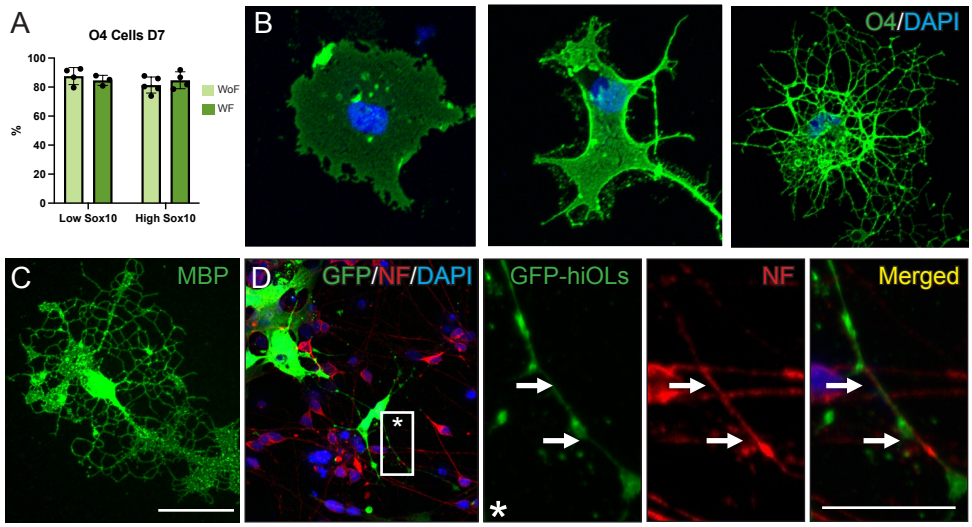

Figure 10. Overexpression of OLIG2 and SOX10 in human lt-NES cells generates mature oligodendrocytes (OLs) *in vitro* capable of interacting with neuronal axons

A, Percentages of $O4^+$ cells analyzed by flow cytometry using different programming protocols for 7 days. **B-C**, Representative confocal images of non-, mid- and highly ramified $O4^+$ cells (**B**) and cells positive to myelin basic protein (**C**). Scale bar in C, 40 µm. **D**, Representative confocal images of co-cultures of hiOLs with induced excitatory neurons at day 21 of programming. Arrows show OL processes in close proximity to neurofilament marker (NF). Scale bar in D, 20 µm. Data is presented as mean ± SD.

To assess the functionality of the generated OLs, we co-cultured hiOLs subjected to 7 days of programming with human neurons derived from two different sources: hES cells and human lt-NES cells. Since grafted OLs in a potential future therapeutic setting must have the ability to myelinate diverse neuronal populations in the brain, co-cultures were conducted with both excitatory neurons, generated by overexpression of NGN2 in hES and human lt-NES cells (*previously described above*, [159]), and inhibitory neurons, generated from hES cells through small molecule differentiation [155]. After 7 days in co-culture (day 14 of hiOLs programming), we found neuronal projections and hiOLs processes in close proximity. Through live cell imaging, we could observe high motility in culture, extending processes towards multiple neuronal projections and surrounding them. Some initially non-myelinated axons were ensheathed by hiOLs processes in less than 24 hours. Co-cultures of hiOLs and lt-NES-iNs at day 21 of reprogramming showed areas in which OL processes (labelled with GFP) were aligned with axons (stained by neurofilament) (Figure 10D). Our results indicate that overexpression of OLIG2 and SOX10 promotes the generation of both pre-myelinating and mature,

myelin-expressing OLs, that interact with human-derived axons, suggesting potential myelination of axons *in vitro*.

Effect of grafted stem cell-derived OLs and cortical neurons in the stroke-injured brain

Transplantation of OLs into the stroke-injured brain (Paper II)

Previously, we have found that 6 months after transplantation 40% of the grafted human lt-NES cells expressed the OL marker SOX10 and most importantly, human-derived MBP was observed close to the transplant suggesting that graft-derived OLs could contribute to remyelination [98]. For this reason, we explored whether intracerebrally transplanted, cortically fated human lt-NES cell-derived progenitors could remyelinate axons after stroke. Rats were subjected to cortical stroke, implanted with cortically primed human lt-NES cells close to the injury after 48 h, and sacrificed 6 months later. In all the animals, the stroke-induced neuronal loss, as determined by lack of NeuN (marker of mature neurons) immunoreactivity, was restricted to the cortex (mostly somatosensory cortex [S1FF and S1BL] and motor area [M1]), sparing subcortical structures [98].

Since we detected $OLIG2^+$ cells of human origin in the corpus callosum (CC), we hypothesized that part of the transplanted lt-NES cell-derived progenitors had become OLs and contributed to the remyelination. We found that, at 6 months after transplantation, around 20% of the $STEM101^+$ cells in the core of the graft expressed OLIG2 and about 20% expressed CNPase, a marker for pre-myelinating and myelinating OLs (Figure 11A,B). We also observed human-derived cells expressing the mature OL marker, CC1, with varying density through the graft. Hardly any grafted cells expressed neuron-glial antigen 2 (NG2), a marker for OPCs.

To provide further evidence for the formation of mature myelinating OLs, we performed ultrastructural analysis 6 months after transplantation of GFP^+ cortically fated human lt-NES cells in stroke-affected animals. Most GFP^+ cells were located in the peri-infarct area, while some were found in the CC and contralateral cortex. Some GFP^+ cells exhibited the morphology of mature myelinating OLs: dark electron-dense rectangular-shaped cytoplasm, heterogeneous nuclear chromatin pattern, and short and wide endoplasmic reticulum cisternae organized in the vicinity of the nucleus (Figure 11C). By iEM, we demonstrated that graft-derived human OLs are able to myelinate host axons as gold particles were found within the membranous sheets of myelin (i.e., graft-derived myelin) (Figure 11D). These findings provide evidence that 6 months after transplantation into the stroke-injured

somatosensory cortex, cortically fated lt-NES cells give rise to mature myelinating OLs.

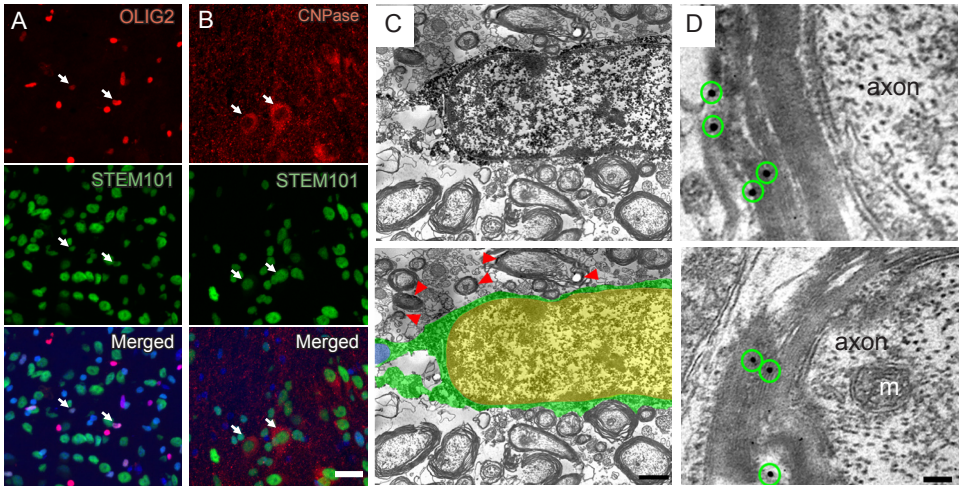

Figure 11. Intracortical transplantation of lt-NES cell-derived progenitors into the stroke injured somatosensory cortex give rise to mature myelinating oligodendrocytes (OLs) after 6 months
A-B,Representative confocal images of the transplantation area showing the expression of pan-oligodendrocyte marker OLIG2 (**A**) and immature and mature oligodendrocyte marker CNPase (**B**). Scale bar, 20 µm. **C**, Representative immuno-electron microscopy images showing GFP/DAB positive cells in the corpus callosum with the morphology of mature myelinating OLs (cytoplasm highlighted in yellow and processes in green). Scale bar, 2 µm. **D**, Electron microscopy image of compact myelin sheaths containing GFP^+ immunogold particles (green circles) showing myelination by the grafted cells. Scale bar, 0.1 µm. Arrows indicate colocalization of OLIG2 or CC1 with the human nuclear marker STEM101. Nuclear staining is included in merged panel (Hoechst). m, mitochondria.

Transplantation of hES-iNs into the stroke-injured brain (Paper III)

In our previous study, we demonstrated that hES-iNs transplanted onto hACtx slices survived and differentiated into functional neurons within 4 weeks and some expressed the upper-layer cortical marker SATB2 [92].

To assess the survival and differentiation of the hES-iNs following intracortical transplantation into rats with cortical ischemic stroke, animals were subjected to dMCAO. MRI was performed 24 hours later to confirm the location of the lesion, which was restricted to the somatosensory cortex in all animals. At 48 hours post-dMCAO, hES-iNs programmed for 7 days were transplanted in close proximity to the damaged cortex, and animals were sacrificed 1 and 3 months later.

Characterization of grafted hES-iNs after intracortical transplantation in the dMCAO model

Grafts predominantly expressed the immature neuronal marker DCX and only a few grafted cells expressed the mature neuronal marker NeuN 1 month after

transplantation. In contrast to these findings, 3 months after transplantation grafted cells expressed markers of both immature (DCX) and mature (NeuN) neurons 3 months after transplantation. Cells expressing these markers were heterogeneously distributed within the transplant core, as some areas only expressed DCX, whereas others solely expressed NeuN. A minority of grafted cells co-expressed DCX and NeuN, suggesting that they were still in an intermediate stage of maturation. These findings demonstrate progressive maturation of the graft over time.

To assess the proliferative status of the graft, we stained for Ki67, a proliferation marker, and STEM101, a human-specific nuclear marker. While at 1 month post-transplantation numerous Ki67-positive cells were observed within the graft, we distinguished two different patterns of Ki67 expression at 3 months after grafting: sparse expression or high proliferative islets, which corresponded with the more immature neuronal areas ($Ki67^{+}DCX^{+}$). Regarding other neural cell types, fewer than 1% of the grafted cells expressed markers for astrocytes, and about 1% expressed the OL lineage marker OLIG2.

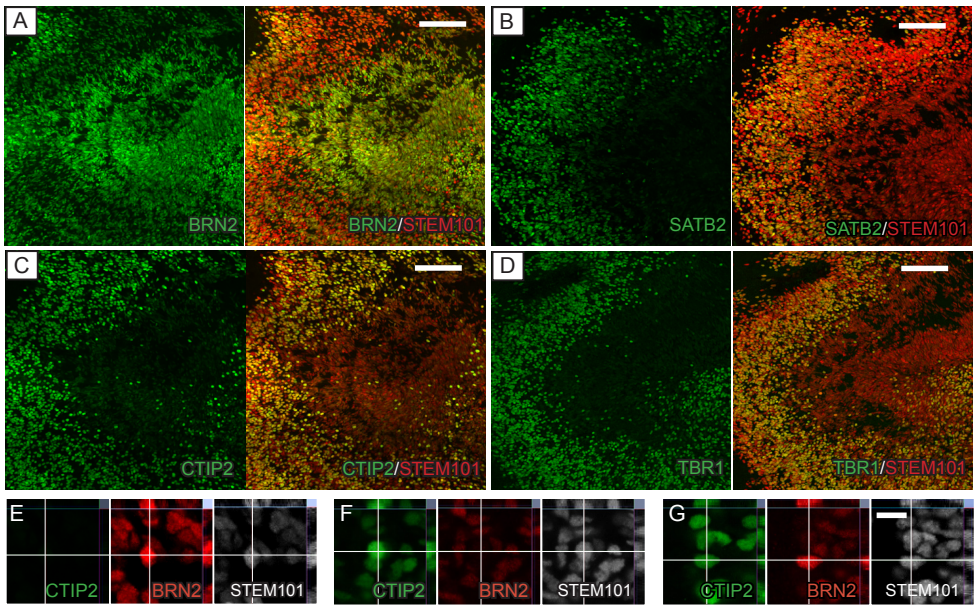

Figure 12. Grafted hES-iNs differentiate into layer-specific cortical neurons 3 months after tranplantation into the rat stroke-injured cortex

A-G, Representative confocal images showing co-expression of the human nuclear marker STEM101 with the upper cortical layer markers BRN2 (**A,E**) and SATB2 (**B**); and deep cortical layer markers CTIP2 (**C,F**) and TBR1 (**D**) in the core of the transplantation. **G**, High magnification conofcal image showing grafted hES-iNS (STEM101) co-expressing CTIP2 (deep) and BRN2 (upper). Scale bar A-D, low magnification 500 µm. Scale bar in E-G high magnification, 10 µm.

To further characterize the transplant, we analyzed whether the grafted cells expressed layer-specific cortical markers that recapitulate the architecture of the cerebral cortex 3 months after grafting. Within the transplant core, hES-iNs expressed either upper cortical layer markers (BRN2 or SABT2, Figure 12A,B,E) or deep cortical layer markers (CTIP2 and TBR1, Figure 12C,D,F). Surprisingly, cells with the same cortical marker were arranged in layers and spatially separated within the graft. In contrast to what we observed *in vitro*, only a few grafted hES-iNs co-expressed markers for upper and deep cortical layers (Figure 12G).

Synaptic integration of grafted hES-iNs into the stroke-injured brain

We next examined the projections of grafted hES-iNs 3 months post-transplantation to identify their target regions. Many graft-derived fibers reached the peri-infarct area, the ipsilateral cortices, throughout the CC and a few reached the contralateral somatosensory cortex. In addition, a few fibers were detected in the caudate-putamen, septum, and internal capsule.

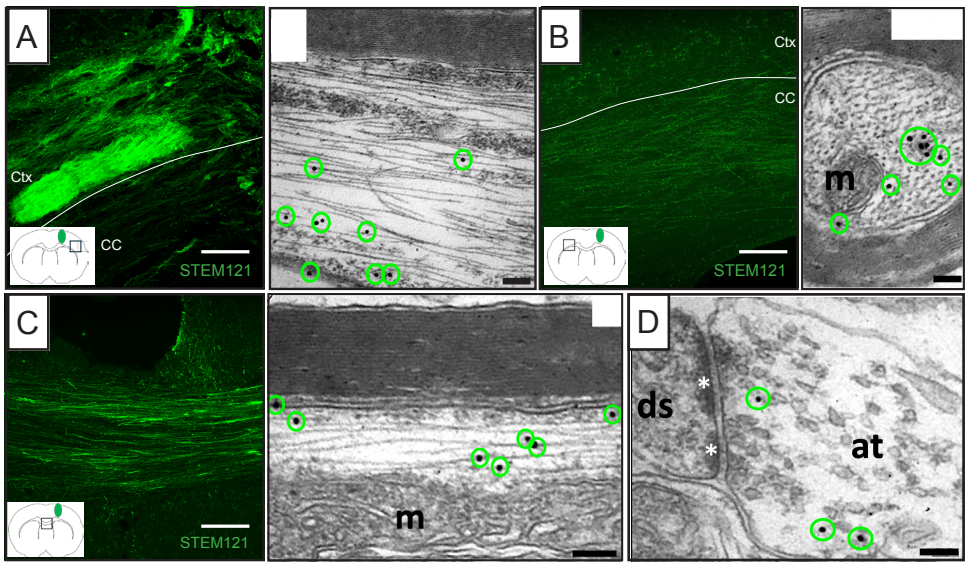

Figure 13. Grafted hES-iNs send wide spread axonal projections, establish functional synapses with host neurons and are myelinated by host oligodendrocytes (OLs) in the stroke-damaged rat brain

A-C, Presence of human fibers (STEM121) in the periinfarct area (**A**), contralateral (**B**) and corpus callosum (**C**) (left). iEM images showing hES-iNs axons myelinated by host OLs in the ipsilateral (**A**) and contralateral (**B**) somatosensory cortex; and corpus callosum (**C**) (right). **D**, Asymetric synapses in host dendritic spines (ds) connected with grafted hES-iNs (at). Ctx, cortex; CC, corpus callosum; m, mitochondria. Gold particles are marked with green circles. Scale bar A-C fluorescence, 100 µm. Scale bars A-C EM, 0.1 µm. Scale bar D, 0.1 µm.

To determine if the axons derived from the grafted hES-iNs had become myelinated and established synaptic connections with host neurons, we performed iEM. In agreement with immunostainings, human STEM121^{+} axons were observed in the CC and ipsilateral and contralateral cortices. Human-derived axons exhibited ultrastructural features similar to those of host axons and were myelinated by STEM121^{-} host OLs (Figure 13A-C). The hES-iN-derived axon terminals had established characteristic synapses with host dendritic spines in the ipsi- and contralateral cortex. Most (89.4%) STEM121^{+} synaptic contacts displayed the ultrastructural characteristics of asymmetric excitatory/glutamatergic synapses. The STEM121^{+} presynaptic terminals had docked synaptic vesicles at the presynaptic membrane, indicating the functional activity of synapses (Figure 13D). Taken together, these results demonstrate that hES-iNs can differentiate into subtypes of cortical neurons and integrate structurally after grafting into stroke-injured rat cortex.

Effect of stem cell transplantation on endogenous myelination and oligodendrogenesis (Paper II-III)

To study the overall distribution of axonal demyelination following stroke, we quantified the expression of MBP as a measure of myelin density. The ischemic insult led to reduced MBP expression in the dorsal striatum, as well as in the middle and lateral regions of the CC and its thickness and peri-infarct areas. Transplantation of cortically primed lt-NES cells resulted in increased MBP expression in the middle part of the CC and dorsal striatum compared with non-grafted rats. However, the thickness of CC remained unchanged between the stroke-affected groups.

It's been previously described that after a stroke, there is an increase of endogenous oligodendrogenesis [103]. With this purpose, we analyzed the effect of lt-NES cells and hES-iNs transplantation on the number of OLs in the corpus callosum after ischemic stroke. More OLIG2^{+} cells were found in the middle part of the corpus callosum in animals subjected to stroke compared with sham-treated animals. In transplanted rats, the increase of OLIG2^{+} cells was even more pronounced. The majority of OLIG2^{+} cells did not co-express the human nuclear marker STEM101 in both transplantations. However, in lt-NES cell-derived grafts, some human cells were also found, even if they contributed only to a fraction of the total number of OLIG2^{+} cells.

Adult human cortex as an *ex vivo* model for human stem cell transplantation

Characterization of cultured organotypic slices of adult human cortical tissue

Organotypic cultures of hACtx offer the possibility to test and validate drugs or different treatments, as stem cell-based therapies. For details on the processing and culture of this tissue, see **Paper I**.

Healthy hACtx tissue is obtained from patients with temporal lobe epilepsy and processed. To study and characterize the starting point of the host tissue as it is important for further experiments, a few slices were fixed after 24 hours in culture. The analysis of different neural cell populations, such as neurons (Figure 14A), OLs, and astrocytes showed optimal preservation of the tissue.

Next step, we wanted to assess how the culture conditions affect the neuronal viability in human tissue. Organotypic slices were cultured for 2 weeks, after which staining for neuronal markers and functionality assessments were performed. The expression of NeuN and MAP2 was still present in the tissue (Figure 14B) and whole-cell patch clamp recordings showed that overall, the cells were slightly less active than in fresh tissue. The majority of the cells were still able to fire at least one, if not multiple, Aps (Figure 14D,E), and fast inward sodium and slow outward potassium currents were present upon step current injections in voltage-clamp mode. Taken together, these recordings indicated that the neurons in organotypic cultures were relatively healthy and exhibited typical neurophysiological intrinsic properties.

Nonetheless, after 4 weeks in culture, the expression of MAP2 decreased by more than 90% and NeuN expression was visually unaffected (Figure 14C). Electrophysiological analysis showed that a couple of neurons were able to fire a single AP, while the majority were unable to generate any (Figure 14F,G). Inability to fire AP corresponded with small inward sodium currents, indicating that sodium conductance was compromised. However, passive membrane properties, such as membrane resistance and resting membrane potential, were still comparable with the values reported in fresh adult human cortical tissue [160], indicating that the neurons are still alive and relatively viable, although not very active. Taken together, our data indicate that the architecture of the adult human cortical tissue is largely preserved, but the functionality of the neuronal network is decreased after 4 weeks in culture.

Transplantation of stem cell-derived progenitors

After characterizing the host tissue, transplantation of the lt-NES cell-derived progenitors was performed. The GFP-lt-NES cells were differentiated for 7 days and grafted into 1-week cultured hACtx tissue. The results were compared with

those of previous transplantations in tissue that was poorly preserved due to a longer time window between resection and plating. In an optimal system, 4 weeks after *ex vivo* transplantation, grafted GFP^{+}-lt-NES cells exhibited extended neurites and extensive and complex arborizations throughout the whole organotypic culture. The poorly preserved tissue did not allow for successful transplantation due to the poor host connectivity. Barely any grafted cells survived; moreover, debris and nonspecific labeling of antibodies on dead cells were broadly observed throughout the human slice. In the case of successful transplantation, the cells became not only morphologically but also functionally active mature neurons with repetitive and often spontaneous APs, fast inward sodium and slow outward potassium currents, and a certain level of synaptic activity, indicating functional integration of the graft with the host tissue 4 weeks post-grafting.

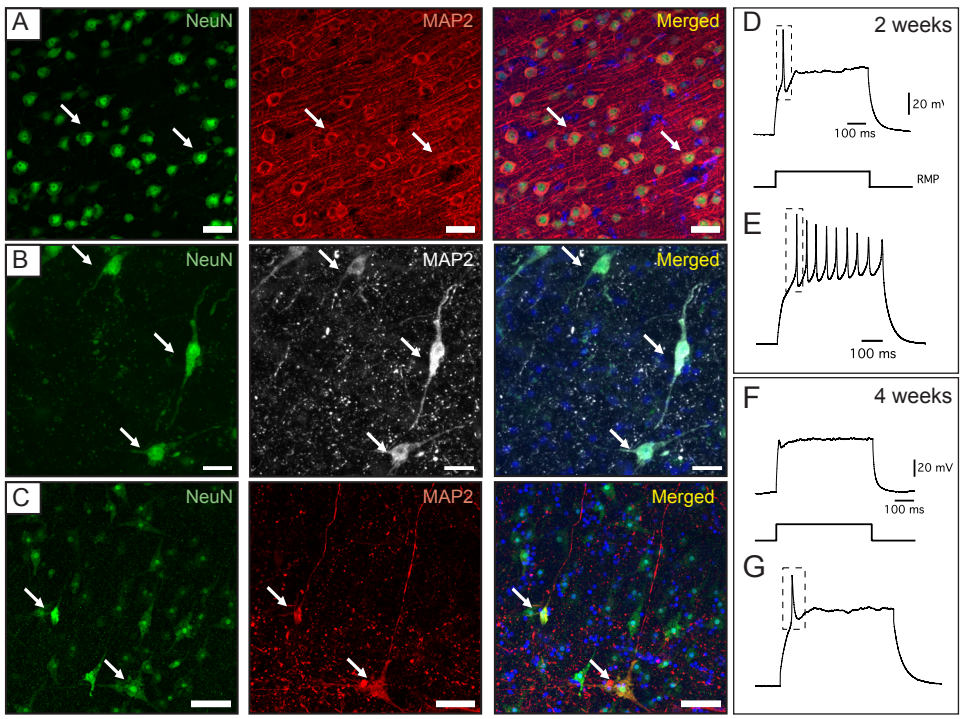

Figure 14. Characterization and electrophysiological properties of human adult cortical (hACtx) neurons after 2 and 4 weeks in organotypic culture

A-C, Representative confocal images of hACtx tissue organotypic slices showing mature neuronal marker NeuN and dendrite mzarker MAP2 after being in culture for 24 hours (**A**), 2 weeks (**B**) and 4 weeks (**C**). Nuclear staining (Hoechst) is included in merged panel. Scale bars A-C, 50 µm. Scale bar B, 20 µm. **D,E**, Whole-cell patch-clamp recordings of two examples of cortical neurons recorded after 2 weeks of culture with single (**D**) or multiple (**E**) action potentials (APs). APs were induced by a 250 pA step current injection at resting membrane potential (RMP). **F,G**, Examples of patch-clamp recordings of cortical neurons 4 weeks after being in culture. The cell is not able to fire APs at step (**F**) while another cell was able to generate a single AP at step (**G**). Arrows indicate colocalization.

Lt-NES cells-derived OLs myelinate host axons in human adult cortical tissue (Paper II)

We used this platform to determine whether grafted human lt-NES cells-derived progenitors, which differentiate to functional neurons after transplantation in hACtx tissue [79], also give rise to mature OLs that can myelinate axons in the adult human cortical environment.

We first evaluated the preservation of OLs in the cortical tissue during the organotypic culture. OLIG2, CNPase, and CC1 were expressed in acute tissue, and MBP expression displayed the characteristic aligned axonal distribution observed in the adult human cortex. After 4 weeks in culture, expression of OLIG2, CNPase, and CC1 was unaffected; however, overall MBP expression was reduced compared with acute tissue.

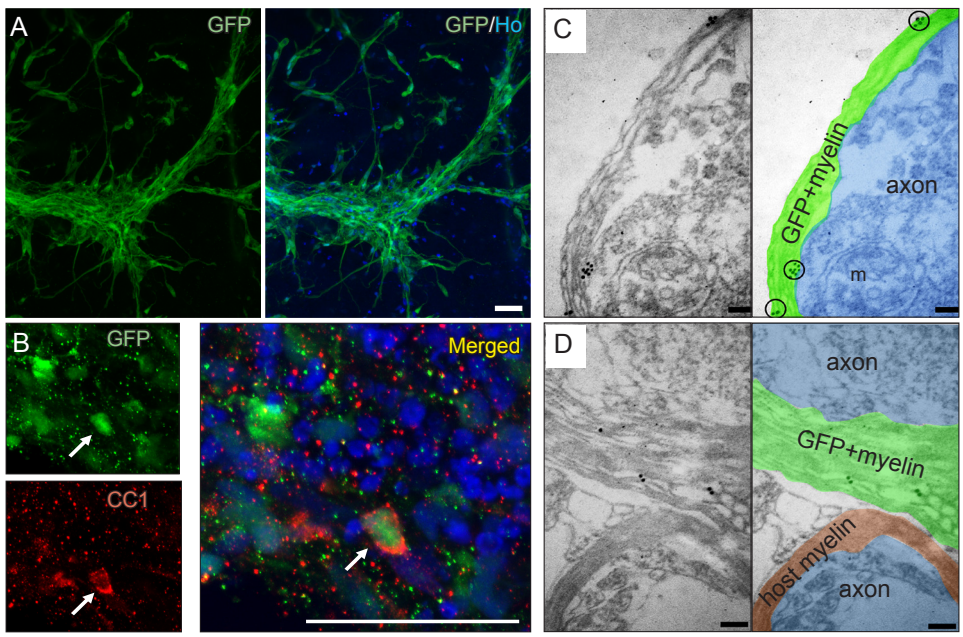

Figure 15. Human lt-NES cell-derived progenitors survive and become mature myelinating oligodendrocytes (OLs) 4 weeks after *ex vivo* transplantation into human adult organotypic (hACtx) tissue organotypic slices

A, Overview of grafted GFP+ human lt-NES cell-derived progenitors 4 weeks after transplantation. **B**, Confocal image showing GFP+ lt-NES cell-derived progenitors express mature OL marker, CC1, 4 weeks after transplantation *ex vivo*. **C,D**, Immuno-electron microscopy images showing grafted derived myelin (green) wrapping human axons (blue) in the hACtx organotypic cultures. Nuclear marker (DAPI) is included in merged image. m, mitochondrion. Arrows indicate colocalization. Scale bars A-B, 50 µm. Scale bar C-D, 0.1 µm.

To assess the capacity of cortically fated GFP$^+$ lt-NES cells to differentiate into myelinating OLs in the adult human cortical environment, they were transplanted onto organotypic slice cultures (Figure 15A). Approximately 40%–50% of the GFP$^+$ cells co-expressed OLIG2. Importantly, colocalization of GFP$^+$ cells with either CNPase or CC1 was also found (Figure 15B), indicating the presence of both immature and mature graft-derived OLs.

We finally determined if the grafted human GFP$^+$ lt-NES cell-derived OLs had formed myelin sheaths. iEM demonstrated the presence of individual or clusters of GFP$^+$ immunogold particles within the membranous sheets of myelin (signifying graft-derived myelin) surrounding non-GFP-labeled host axons (Figure 15C,D). These results provide strong evidence that lt-NES cell-derived OLs can myelinate human-derived axons after transplantation into adult human cortical tissue.

hiOLs myelinate host axons in adult cortical tissue (Paper IV)

To assess the capacity of the transcription factor programmed hiOLs to survive, differentiate and become myelinating in an adult human *ex vivo* system, GFP-labeled hiOLs programmed for 7 days were transplanted onto hACtx organotypic slices and characterized 4 weeks after grafting.

Characterization of grafted hiOLs and myelination capacity

We identified graft-derived GFP$^+$ cells distributed throughout the tissue, showing a complex morphology and arborization 1 month after transplantation (Figure 16A). To confirm that the transplanted cells kept their OL identity after transplantation, we checked for the expression of OLIG2 and SOX10. OLIG2 was expressed in the vast majority of the grafted cells, while SOX10 expression was only present in about 20%. Importantly, we could observe some colocalizations of GFP$^+$ cells with CC1, showing the presence of mature graft-derived OLs. To validate the immuno-histochemical results, we performed ultrastructural characterization of the grafted cells. Using iEM, we found that GFP$^+$ immunogold-labeled cells exhibited the morphology characteristic of mature myelinating OLs such as uneven distribution of chromatin, granular endoplasmic reticulum organized in stacks, and microtubules were numerous, particularly in the processes, where they were aligned longitudinally.

Importantly, GFP$^+$ immunogold-labeled processes formed myelin sheaths around host axons. We observed host axons at initial and intermediate stages of myelination by GFP$^+$ processes (Figure 16B,C), as well as axons enclosed by compact myelin sheaths (Figure 16D).

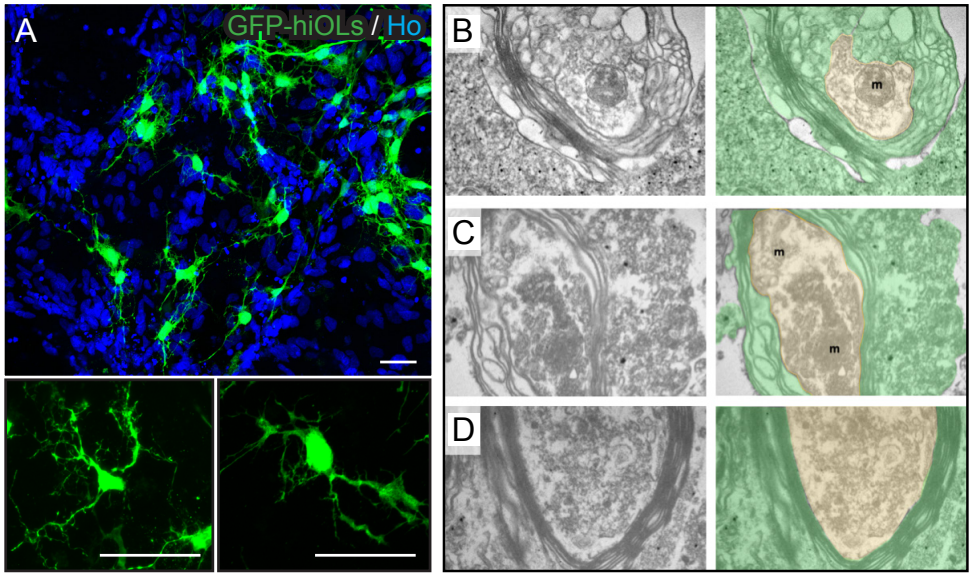

Figure 16. Human lt-NES cell-induced OLs (hiOLs) survive and differentiate into mature myelinating oligodendrocytes after transplantation into human adult cortical (hACtx) organotypic cultures

A, Overview of grafted GFP$^+$ hiOLs 4 weeks after transplantation. **B-D**, Representative immuno electron microscopy images showing host axons (yellow) at different stages of myelination by GFP$^+$ hiOLs processes (green). **B,C**, intermediate stage of myelination. **D**, Compact myelin sheaths. m, mitochondria. Scale bars, 50 µm.

Discussion and future perspectives

This thesis describes the use of cortical neurons and OLs derived from stem cells to restore the damaged brain, along with a human-based platform to bridge the gap between preclinical and clinical studies. We report four main findings. First, structurally and functionally well-preserved hACtx organotypic cultures serve as a valuable *ex vivo* platform for the assessment of stem cell-based therapies. Second, human lt-NES cells derived from hiPS cells, primed toward a cortical phenotype, differentiate into functional cortical neurons and myelinating OLs that survive and myelinate host axons after transplantation into the rodent stroke-injured cortex and hACtx organotypic cultures. Third, by forcing the expression of OLIG2 and SOX10, involved in OLs specification, human lt-NES cells can be quickly and efficiently reprogrammed into OLs within 7 days, which can myelinate human axons after grafting into hACtx tissue slices. Fourth, NGN2-programmed hES cells give rise to excitatory neurons at different stages of maturation *in vitro*, which, after intracortical grafting, into the stroke-lesioned cortex, matured into layer-specific cortical neurons, sent widespread projections to the host brain, were myelinated by host OLs and established efferent synaptic connections with host neurons.

Targeting the cerebral cortex: a stem cell-based therapy for post-stroke regeneration

Neurodegenerative disorders are characterized by the death of neurons, causing neurological impairments [5]. In a clinical study, it has been shown that lesions in the cerebral cortex are associated with long-lasting neurological deficits in a majority of stroke patients [14]. Supplying the cerebral cortex with hPS cell-derived neurons is a warranted therapy that could have a major impact on the functional recovery, and it has been hypothesized that functional recovery after stroke can improve if the transcallosal pathways are reconstructed, restoring interhemispheric communication via transplantation of a rich population of transcallosal projection neurons [161].

In this thesis, in **Paper III**, using a protocol for the overexpression of NGN2 in hES cells without mouse glia coculture, we found at day 7 that approximately 95% of the cells were of excitatory phenotype and co-expression of upper and deep cortical

layer markers was found in 95% of the cells. In contrast, previous protocols have shown that the sole overexpression of NGN2 or in combination with neural induction generated mainly upper cortical layer neurons after 3 weeks [89, 162]. These variations may be attributed to methodological differences in our protocol, such as coculture with mouse glia and the timing of their addition. Nonetheless, our findings are consistent with those from Miskinyte and collaborators, who reported that single 8-week hES-iNs, cocultured with mouse glia, expressed both upper and deep cortical layer makers, which resembled the expression profile of human fetal cortical cells [92]. Comparable results in mouse neural precursors [163] further suggest that hES-iNs are primed towards cortical neurons of diverse subtypes. Moreover, overexpression of NGN2 together with either SATB2 or CUX1, to further differentiate them towards upper or deep cortical layer neurons, did not show any differences in their morphology, molecular signature or electrical activity when compared to only NGN2 overexpression [92]. These findings suggest that additional cues are required to achieve specificity towards a defined cortical layer.

Concerning functional maturation, 65% of the NGN2-programmed hES-iNs generated multiple action potentials on day 7 with our protocol, while protocols involving neural induction take up to 2 months and the combination of overexpression of NGN2 and neural induction takes up to 14 days [86, 162]. To date, our protocol for the generation of cortical neurons is the fastest in terms of neuronal maturation and functionality. Nonetheless, the electrophysiological properties of the programmed hES-iNs at day 7 indicate they are still young neurons, making this an optimal time point for transplantation, as the early developmental stage often leads to higher graft survival and integration into host circuitries when compared to mature neurons [164].

Transplantation of hPS cell-derived cortical progenitors, generated via neural induction, has been previously reported. 2 months post-transplantation into the injured visual or somatosensory cortex, grafts were mainly immature and proliferative and only a small percentage expressed the mature neuronal marker NeuN [78, 95]. Our transplantation study, in **Paper III**, is the first one with transcription factor-programmed cortical neurons generated. In line with previous findings, hES-iNs grafts after 1 month were predominantly immature and by 3 months, 40% of the graft was composed of immature neurons and around 35% of mature neurons. It is important to consider the persistence of immature neurons in the graft, as, in principle, it may limit their contribution to neuronal circuit repair. However, evidence from other cell sources indicates that graft maturation can occur over time [78, 98].

Reconstructing the cerebral cortex cytoarchitecture is crucial for restoring the neuronal connectivity with other brain regions. In 3-month hES-iNs grafts transplanted into the stroke-injured cortex, numerous transplanted cells expressed either upper or deep cortical layer markers organized in a layer-specific manner, suggesting some level of organization within the graft. By contrast, transplantation

of hiPS cell-derived cortical progenitors into the healthy cortex showed no evident cytoarchitecture organization 3 months after transplantation. Nonetheless, by 5 months, clear segregation of the upper and deep cortical layers was observed [165]. On the contrary, hES or hiPS cell-derived cortical progenitor transplantations into the damaged cortex of adult rodents displayed no segregation of cortical neurons 6 months after grafting [78, 95, 98]. Interestingly, we have previously reported that NGN2-programmed hES-iNs co-expressed both upper and deep cortical layer markers after 2 months in culture [92], contrary to what we observed *in vivo*. Our findings suggest that the lack of specific cortical layer neurons generated *in vitro* may be attributed to the absence of extracellular signaling cues that are inherently provided by the *in vivo* environment.

Consistent with previous studies, grafting hPS cell-derived cortical neurons into the stroke-injured brain, hES-iNs expended axonal projections to both ipsi- and contralateral hemispheres [98]. Regarding integration into the host circuitry, hES-iNs grafts after 3 months form synapses with the host cortical neurons and are myelinated by host OLs. These grafts resemble those of cortical neurons generated via neural induction protocols at 6-8 months [95, 98]. These differences may be due to the method used for the generation of cortical neurons. Neural induction, through small molecules, follows embryonic development, where deep cortical layer neurons form first and could explain the slow maturation of the generated cells. On the other hand, transcription factor programming drives cells towards a mature state while bypassing certain developmental stages.

Our findings in **Paper III** demonstrates that intracortical grafts generated through transcription factor programming of hES cells undergo faster and more organized cortical maturation compared to those derived from neural induction protocols Despite the outcomes in **Paper III** show the potential of hES-iNs as a stem cell source for transplantation, further behavioral assessments and functional characterization will be essential to evaluate their usefulness for clinical translation. It should be highlighted that the transcription factor programming protocol used in this paper predominantly generates cortical neurons, whereas small-molecule-based differentiation can give rise to a broader range of cell types, such as OLs, which are essential for the regeneration of the stroke-injured brains. The absence of myelinating cells in hES-iNs grafts could potentially be solved by co-transplanting these cells with stem cell-derived OLs to provide the injured brain with both neuronal and glial populations.

Generally, neural differentiation protocols are used for the production of functional neurons, while human NPCs or stem cell-derived OPCs are used to generate myelinating OLs. The simultaneous generation of mature neurons and OLs *in vitro* is a challenge due to differences in their development timing [166]. Previous studies have shown that differentiation of hiPS cell-derived lt-NES cells into neuronal and glial linages produces mature neurons, while OL markers were expressed neither OL-like morphology nor axonal myelination was observed after 4 weeks in culture

[167]. On the contrary, Ehlrich and collaborators generated 70% OLs and 20% neurons after 28 days of overexpressing the transcription factors SOX10, OLIG2 and NKX6.2 in hPS cell-derived NPCs with the addition of a cocktail of factors to fast-track OL differentiation [113, 168]. Similarly, exposing neuroectodermal-cell-derived organoids to a cocktail of factors (T3, NT3, IGF, PDGFa and cAMP) yields 29% neurons, 20% immature OLs and 40% myelinating OLs [168]. In **Paper II** of this thesis, we use hiPS cell-derived lt-NES cells as a cell source. They share characteristics with hPS cells such as stability, long-term expansion and the ability to generate neurons and glial cells. One main difference is that lt-NES cells lack tumorigenic potential, making them a great stem cell source for the generation of neural cells. We found that cortical priming of hiPS cell-derived lt-NES cells produced a low percentage of OLs (5%). Nevertheless, after 21 days of differentiation myelin-ensheathed human axons were observed, which is earlier than the previously mentioned protocols. In principle, the number of OLs in our cultures could be increased by adding some of the factors mentioned before.

Current knowledge about myelination of axons from human stem cell-derived transplants after stroke is limited. However, there are several studies showing remyelination after OPC transplantation for other diseases such as MS or leukodystrophies [115, 169]. In the demyelinated spinal cord of rodents, grafting of hiPS cell-derived O4+ cells gives rise to 80% OLs and 2% neurons [113]. Similarly, 94% OLs were observed after transplantation of hES cell-derived OPCs in the damaged spinal cord [170]. On the other hand, other hPS cell-derived grafts only gave rise to neurons. In Steinbeck and collaborators' study, 80% of the grafted cells were neurons and no OL marker was found after the transplantation of human lt-NES cells into the rodent motor cortex [93]. Likewise, 7-week grafts of hiPS cell-derived lt-NES cells in the spinal cord-injured mice showed 75% of mature neurons and less than 1% were OLs [171].

The simultaneous generation of neurons and OLs from human stem cell grafts in the context of stroke remains poorly understood. Intracortical transplantations of hiPS cell-derived NSCs in a pig model of cortical stroke gave rise to 75% neurons and 25% OLs [172]. A previous study from our lab showed that 6 months after intracortical transplantation of human lt-NES cell-derived cortical progenitors into the rodent stroke-lesioned cortex, 40% of the grafted cells expressed SOX10 and human-derived myelin was observed [98]. We performed an extended OL characterization of these grafts showing that 6 months after transplantation, 40% of the cells became mature neurons and 20% became pre-myelinating and myelinating OLs, which are consistent with previous reported percentages. We demonstrate for the first time that grafted hPS cell-derived OLs are functional and capable of myelinating rodent host axons. Most importantly, we show the survival and capacity of myelination of these OLs following transplantation in an *ex vivo* model of hACtx tissue.

It is known that endogenous oligodendrogenesis increases after ischemic stroke, as a self-repair process [173]. We observed an increase on oligodendrogenesis in response to ischemic stroke, as reported previously [174]. In **Papers II and III**, transplantation of human lt-NES cell-derived cortical progenitors, as well as hES-iNs, respectively enhanced endogenous oligodendrogenesis and increased axonal remyelination. In line with our findings, increased oligodendrogenesis has been observed after transplantation of other human stem cell types such as hiPS cell-derived astroglia and hMSCs [102, 175]. This phenomenon is attributed to the so-called bystander effect, as only a minor portion of the new OLs were grafted. Nonetheless, it should be considered that endogenous oligodendrogenesis and remyelination efficiency decline with age [176, 177], suggesting that replacing them with grafted OLs could be more accurate for remyelination in older patients, who are most often affected by stroke, and in primary demyelinating diseases these processes are impaired.

Stem cell-based therapies that have reached the clinic for the treatment of stroke aim at stimulating plasticity and reducing inflammation [123, 178, 179]. Results from these trials have shown minor or no improvement at all. From a clinical perspective, a human stem cell source that has the capacity to repair the injured neural circuitries, remyelinate the damaged axons and increase neuronal plasticity can be a step towards an effective therapy not only for stroke patients but for other brain injuries. Still, significant work remains to select the optimal stem cell source for the generation of both neurons and glia needed to effectively reconstruct stroke lesions and accomplish functional recovery.

Optimizing OLs generation for stem cell-based remyelinating therapies

In demyelinating diseases, the myelin sheath that wraps the axons is damaged or lost. OLs, the CNS cells in charge of producing myelin, are directly affected through injury or death and their capacity for regeneration and remyelination of damaged axons is impaired. Over time, the loss of myelin and failure to repair contribute to axonal degeneration and progressive neurological deficits.

OL generation protocols from hPS cells or human fibroblasts have been previously described, but they are either lengthy or inefficient in terms of yield [68, 83, 107, 108, 113, 114]. In this thesis, in **Paper IV**, we present a consistent and highly reproducible protocol for the generation of human OLs from lt-NES cells via the overexpression of the transcription factors OLIG2 and SOX10. Using transcription factor programming, Garcia-Leon and collaborators, as well as Ehrlich and colleagues, have shown that the induction of SOX10 or a combination of SOX10, OLIG2 and NKX6.2, respectively in human NPCs gives rise to $O4^+$ OLs after 10

days (50%) or 28 days (70%) [113, 114]. Similarly, we found that OLIG2 and SOX10 overexpression in lt-NES cells programs them toward OL lineage with 80% of the cells expressing O4 after just 7 days, being the fastest and most efficient protocol to date.

Regarding maturation, we found expression of mature OLs markers, such as MBP, PLP and CC1, after 10 days of programming. When co-cultured with both hPS cell-derived excitatory or inhibitory neurons, OLs processes were in close proximity to neuronal axons after 7 days, suggesting their myelination. Our findings show earlier maturation when compared with previous protocols that take 35 days to observe mature myelinating OLs [113, 114]. To improve the efficiency of generation of OLs, previous studies have relied on the addition of factors to enhance glial proliferation and differentiation (PDGFR, SAG, T3, AA, NT3, IGF-1) [113, 114]. Interestingly, with our protocol, the presence of a cocktail of maturation factors did not affect the efficiency of OL production.

Intracerebral transplantation of OLs in preclinical models has shown great promise to repair the demyelinated brain and improve functional impairments [180, 181]. However, translating results from animal models to humans presents significant challenges. To strengthen clinical translation, the use of human adult tissue as a platform for allotransplantation can give insight into the mechanism of action of grafted OLs in a human environment. In **Paper IV**, we demonstrate that OLs generated from hPS cell-derived lt-NES cells survive, differentiate and myelinate human host axons after grafting into human adult organotypic slices.

To date, only two clinical trials are ongoing to explore the therapeutic use of intracerebral transplantation of human stem cell-derived cells in MS patients [139, 140]. Still, further investigation on the remyelination process of human axons by transplanted stem cell-derived OLs is needed. The findings presented in this thesis, **Paper IV**, hold significant implications for advancing stem cell-based therapies for demyelinating diseases towards clinical application.

Bridging the gap: advancing toward clinical translation

Most of the knowledge about stem cell transplantation in neurodegenerative disorders relies on the use of animal models. Even though these systems are highly valuable for assessing the effect of transplanted cells in the damaged host circuitry, most therapies fail when translated to the clinic due to clear differences between rodents and humans [141, 182]. In this thesis, in **Paper I**, we assessed hACtx tissue organotypic slices as an *ex vivo* platform.

The use of organotypic cultures of hACtx tissue has become a useful tool to study the functional and morphological integration of grafted cells into adult human

neuronal networks. This model represents a cellular network that preserved 3D architecture, synaptic connectivity and microenvironment. Nonetheless, this system has some limitations that must be taken into account. When cutting, axons are dissected, inducing neuronal damage that will lead to microglia activation. Even if the tissue is considered healthy when resected from the brain, the neural cell behaviour in culture could be different due to the manipulation and preparation of the tissue slices [183, 184]. Although we observed microglia activation after 2 weeks in culture due to the change of environment, neurons were still viable and functional, keeping their electrophysiological properties. Still, culture conditions can be optimized to ameliorate injury response after cutting [147].

The present model holds certain advantages when compared with other 3D culture systems such as organoids. This tissue comes from a fully developed human brain, which means that the cellular composition and matrix are the same we find in the adult human brain [148, 150, 185]. While organoids are similar to fetal tissues, which is optimal for modelling developmental disorders, for example[186]. To study the potential of stem cell-based therapies for neurodegenerative diseases, which affect the adult population, organotypic cultures of human adult tissue is the best system to validate them.

Concluding remarks

In this thesis, we show the production of myelinating OLs by two different methods from hiPS cell-derived lt-NES cells: small molecule differentiation, generating both cortical neurons and OLs; and transcription factor programming. Regardless of the generation protocol we have produced *bona fide* functional OLs based on the following findings: *in vitro,* the generated cells express OL markers such as O4, CNPase, CC1, MBP and PLP as shown by flow cytometry and immunocytochemistry (**Papers II, IV**) and EM showed the presence of mature OLs and myelin surrounding lt-NES cell-derived axons after 21 days *in vitro (***Paper II***)*. *In vivo*, 6 months after transplantation into the stroke-injured rat cortex, 20% of the grafted cells became OLs and graft-derived myelin was observed in areas of demyelination (**Paper II**) and *ex vivo*, 1 month after transplantation into hACtx tissue organotypic slices, grafted cells presented ultrastructural characteristics of mature OLs as well as expression of mature OL markers (**Paper II, IV**). Most importantly, graft-derived myelin sheets were found surrounding human host axons in this human *ex vivo* system by iEM (**Papers II, IV**). One remaining challenge is to study the capacity of migration of the generated OLs towards demyelinated lesions, as it is important when moving towards clinical translation.

In **Paper III**, we introduced a new hPS cell-derived neural cell source to be considered for stem cell-based therapies of the injured cortex. hES-iNs structurally integrate into host neuronal circuitries after transplantation into the stroke-injured rat brain, nonetheless, functional integration of this source is yet to be investigated.

With the aim of bridging the gap between preclinical research and clinical application, in this thesis, we present a human adult *ex vivo* platform for validating stem cell-based therapies and demonstrate its utility to further investigate graft-host interactions (**Paper I**). This approach represents a promising strategy to advance the clinical translation of cell replacement therapies aimed at promoting functional recovery in the injured brain.

Altogether, the findings presented in this thesis represent a step towards the clinical translation of transplantation of neural cells to promote functional recovery in neurodegenerative diseases.

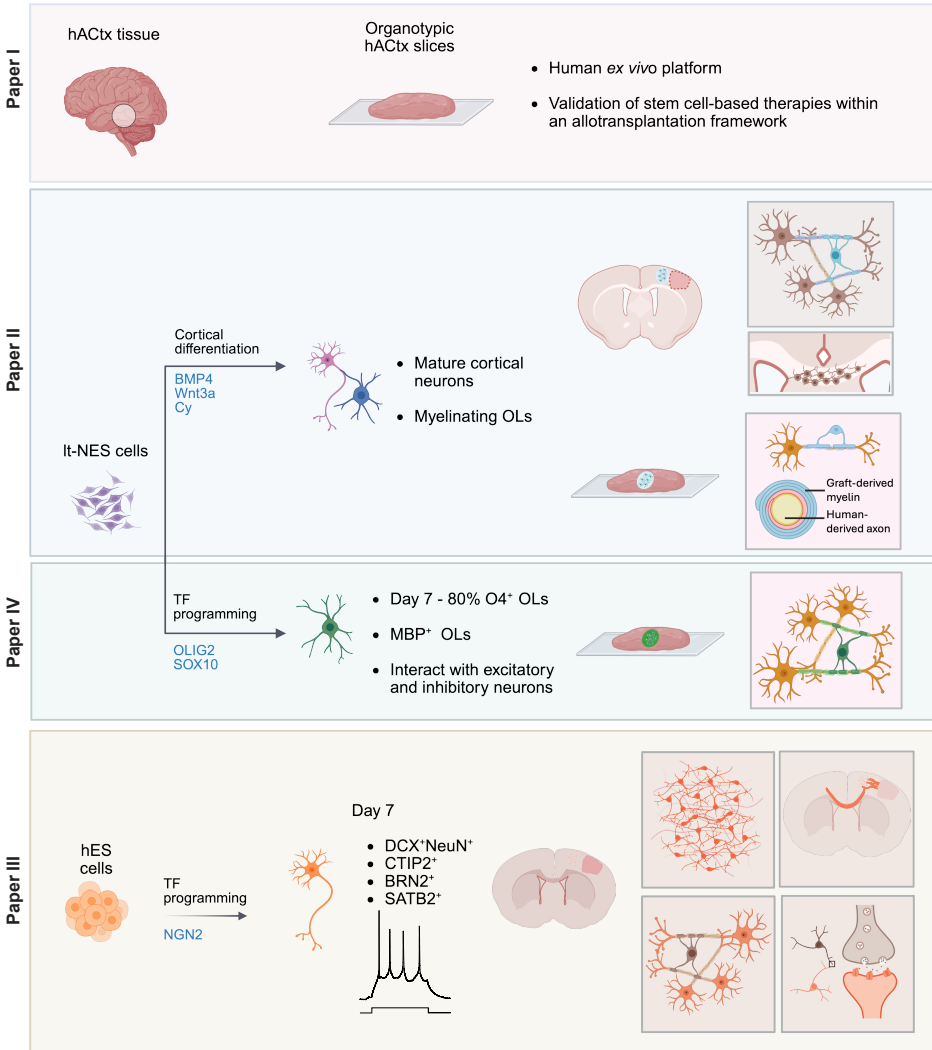

Figure 17. Schematic of studies included in this thesis

Each row represents the main highlights of each paper. We used different starting material: human adult cortical (hACtx) tissue (**Paper I**), long-termn neuroepithelial stem (lt-NES) cells (**Paper II, IV**) and human embryonic stem (ES) cells (**Paper III**). hACtx tissue was sectioned to obatined organotypic slices which serve as a human *ex vivo* platform to test stem cell-based therapies in a human-to-human setting before translation to the clinic. lt-NES cells, cortically differenatiated, produced mature cortical neurons and oligodendrocytes (OLs) that upon transplantation onto a stroke animal model and hACtx organotypic slices, matured and myelinated both, rat and human host axons. In the stroke animal model, transplantation increased the endogenous oligodendrogenesis. Transcription factor (TF) programming of lt-NES cells (OLIG2 and SOX10) produced a pure population of OLs within 7 days that myelinated human axons after transplantation onto hACtx organotypic slices. Induced neurons were generated from human embryonic stem (hES) cells (hES-iNs) that following transplantation into a stroke animal model survived, differentiated into cortical neurons, extended widespread projections, became myelinated by host oligodendrocytes, and established synapses with host neurons. Figure made with BioRender.

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– Stephen Schwartz

Now, it is time to try defying gravity.

Os quiero

References

1. Wilson, D.M., 3rd, et al., *Hallmarks of neurodegenerative diseases.* Cell, 2023. **186**(4): p. 693-714.
2. Allan, S.M. and N.J. Rothwell, *Cytokines and acute neurodegeneration.* Nat Rev Neurosci, 2001. **2**(10): p. 734-44.
3. Lamptey, R.N.L., et al., *A Review of the Common Neurodegenerative Disorders: Current Therapeutic Approaches and the Potential Role of Nanotherapeutics.* Int J Mol Sci, 2022. **23**(3).
4. Reddy, D.S. and H.N. Abeygunaratne, *Experimental and Clinical Biomarkers for Progressive Evaluation of Neuropathology and Therapeutic Interventions for Acute and Chronic Neurological Disorders.* Int J Mol Sci, 2022. **23**(19).
5. Verkhratskiĭ, A.N., A. Butt, and ScienceDirect, *Neuroglia : function and pathology*. 2023, London: Academic Press.
6. Aho, K., et al., *Cerebrovascular disease in the community: results of a WHO collaborative study.* Bull World Health Organ, 1980. **58**(1): p. 113-30.
7. Feigin, V.L., et al., *World Stroke Organization: Global Stroke Fact Sheet 2025.* Int J Stroke, 2025. **20**(2): p. 132-144.
8. Collaborators, G.B.D.S., *Global, regional, and national burden of stroke and its risk factors, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019.* Lancet Neurol, 2021. **20**(10): p. 795-820.
9. Feigin, V.L., M.O. Owolabi, and G. World Stroke Organization-Lancet Neurology Commission Stroke Collaboration, *Pragmatic solutions to reduce the global burden of stroke: a World Stroke Organization-Lancet Neurology Commission.* Lancet Neurol, 2023. **22**(12): p. 1160-1206.
10. Bamford, J., et al., *Classification and natural history of clinically identifiable subtypes of cerebral infarction.* Lancet, 1991. **337**(8756): p. 1521-6.
11. Pu, L., et al., *Projected Global Trends in Ischemic Stroke Incidence, Deaths and Disability-Adjusted Life Years From 2020 to 2030.* Stroke, 2023. **54**(5): p. 1330-1339.
12. Zhao, H.L. and Y. Huang, *Lifetime Risk of Stroke in the Global Burden of Disease Study.* N Engl J Med, 2019. **380**(14): p. 1377-1378.
13. Kessner, S.S., et al., *Somatosensory Deficits After Ischemic Stroke.* Stroke, 2019. **50**(5): p. 1116-1123.
14. Delavaran, H., et al., *Proximity of brain infarcts to regions of endogenous neurogenesis and involvement of striatum in ischaemic stroke.* Eur J Neurol, 2013. **20**(3): p. 473-479.
15. Wicks, E.E., et al., *The Translational Potential of Microglia and Monocyte-Derived Macrophages in Ischemic Stroke.* Front Immunol, 2022. **13**: p. 897022.

16. Fang, J., Z. Wang, and C.Y. Miao, *Angiogenesis after ischemic stroke.* Acta Pharmacol Sin, 2023. **44**(7): p. 1305-1321.
17. Pekna, M., M. Pekny, and M. Nilsson, *Modulation of neural plasticity as a basis for stroke rehabilitation.* Stroke, 2012. **43**(10): p. 2819-28.
18. Carmichael, S.T., *The 3 Rs of Stroke Biology: Radial, Relayed, and Regenerative.* Neurotherapeutics, 2016. **13**(2): p. 348-59.
19. Ramos-Cabrer, P., et al., *Targeting the ischemic penumbra.* Stroke, 2011. **42**(1 Suppl): p. S7-11.
20. Cao, Z., et al., *Inflammatory Responses in the Secondary Thalamic Injury After Cortical Ischemic Stroke.* Front Neurol, 2020. **11**: p. 236.
21. Brunelli, S., et al., *Secondary neurodegeneration following Stroke: what can blood biomarkers tell us?* Front Neurol, 2023. **14**: p. 1198216.
22. Duncan, I.D., et al., *The adult oligodendrocyte can participate in remyelination.* Proc Natl Acad Sci U S A, 2018. **115**(50): p. E11807-E11816.
23. Franklin, R.J.M., J. Frisen, and D.A. Lyons, *Revisiting remyelination: Towards a consensus on the regeneration of CNS myelin.* Semin Cell Dev Biol, 2021. **116**: p. 3-9.
24. Marin, M.A. and S.T. Carmichael, *Mechanisms of demyelination and remyelination in the young and aged brain following white matter stroke.* Neurobiol Dis, 2019. **126**: p. 5-12.
25. Cheng, Y.J., et al., *Prolonged myelin deficits contribute to neuron loss and functional impairments after ischaemic stroke.* Brain, 2024. **147**(4): p. 1294-1311.
26. Groan, M., et al., *Time-Based Decision Making for Reperfusion in Acute Ischemic Stroke.* Front Neurol, 2021. **12**: p. 728012.
27. Saver, J.L., et al., *Time to treatment with intravenous tissue plasminogen activator and outcome from acute ischemic stroke.* JAMA, 2013. **309**(23): p. 2480-8.
28. Moskowitz, M.A., E.H. Lo, and C. Iadecola, *The science of stroke: mechanisms in search of treatments.* Neuron, 2010. **67**(2): p. 181-98.
29. Kokaia, Z., D. Tornero, and O. Lindvall, *Transplantation of reprogrammed neurons for improved recovery after stroke.* Prog Brain Res, 2017. **231**: p. 245-263.
30. Veerbeek, J.M., et al., *What is the evidence for physical therapy poststroke? A systematic review and meta-analysis.* PLoS One, 2014. **9**(2): p. e87987.
31. Saunders, D.E., A.G. Clifton, and M.M. Brown, *Measurement of infarct size using MRI predicts prognosis in middle cerebral artery infarction.* Stroke, 1995. **26**(12): p. 2272-6.
32. El-Koussy, M., et al., *Imaging of acute ischemic stroke.* Eur Neurol, 2014. **72**(5-6): p. 309-16.
33. Gao, R., et al., *Myelin debris phagocytosis in demyelinating disease.* Glia, 2024. **72**(11): p. 1934-1954.
34. Osso, L.A. and E.G. Hughes, *Dynamics of mature myelin.* Nat Neurosci, 2024. **27**(8): p. 1449-1461.
35. Aber, E.R., et al., *Oligodendroglial macroautophagy is essential for myelin sheath turnover to prevent neurodegeneration and death.* Cell Rep, 2022. **41**(3): p. 111480.
36. Coutinho Costa, V.G., et al., *Central nervous system demyelinating diseases: glial cells at the hub of pathology.* Front Immunol, 2023. **14**: p. 1135540.

37. Ryan, M., M. Ibrahim, and H.A. Parmar, *Secondary demyelination disorders and destruction of white matter.* Radiol Clin North Am, 2014. **52**(2): p. 337-54.
38. Barkhof, F. and K.K. Koeller, *Demyelinating Diseases of the CNS (Brain and Spine)*, in *Diseases of the Brain, Head and Neck, Spine 2020-2023: Diagnostic Imaging*, J. Hodler, R.A. Kubik-Huch, and G.K. von Schulthess, Editors. 2020: Cham (CH). p. 165-76.
39. Bierhansl, L., et al., *Thinking outside the box: non-canonical targets in multiple sclerosis.* Nat Rev Drug Discov, 2022. **21**(8): p. 578-600.
40. Walton, C., et al., *Rising prevalence of multiple sclerosis worldwide: Insights from the Atlas of MS, third edition.* Mult Scler, 2020. **26**(14): p. 1816-1821.
41. Jakimovski, D., et al., *Multiple sclerosis.* Lancet, 2024. **403**(10422): p. 183-202.
42. Filippi, M., et al., *Multiple sclerosis.* Nat Rev Dis Primers, 2018. **4**(1): p. 43.
43. Strijbis, E.M.M., et al., *Cortical Remyelination Is Heterogeneous in Multiple Sclerosis.* J Neuropathol Exp Neurol, 2017. **76**(5): p. 390-401.
44. Patrikios, P., et al., *Remyelination is extensive in a subset of multiple sclerosis patients.* Brain, 2006. **129**(Pt 12): p. 3165-72.
45. Yeung, M.S.Y., et al., *Publisher Correction: Dynamics of oligodendrocyte generation in multiple sclerosis.* Nature, 2019. **566**(7744): p. E9.
46. Goldschmidt, T., et al., *Remyelination capacity of the MS brain decreases with disease chronicity.* Neurology, 2009. **72**(22): p. 1914-21.
47. Boyd, A., H. Zhang, and A. Williams, *Insufficient OPC migration into demyelinated lesions is a cause of poor remyelination in MS and mouse models.* Acta Neuropathol, 2013. **125**(6): p. 841-59.
48. Neely, S.A., et al., *New oligodendrocytes exhibit more abundant and accurate myelin regeneration than those that survive demyelination.* Nat Neurosci, 2022. **25**(4): p. 415-420.
49. Bacmeister, C.M., et al., *Motor learning promotes remyelination via new and surviving oligodendrocytes.* Nat Neurosci, 2020. **23**(7): p. 819-831.
50. Mezydlo, A., et al., *Remyelination by surviving oligodendrocytes is inefficient in the inflamed mammalian cortex.* Neuron, 2023. **111**(11): p. 1748-1759 e8.
51. Filippini, G., et al., *Immunomodulators and immunosuppressants for multiple sclerosis: a network meta-analysis.* Cochrane Database Syst Rev, 2013. **2013**(6): p. CD008933.
52. Kappos, L., et al., *Siponimod versus placebo in secondary progressive multiple sclerosis (EXPAND): a double-blind, randomised, phase 3 study.* Lancet, 2018. **391**(10127): p. 1263-1273.
53. Montalban, X., et al., *Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis.* N Engl J Med, 2017. **376**(3): p. 209-220.
54. Green, A.J., et al., *Clemastine fumarate as a remyelinating therapy for multiple sclerosis (ReBUILD): a randomised, controlled, double-blind, crossover trial.* Lancet, 2017. **390**(10111): p. 2481-2489.
55. Lindvall, O., et al., *Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease. A detailed account of methodology and a 6-month follow-up.* Arch Neurol, 1989. **46**(6): p. 615-31.

56. Lindvall, O., et al., *Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease.* Science, 1990. **247**(4942): p. 574-7.
57. Lindvall, O., *Treatment of Parkinson's disease using cell transplantation.* Philos Trans R Soc Lond B Biol Sci, 2015. **370**(1680): p. 20140370.
58. Barker, R.A., et al., *Fetal dopaminergic transplantation trials and the future of neural grafting in Parkinson's disease.* Lancet Neurol, 2013. **12**(1): p. 84-91.
59. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
60. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-72.
61. Wernig, M., et al., *In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state.* Nature, 2007. **448**(7151): p. 318-24.
62. Okita, K., T. Ichisaka, and S. Yamanaka, *Generation of germline-competent induced pluripotent stem cells.* Nature, 2007. **448**(7151): p. 313-7.
63. Palma-Tortosa, S., et al., *Neuronal Replacement in Stem Cell Therapy for Stroke: Filling the Gap.* Front Cell Dev Biol, 2021. **9**: p. 662636.
64. Brennand, K.J., et al., *Modelling schizophrenia using human induced pluripotent stem cells.* Nature, 2011. **473**(7346): p. 221-5.
65. Doi, D., et al., *Isolation of human induced pluripotent stem cell-derived dopaminergic progenitors by cell sorting for successful transplantation.* Stem Cell Reports, 2014. **2**(3): p. 337-50.
66. Kim, T.W., et al., *Biphasic Activation of WNT Signaling Facilitates the Derivation of Midbrain Dopamine Neurons from hESCs for Translational Use.* Cell Stem Cell, 2021. **28**(2): p. 343-355 e5.
67. Autar, K., et al., *A functional hiPSC-cortical neuron differentiation and maturation model and its application to neurological disorders.* Stem Cell Reports, 2022. **17**(1): p. 96-109.
68. Wang, S., et al., *Human iPSC-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination.* Cell Stem Cell, 2013. **12**(2): p. 252-64.
69. Czepiel, M., et al., *Differentiation of induced pluripotent stem cells into functional oligodendrocytes.* Glia, 2011. **59**(6): p. 882-92.
70. Canals, I., et al., *Rapid and efficient induction of functional astrocytes from human pluripotent stem cells.* Nat Methods, 2018. **15**(9): p. 693-696.
71. Nicholas, C.R., et al., *Functional maturation of hPSC-derived forebrain interneurons requires an extended timeline and mimics human neural development.* Cell Stem Cell, 2013. **12**(5): p. 573-86.
72. Kirkeby, A., et al., *Predictive Markers Guide Differentiation to Improve Graft Outcome in Clinical Translation of hESC-Based Therapy for Parkinson's Disease.* Cell Stem Cell, 2017. **20**(1): p. 135-148.
73. Studer, L., *Strategies for bringing stem cell-derived dopamine neurons to the clinic-The NYSTEM trial.* Prog Brain Res, 2017. **230**: p. 191-212.
74. Takahashi, J., *Strategies for bringing stem cell-derived dopamine neurons to the clinic: The Kyoto trial.* Prog Brain Res, 2017. **230**: p. 213-226.

75. Koch, P., et al., *A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration.* Proc Natl Acad Sci U S A, 2009. **106**(9): p. 3225-30.
76. Falk, A., et al., *Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons.* PLoS One, 2012. **7**(1): p. e29597.
77. Voulgaris, D., P. Nikolakopoulou, and A. Herland, *Generation of Human iPSC-Derived Astrocytes with a mature star-shaped phenotype for CNS modeling.* Stem Cell Rev Rep, 2022. **18**(7): p. 2494-2512.
78. Tornero, D., et al., *Human induced pluripotent stem cell-derived cortical neurons integrate in stroke-injured cortex and improve functional recovery.* Brain, 2013. **136**(Pt 12): p. 3561-77.
79. Gronning Hansen, M., et al., *Grafted human pluripotent stem cell-derived cortical neurons integrate into adult human cortical neural circuitry.* Stem Cells Transl Med, 2020. **9**(11): p. 1365-1377.
80. Oki, K., et al., *Human-induced pluripotent stem cells form functional neurons and improve recovery after grafting in stroke-damaged brain.* Stem Cells, 2012. **30**(6): p. 1120-33.
81. Vierbuchen, T., et al., *Direct conversion of fibroblasts to functional neurons by defined factors.* Nature, 2010. **463**(7284): p. 1035-41.
82. Pang, Z.P., et al., *Induction of human neuronal cells by defined transcription factors.* Nature, 2011. **476**(7359): p. 220-3.
83. Chanoumidou, K., et al., *One-step Reprogramming of Human Fibroblasts into Oligodendrocyte-like Cells by SOX10, OLIG2, and NKX6.2.* Stem Cell Reports, 2021. **16**(4): p. 771-783.
84. Quist, E., et al., *Transcription factor-based direct conversion of human fibroblasts to functional astrocytes.* Stem Cell Reports, 2022. **17**(7): p. 1620-1635.
85. Cao, S.Y., et al., *Enhanced derivation of human pluripotent stem cell-derived cortical glutamatergic neurons by a small molecule.* Sci Rep, 2017. **7**(1): p. 3282.
86. Shi, Y., et al., *Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses.* Nat Neurosci, 2012. **15**(3): p. 477-86, S1.
87. Espuny-Camacho, I., et al., *Pyramidal neurons derived from human pluripotent stem cells integrate efficiently into mouse brain circuits in vivo.* Neuron, 2013. **77**(3): p. 440-56.
88. van den Ameele, J., et al., *Thinking out of the dish: what to learn about cortical development using pluripotent stem cells.* Trends Neurosci, 2014. **37**(6): p. 334-42.
89. Zhang, Y., et al., *Rapid single-step induction of functional neurons from human pluripotent stem cells.* Neuron, 2013. **78**(5): p. 785-98.
90. Wang, C., et al., *Scalable Production of iPSC-Derived Human Neurons to Identify Tau-Lowering Compounds by High-Content Screening.* Stem Cell Reports, 2017. **9**(4): p. 1221-1233.
91. Hulme, A.J., et al., *Making neurons, made easy: The use of Neurogenin-2 in neuronal differentiation.* Stem Cell Reports, 2022. **17**(1): p. 14-34.

92. Miskinyte, G., et al., *Transcription factor programming of human ES cells generates functional neurons expressing both upper and deep layer cortical markers.* PLoS One, 2018. **13**(10): p. e0204688.
93. Steinbeck, J.A., et al., *Human embryonic stem cell-derived neurons establish region-specific, long-range projections in the adult brain.* Cell Mol Life Sci, 2012. **69**(3): p. 461-70.
94. Denham, M., et al., *Neurons derived from human embryonic stem cells extend long-distance axonal projections through growth along host white matter tracts after intracerebral transplantation.* Front Cell Neurosci, 2012. **6**: p. 11.
95. Espuny-Camacho, I., et al., *Human Pluripotent Stem-Cell-Derived Cortical Neurons Integrate Functionally into the Lesioned Adult Murine Visual Cortex in an Area-Specific Way.* Cell Rep, 2018. **23**(9): p. 2732-2743.
96. Somaa, F.A., et al., *Peptide-Based Scaffolds Support Human Cortical Progenitor Graft Integration to Reduce Atrophy and Promote Functional Repair in a Model of Stroke.* Cell Rep, 2017. **20**(8): p. 1964-1977.
97. Tornero, D., et al., *Synaptic inputs from stroke-injured brain to grafted human stem cell-derived neurons activated by sensory stimuli.* Brain, 2017. **140**(3): p. 692-706.
98. Palma-Tortosa, S., et al., *Activity in grafted human iPS cell-derived cortical neurons integrated in stroke-injured rat brain regulates motor behavior.* Proc Natl Acad Sci U S A, 2020. **117**(16): p. 9094-9100.
99. Pasca, A.M., et al., *Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture.* Nat Methods, 2015. **12**(7): p. 671-8.
100. Jgamadze, D., et al., *Structural and functional integration of human forebrain organoids with the injured adult rat visual system.* Cell Stem Cell, 2023. **30**(2): p. 137-152 e7.
101. Cao, S.Y., et al., *Cerebral organoids transplantation repairs infarcted cortex and restores impaired function after stroke.* NPJ Regen Med, 2023. **8**(1): p. 27.
102. Jiang, P., et al., *Human iPSC-Derived Immature Astroglia Promote Oligodendrogenesis by Increasing TIMP-1 Secretion.* Cell Rep, 2016. **15**(6): p. 1303-15.
103. Llorente, I.L., et al., *Patient-derived glial enriched progenitors repair functional deficits due to white matter stroke and vascular dementia in rodents.* Sci Transl Med, 2021. **13**(590).
104. Laterza, C., et al., *iPSC-derived neural precursors exert a neuroprotective role in immune-mediated demyelination via the secretion of LIF.* Nat Commun, 2013. **4**: p. 2597.
105. Chen, L., et al., *Human neural precursor cells promote neurologic recovery in a viral model of multiple sclerosis.* Stem Cell Reports, 2014. **2**(6): p. 825-37.
106. McIntyre, L.L., et al., *Regulatory T cells promote remyelination in the murine experimental autoimmune encephalomyelitis model of multiple sclerosis following human neural stem cell transplant.* Neurobiol Dis, 2020. **140**: p. 104868.
107. Douvaras, P., et al., *Efficient generation of myelinating oligodendrocytes from primary progressive multiple sclerosis patients by induced pluripotent stem cells.* Stem Cell Reports, 2014. **3**(2): p. 250-9.

108. McCaughey-Chapman, A. and B. Connor, *Cell reprogramming for oligodendrocytes: A review of protocols and their applications to disease modeling and cell-based remyelination therapies.* J Neurosci Res, 2023. **101**(6): p. 1000-1028.
109. Livesey, M.R., et al., *Maturation and electrophysiological properties of human pluripotent stem cell-derived oligodendrocytes.* Stem Cells, 2016. **34**(4): p. 1040-53.
110. Kim, D.S., et al., *Rapid generation of OPC-like cells from human pluripotent stem cells for treating spinal cord injury.* Exp Mol Med, 2017. **49**(7): p. e361.
111. Piao, J., et al., *Human embryonic stem cell-derived oligodendrocyte progenitors remyelinate the brain and rescue behavioral deficits following radiation.* Cell Stem Cell, 2015. **16**(2): p. 198-210.
112. Stacpoole, S.R., et al., *High yields of oligodendrocyte lineage cells from human embryonic stem cells at physiological oxygen tensions for evaluation of translational biology.* Stem Cell Reports, 2013. **1**(5): p. 437-50.
113. Ehrlich, M., et al., *Rapid and efficient generation of oligodendrocytes from human induced pluripotent stem cells using transcription factors.* Proc Natl Acad Sci U S A, 2017. **114**(11): p. E2243-E2252.
114. Garcia-Leon, J.A., et al., *SOX10 Single Transcription Factor-Based Fast and Efficient Generation of Oligodendrocytes from Human Pluripotent Stem Cells.* Stem Cell Reports, 2018. **10**(2): p. 655-672.
115. Windrem, M.S., et al., *Human Glial Progenitor Cells Effectively Remyelinate the Demyelinated Adult Brain.* Cell Rep, 2020. **31**(7): p. 107658.
116. Thiruvalluvan, A., et al., *Survival and Functionality of Human Induced Pluripotent Stem Cell-Derived Oligodendrocytes in a Nonhuman Primate Model for Multiple Sclerosis.* Stem Cells Transl Med, 2016. **5**(11): p. 1550-1561.
117. Wagstaff, L.J., et al., *CRISPR-edited human ES-derived oligodendrocyte progenitor cells improve remyelination in rodents.* Nat Commun, 2024. **15**(1): p. 8570.
118. Clayton, B.L.L., et al., *Patient iPSC models reveal glia-intrinsic phenotypes in multiple sclerosis.* Cell Stem Cell, 2024. **31**(11): p. 1701-1713 e8.
119. Lopez-Caraballo, L., et al., *iPS-Derived Early Oligodendrocyte Progenitor Cells from SPMS Patients Reveal Deficient In Vitro Cell Migration Stimulation.* Cells, 2020. **9**(8).
120. Mozafari, S., et al., *Multiple sclerosis iPS-derived oligodendroglia conserve their properties to functionally interact with axons and glia in vivo.* Sci Adv, 2020. **6**(49).
121. Li, W., et al., *Oligodendrocyte Precursor Cells Transplantation Improves Stroke Recovery via Oligodendrogenesis, Neurite Growth and Synaptogenesis.* Aging Dis, 2021. **12**(8): p. 2096-2112.
122. Wang, L., et al., *Oligodendrocyte precursor cells transplantation protects blood-brain barrier in a mouse model of brain ischemia via Wnt/beta-catenin signaling.* Cell Death Dis, 2020. **11**(1): p. 9.
123. de Celis-Ruiz, E., et al., *Allogeneic adipose tissue-derived mesenchymal stem cells in ischaemic stroke (AMASCIS-02): a phase IIb, multicentre, double-blind, placebo-controlled clinical trial protocol.* BMJ Open, 2021. **11**(8): p. e051790.
124. Jaillard, A., et al., *Autologous Mesenchymal Stem Cells Improve Motor Recovery in Subacute Ischemic Stroke: a Randomized Clinical Trial.* Transl Stroke Res, 2020. **11**(5): p. 910-923.

125. Law, Z.K., et al., *The effects of intravenous infusion of autologous mesenchymal stromal cells in patients with subacute middle cerebral artery infarct: a phase 2 randomized controlled trial on safety, tolerability and efficacy.* Cytotherapy, 2021. **23**(9): p. 833-840.
126. Levy, M.L., et al., *Phase I/II Study of Safety and Preliminary Efficacy of Intravenous Allogeneic Mesenchymal Stem Cells in Chronic Stroke.* Stroke, 2019. **50**(10): p. 2835-2841.
127. Chiu, T.L., et al., *Intracerebral transplantation of autologous adipose-derived stem cells for chronic ischemic stroke: A phase I study.* J Tissue Eng Regen Med, 2022. **16**(1): p. 3-13.
128. Steinberg, G.K., et al., *Clinical Outcomes of Transplanted Modified Bone Marrow-Derived Mesenchymal Stem Cells in Stroke: A Phase 1/2a Study.* Stroke, 2016. **47**(7): p. 1817-24.
129. Zhang, G., et al., *Stable Intracerebral Transplantation of Neural Stem Cells for the Treatment of Paralysis Due to Ischemic Stroke.* Stem Cells Transl Med, 2019. **8**(10): p. 999-1007.
130. Muir, K.W., et al., *Intracerebral implantation of human neural stem cells and motor recovery after stroke: multicentre prospective single-arm study (PISCES-2).* J Neurol Neurosurg Psychiatry, 2020. **91**(4): p. 396-401.
131. Moniche, F., et al., *Safety and efficacy of intra-arterial bone marrow mononuclear cell transplantation in patients with acute ischaemic stroke in Spain (IBIS trial): a phase 2, randomised, open-label, standard-of-care controlled, multicentre trial.* Lancet Neurol, 2023. **22**(2): p. 137-146.
132. Houkin, K., et al., *Allogeneic Stem Cell Therapy for Acute Ischemic Stroke: The Phase 2/3 TREASURE Randomized Clinical Trial.* JAMA Neurol, 2024. **81**(2): p. 154-162.
133. Nicholas, R.S., et al., *Autologous Hematopoietic Stem Cell Transplantation in Active Multiple Sclerosis: A Real-world Case Series.* Neurology, 2021. **97**(9): p. e890-e901.
134. Silfverberg, T., et al., *Haematopoietic stem cell transplantation for treatment of relapsing-remitting multiple sclerosis in Sweden: an observational cohort study.* J Neurol Neurosurg Psychiatry, 2024. **95**(2): p. 125-133.
135. Freedman, M.S., et al., *The therapeutic potential of mesenchymal stem cell transplantation as a treatment for multiple sclerosis: consensus report of the International MSCT Study Group.* Mult Scler, 2010. **16**(4): p. 503-10.
136. Petrou, P., et al., *Effects of Mesenchymal Stem Cell Transplantation on Cerebrospinal Fluid Biomarkers in Progressive Multiple Sclerosis.* Stem Cells Transl Med, 2022. **11**(1): p. 55-58.
137. Petrou, P., et al., *Beneficial effects of autologous mesenchymal stem cell transplantation in active progressive multiple sclerosis.* Brain, 2020. **143**(12): p. 3574-3588.
138. Shokati, A., et al., *Cell therapy with placenta-derived mesenchymal stem cells for secondary progressive multiple sclerosis patients in a phase 1 clinical trial.* Sci Rep, 2025. **15**(1): p. 16005.
139. Leone, M.A., et al., *Phase I clinical trial of intracerebroventricular transplantation of allogeneic neural stem cells in people with progressive multiple sclerosis.* Cell Stem Cell, 2023. **30**(12): p. 1597-1609 e8.

140. Genchi, A., et al., *Neural stem cell transplantation in patients with progressive multiple sclerosis: an open-label, phase 1 study.* Nat Med, 2023. **29**(1): p. 75-85.
141. Akhtar, A., *The flaws and human harms of animal experimentation.* Camb Q Healthc Ethics, 2015. **24**(4): p. 407-19.
142. Robinson, N.B., et al., *The current state of animal models in research: A review.* Int J Surg, 2019. **72**: p. 9-13.
143. Xu, N., et al., *Functional Connectivity of the Brain Across Rodents and Humans.* Front Neurosci, 2022. **16**: p. 816331.
144. Bayes, A., et al., *Comparative study of human and mouse postsynaptic proteomes finds high compositional conservation and abundance differences for key synaptic proteins.* PLoS One, 2012. **7**(10): p. e46683.
145. Koopmans, F., et al., *Comparative Hippocampal Synaptic Proteomes of Rodents and Primates: Differences in Neuroplasticity-Related Proteins.* Front Mol Neurosci, 2018. **11**: p. 364.
146. Weber, R.Z., R. Rust, and C. Tackenberg, *How neural stem cell therapy promotes brain repair after stroke.* Stem Cell Reports, 2025. **20**(6): p. 102507.
147. Eugene, E., et al., *An organotypic brain slice preparation from adult patients with temporal lobe epilepsy.* J Neurosci Methods, 2014. **235**: p. 234-44.
148. Jones, R.S., et al., *Human brain slices for epilepsy research: Pitfalls, solutions and future challenges.* J Neurosci Methods, 2016. **260**: p. 221-32.
149. Kalmbach, B.E., et al., *Signature morpho-electric, transcriptomic, and dendritic properties of human layer 5 neocortical pyramidal neurons.* Neuron, 2021. **109**(18): p. 2914-2927 e5.
150. Schwarz, N., et al., *Long-term adult human brain slice cultures as a model system to study human CNS circuitry and disease.* Elife, 2019. **8**.
151. Gonzalez-Ramos, A., et al., *Human stem cell-derived GABAergic neurons functionally integrate into human neuronal networks.* Sci Rep, 2021. **11**(1): p. 22050.
152. Noraberg, J., et al., *Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair.* Curr Drug Targets CNS Neurol Disord, 2005. **4**(4): p. 435-52.
153. Barth, M., et al., *Microglial inclusions and neurofilament light chain release follow neuronal alpha-synuclein lesions in long-term brain slice cultures.* Mol Neurodegener, 2021. **16**(1): p. 54.
154. Schwarz, N., et al., *Human Cerebrospinal fluid promotes long-term neuronal viability and network function in human neocortical organotypic brain slice cultures.* Sci Rep, 2017. **7**(1): p. 12249.
155. Bruzelius, A., et al., *Three-dimensional co-culturing reveals human stem cell-derived somatostatin interneurons with subclass expression.* Stem Cell Reports, 2025. **20**(9): p. 102634.
156. Chen, S.T., et al., *A model of focal ischemic stroke in the rat: reproducible extensive cortical infarction.* Stroke, 1986. **17**(4): p. 738-43.
157. Renier, N., et al., *iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging.* Cell, 2014. **159**(4): p. 896-910.

158. Martinez-Curiel, R., et al., *Oligodendrocytes in human induced pluripotent stem cell-derived cortical grafts remyelinate adult rat and human cortical neurons.* Stem Cell Reports, 2023. **18**(8): p. 1643-1656.
159. Martinez-Curiel, R., et al., *Human cortical neurons rapidly generated by embryonic stem cell programming integrate into the stroke-injured rat cortex.* Stem Cells, 2025.
160. Avaliani, N., et al., *Optogenetics reveal delayed afferent synaptogenesis on grafted human-induced pluripotent stem cell-derived neural progenitors.* Stem Cells, 2014. **32**(12): p. 3088-98.
161. Chen, J.L. and G. Schlaug, *Resting state interhemispheric motor connectivity and white matter integrity correlate with motor impairment in chronic stroke.* Front Neurol, 2013. **4**: p. 178.
162. Nehme, R., et al., *Combining NGN2 Programming with Developmental Patterning Generates Human Excitatory Neurons with NMDAR-Mediated Synaptic Transmission.* Cell Rep, 2018. **23**(8): p. 2509-2523.
163. Zahr, S.K., et al., *A Translational Repression Complex in Developing Mammalian Neural Stem Cells that Regulates Neuronal Specification.* Neuron, 2018. **97**(3): p. 520-537 e6.
164. Payne, S.L., et al., *Initial cell maturity changes following transplantation in a hyaluronan-based hydrogel and impacts therapeutic success in the stroke-injured rodent brain.* Biomaterials, 2019. **192**: p. 309-322.
165. Real, R., et al., *In vivo modeling of human neuron dynamics and Down syndrome.* Science, 2018. **362**(6416).
166. Qian, X., et al., *Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells.* Neuron, 2000. **28**(1): p. 69-80.
167. Isoda, M., et al., *Robust production of human neural cells by establishing neuroepithelial-like stem cells from peripheral blood mononuclear cell-derived feeder-free iPSCs under xeno-free conditions.* Neurosci Res, 2016. **110**: p. 18-28.
168. Shaker, M.R., et al., *Rapid and Efficient Generation of Myelinating Human Oligodendrocytes in Organoids.* Front Cell Neurosci, 2021. **15**: p. 631548.
169. Kondo, Y. and I.D. Duncan, *Transplantation of oligodendrocyte progenitor cells in animal models of leukodystrophies.* Methods Mol Biol, 2009. **549**: p. 175-85.
170. Sharp, J., et al., *Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury.* Stem Cells, 2010. **28**(1): p. 152-63.
171. Fujimoto, Y., et al., *Treatment of a mouse model of spinal cord injury by transplantation of human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem cells.* Stem Cells, 2012. **30**(6): p. 1163-73.
172. Baker, E.W., et al., *Induced Pluripotent Stem Cell-Derived Neural Stem Cell Therapy Enhances Recovery in an Ischemic Stroke Pig Model.* Sci Rep, 2017. **7**(1): p. 10075.
173. Itoh, K., et al., *Mechanisms of cell-cell interaction in oligodendrogenesis and remyelination after stroke.* Brain Res, 2015. **1623**: p. 135-49.
174. Hernandez, I.H., et al., *Glial Cells as Therapeutic Approaches in Brain Ischemia-Reperfusion Injury.* Cells, 2021. **10**(7).

175. Bakhtiari, M., et al., *Evaluation of Edaravone effects on the differentiation of human adipose derived stem cells into oligodendrocyte cells in multiple sclerosis disease in rats.* Life Sci, 2021. **282**: p. 119812.
176. Segel, M., et al., *Niche stiffness underlies the ageing of central nervous system progenitor cells.* Nature, 2019. **573**(7772): p. 130-134.
177. Shen, S., et al., *Age-dependent epigenetic control of differentiation inhibitors is critical for remyelination efficiency.* Nat Neurosci, 2008. **11**(9): p. 1024-34.
178. Gong, P., et al., *Classification and Characteristics of Mesenchymal Stem Cells and Its Potential Therapeutic Mechanisms and Applications against Ischemic Stroke.* Stem Cells Int, 2021. **2021**: p. 2602871.
179. Hess, D.C., et al., *Safety and efficacy of multipotent adult progenitor cells in acute ischaemic stroke (MASTERS): a randomised, double-blind, placebo-controlled, phase 2 trial.* Lancet Neurol, 2017. **16**(5): p. 360-368.
180. Christodoulou, M.V., et al., *Cell replacement therapy with stem cells in multiple sclerosis, a systematic review.* Hum Cell, 2024. **37**(1): p. 9-53.
181. Rosenzweig, E.S., et al., *Restorative effects of human neural stem cell grafts on the primate spinal cord.* Nat Med, 2018. **24**(4): p. 484-490.
182. Mak, I.W., N. Evaniew, and M. Ghert, *Lost in translation: animal models and clinical trials in cancer treatment.* Am J Transl Res, 2014. **6**(2): p. 114-8.
183. Verwer, R.W., et al., *Injury Response of Resected Human Brain Tissue In Vitro.* Brain Pathol, 2015. **25**(4): p. 454-68.
184. Verwer, R.W., et al., *Altered Loyalties of Neuronal Markers in Cultured Slices of Resected Human Brain Tissue.* Brain Pathol, 2016. **26**(4): p. 523-32.
185. Qi, X.R., et al., *Human Brain Slice Culture: A Useful Tool to Study Brain Disorders and Potential Therapeutic Compounds.* Neurosci Bull, 2019. **35**(2): p. 244-252.
186. Lancaster, M.A. and J.A. Knoblich, *Organogenesis in a dish: modeling development and disease using organoid technologies.* Science, 2014. **345**(6194): p. 1247125.