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Stem cells and optogenetics:
novel approaches for the treatment of focal ischemic stroke in adult mice

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Graduate Division of Biological and Biomedical Science (GDBBS)
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ABSTRACT

Stem cells and optogenetics:
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By Osama Mohamad

Stroke is the fourth leading cause of death and the number one cause of disability in the adult population in the United States in 2011. Despite the economic, healthcare and social burden of stroke, therapy is still limited to one FDA approved drug (tPA), mechanical thrombolysis, supportive care and rehabilitation. Stem cell-based therapies especially human induced pluripotent stem (iPS) cells offer new hope for stroke and many other degenerative diseases. The rationale behind using stem cells in the treatment of stroke arises from their potential to foster endogenous host repair after the injury and their ability to differentiate to neurons and integrate into host circuits. However, the mechanisms of stem cell-induced benefit after transplantation are not fully understood and the neuronal differentiation and post-injury transplantation protocols have not been optimized yet.

In this dissertation, we first tested the therapeutic potential of vector-free human induced pluripotent stem (hiPS) cells in a mouse model of focal ischemic stroke. hiPS cells differentiated into functional neurons *in vitro* staining positively for mature neuronal markers and firing trains of action potentials. After transplantation in stroke mice, hiPS cells survived and differentiated to neurons with no signs of tumor formation. Transplantation of hiPS cell-derived neural precursors alleviated sensorimotor deficits, increased trophic support and restored neurovascular architecture after stroke. While whisker stimulation alone alleviated functional deficits of stroke, combining hiPS cell transplantation with whisker stimulation did not further increase benefits. We also developed optogenetics tools to be applied in stroke research and stem cell therapies. Channelrhodopsin-2 (ChR2) was over-expressed in cortical neurons *in vitro* and *in vivo* and in mouse iPS cell-derived neurons. Functional responses to light stimulation were recorded *in vitro* and in brain slices. Pre- and post-stroke light stimulation in the penumbra of ChR2 expressing mice reduced infarct volume and accelerated behavioral recovery, respectively.

Second, we developed a new protocol for the neuronal differentiation of mouse pluripotent stem cells. Culturing embryoid bodies (EBs) from D3 mouse ES cells on a rotary shaker produced smaller and more uniform neurospheres and increased the yield of neurons after the 4-/4+ differentiation protocol by 4-5 times. Terminally differentiated neurons were morphologically, functionally and immunohistochemically similar to those produced with static cultures. The rotary culture differentiation protocol also increased the efficacy of neuronal differentiation of mouse iPS cells.

Collectively, these data suggest that stem cell transplantation is a promising therapy for stroke and that optogenetics provide valuable tools for stroke and stem cell research. More work, however, is required to optimize neuronal differentiation and transplantation protocols before transitioning to human clinical trials.

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List of Abbreviations

FDA	Food and Drug Administration
tPA	Tissue plasminogen activator
EB	Embryoid body
ES	Embryonic stem
iPS	Induced pluripotent stem
AP	Action potential
Na^+	Sodium ion
K^+	Potassium ion
ATP	Adenosine-5'-triphosphate
Ca^{2+}	Calcium ion
NMDA	N-Methyl-D-Aspartate
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
IACUC	Institutional Animal Care and Use Committee
ANOVA	Analysis of variance
ROS	Reactive oxygen species
AIF	Apoptosis inducing factor
ICAM-1	Intercellular adhesion molecule 1
BBB	Blood brain barrier
GABA	Gamma-aminobutyric acid
GAT	GABA transporter
SDF-1α	Stromal cell-derived factor-1
BDNF	Brain-derived neurotrophic factor
GDNF	Glial cell-derived neurotrophic factor
VEGF	Vascular endothelial growth factor (
SVZ	Subventricular zone
SGZ	Subgranular zone
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor
Ang1/2	Angiopoietin1/2
Tie1/2	Tyrosine kinase with immunoglobulin-like and EGF-like domains
TNF-α.	Tumor necrosis factor -α
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
ChR2	Channelrhodopsin-2
eNpHR	Halorhodopsin
WS	Whisker stimulation
iPS	Induced pluripotent stem
ES	Embryonic stem
hiPS	human iPS
miPS	mouse iPS
ICH	Intracranial hemorrhage
MMP	Matrix metalloproteases
MSC	Mesenchymal stem cells
BMSC	Bone marrow-derived stem cells
HP	Hypoxic preconditioning
MI	Myocardial infarction
IV	Intravenous
IA	Intra-arterial
SSFO	Stabilized step function opsins
DIO	Double-floxed inverted open reading frame

Chapter I
Ischemic Stroke

A. Stroke facts

1. Definition: The WHO MONICA (World Health Organization Monitoring Trends and Determinants in Cardiovascular Disease) project defines stroke as "rapidly developed signs of focal (or global) disturbance of cerebral function lasting >24 hours (unless interrupted by surgery or death), with no apparent nonvascular cause … including patients presenting with clinical signs and symptoms suggestive of subarachnoid hemorrhage, intracerebral hemorrhage, or cerebral ischemic infarction" [1]. In simpler terms, stroke is defined as the clinical neurologic condition resulting from sudden or gradual reduction in blood supply to a particular brain region [2]. This reduction in local blood flow will lead to neuronal energy failure, the inability of neurons to maintain their ionic and homeostatic balance and eventually cell death. While different stroke definitions may include or exclude other types of stroke such as transient ischemic attacks, stroke is a devastating pandemic affecting around 6 million people worldwide [3].

2. Epidemiology: According to a joint report by the American Heart Association (AHA), the Center for Disease Control and Protection (CDC) and the National Institute of Health (NIH), stroke is the fourth leading cause of death and the number one cause of disability in the adult population in the United States [4]. According to this report, there is one new stroke occurring every 40 seconds in the United States and one death from stroke every 4 minutes. With 7 million American stroke patients, the overall stroke prevalence is almost 3%. The prevalence of silent stroke is estimated to range from 6 to 28%. Of the 795,000 patients who suffer a stroke annually, 610,000 suffer a first attack while the rest have recurrent attacks. The actual number of stroke deaths has significantly dropped in the past decade because of the tremendous improvement in health care delivery to stroke patients. However, stroke still accounts for 1 out of every 18 deaths in the United States and both the incidence and prevalence of stroke are expected to rise. Gender

differences also exist in stroke pathologies with women having higher lifetime risk and worse outcomes despite being older than men at the time of their first stroke [5-7]. In terms of disability, stroke is the leading cause of grave long-term disabilities with over 50% of patients suffering hemiparesis and 35% having depressive symptoms even 6 months after stroke onset. Despite these staggering statistics, less than one third of patients receive rehabilitation, one of the very few available treatments after stroke. Given the reduced number of stroke deaths and the increase time of hospitalizations and procedures for stroke treatment, the cost of stroke is projected to surpass $2 trillion from 2005 to 2050 [8]. In short, stroke represents a clinical entity that definitely requires more focused research to develop drugs or techniques to limit its debilitating effects on patients, the healthcare system and the national economy.

3. Causes: Among all strokes patients, 87% suffer ischemic stroke, 10% intra-cerebral hemorrhage and 3% suffer subarachnoid hemorrhage [4]. Normally in primates, cerebral blood flow is about 50-60 ml/100g per minute [9]. In ischemic stroke, a clot occludes a brain vessel (most commonly the middle cerebral artery or one of its branches) and blood flow to the brain region supplied by that vessel drops below 10-15 ml/100g per minute, causing a cascade of toxic signals ultimately leading to energy failure, acidosis, excitotoxicity, glutamate release, elevated Ca^{2+} levels, generation of free radicals (especially after reperfusion), blood brain barrier (BBB) disruption, inflammation and eventually massive cell death. Hemorrhagic stroke, on the other hand, occurs when a blood vessel ruptures in the brain leading to intracranial hemorrhage (ICH) - intra-cerebral or subarachnoid. ICH, with the associated edema, makes the outcome of hemorrhagic stroke more serious than ischemic stroke with only 38% of ICH victims surviving the first year [10]. Despite the severity of ICH, all of the experiments carried out in this

dissertation utilize a mouse model of focal ischemic stroke and this will be the focus of any further discussion.

B. Ischemic injury

1. Molecular events following ischemia: Ischemic injuries cause a disruption in cellular metabolism, cell signaling, signal transduction, and gene expression. In brief, shortage in blood supply to a particular brain region causes a cascade of toxic signals and ultimately leads to energy failure, acidosis, excitotoxicity, glutamate release, elevated Ca^{2+} levels, generation of free radicals, blood brain barrier (BBB) disruption, and inflammation.

The severity of the ischemic damage depends on the distance from the occluded vessel and the duration and severity of the occlusion [9]. Centrifugally from the site of occlusion, metabolic changes occur in a gradient fashion with the most severe outcomes occurring closest to the occluded blood vessel leading to irreversible and massive cell death. This is called the infarct core. Tissue further away from the infarct is partially supplied by collateral blood supply. This is called the penumbra. Penumbral damage can be reversed and tissue could be salvaged, if appropriately treated. If not treated early, penumbra tissue may turn into additional infarct core tissue [11]. It seems that the pathophysiological processes occurring in the core and penumbra are notably different. It is commonly accepted that necrosis is the dominant mechanism of cell death in the core whereas apoptosis is more common in the penumbra [12]. It is important to mention that blood reperfusion, after spontaneous, mechanical or pharmacological lysis of the blood clot, can be beneficial or harmful. Reperfusion can initiate recovery in areas with reversible damage by restoring oxygen and glucose needs [13] or increase the production of ROS leading to an amplification of ischemic injury [14].

Within minutes of oxygen and glucose shortage, a complex series of overlapping events start to occur. These events will extend over a period of weeks and is affected by different regulatory/modulatory mechanisms especially blood reperfusion. Figure 1.1 illustrates the molecular events associated with cerebral ischemia. Despite the fact that it is only 2% of the total body weight, the brain receives >10% of the cardiac output and almost 20% of the total oxygen consumption [15]. Being devoid of energy stores, shortage in blood supply in the brain will cause acute energy failure and accumulation of byproducts of anaerobic metabolism due to the depletion of oxygen and glucose. Energy failure disrupts the proper functioning of the Na^+/K^+ ATPase pump and the Na^+/Ca^{2+} exchanger leading to increased intracellular Na^+ and Ca^{2+}, reduced intracellular K^+ levels and loss of membrane potential to a general state of depolarization [16]. This increase in intracellular Na^+ and Ca^+ also causes cytotoxic edema. After membrane depolarization, the excitatory amino acid glutamate is released into the extracellular milieu causing widespread activation of NMDA and AMPA receptors and further increasing the intracellular levels of Na^+ and Ca^{2+}. After ischemic injury, Ca^{2+} enters neuronal cytoplasm via two main routes: 1) from the extracellular compartment via NMDA and AMPA receptors, voltage-gated Ca^{2+} channels and the Na^+/Ca^{2+} exchanger, and 2) from the intracellular calcium storage compartments such as the endoplasmic reticulum and the mitochondria. The rise in intracellular Ca^{2+} plays a pivotal role in ischemic injury. Ca^{2+} activates several Ca^{2+} dependant enzymes such as protein kinase C, phospholipases, calpain, and cyclo-oxygenases among many others. The activation of these enzymes will increase the production of ROS. ROS are also released from the mitochondria and generated during the inflammatory process. ROS interact with proteins, lipids and DNA causing serious membrane and DNA damage and further adding to the severity of the ischemic insult [17].

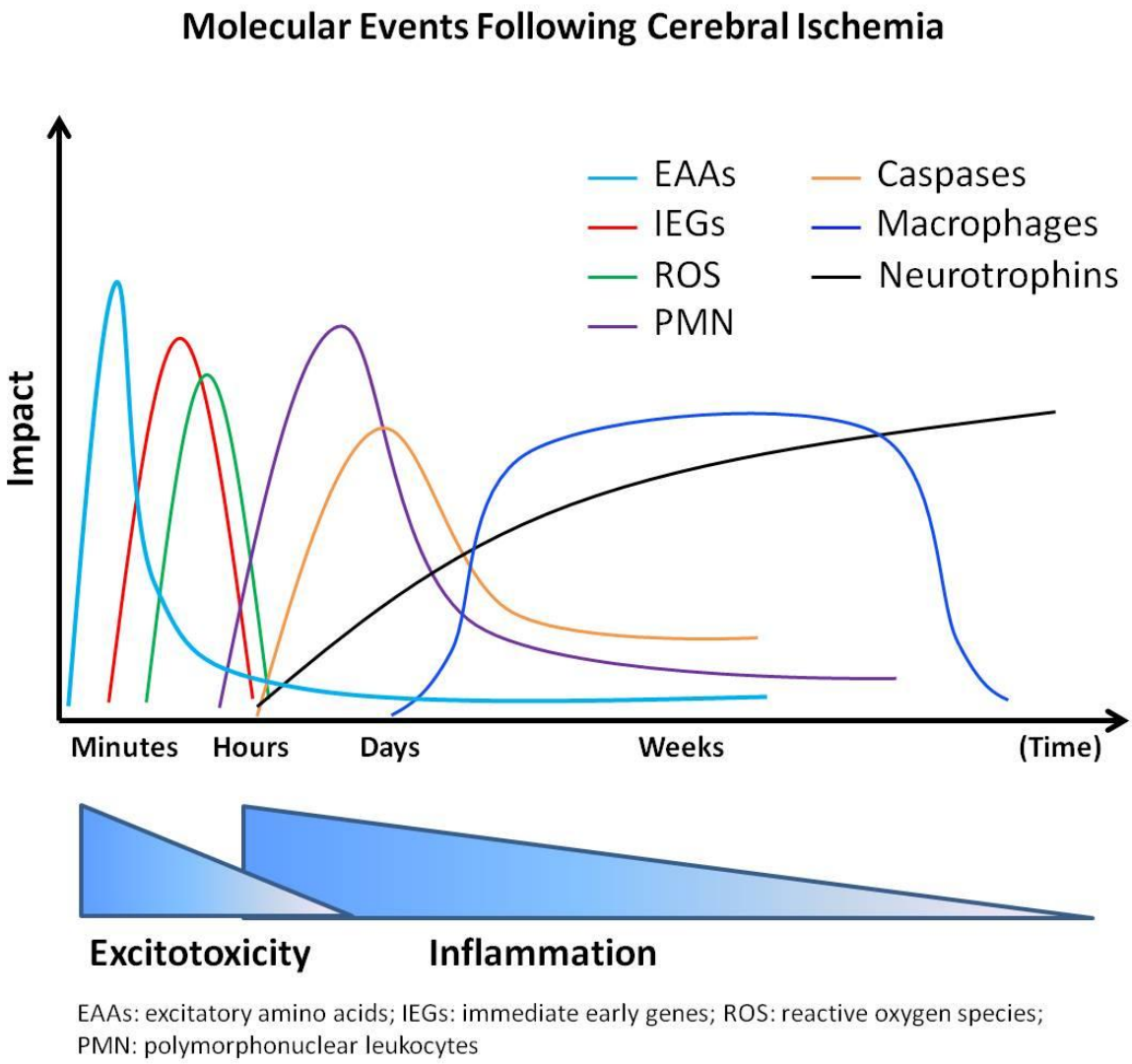

Figure 1.1. Molecular events following ischemia. Within few minutes after ischemia, there is a rapid surge of excitatory amino acid release, followed by changes in gene expression and increase in ROS production. A cascade of cell death, including both apoptosis and necrosis, starts within minutes of stroke onset. Inflammation occurs causing a rise in inflammatory cytokines and leukocyte infiltration to brain parenchyma. The x-axis shows the development of the events over time, while the y-axis demonstrates the impact of each event.

Excitotoxicity, mitochondrial damage, ROS formation and the rise in intracellular Ca^{2+} are all capable of inducing cell death even in the penumbra where the blood flow is not totally blocked [18]. Apoptosis is the dominant mechanism of cell death in the peri-infarct area of ischemic stroke [19]. There are two pathways for apoptotic cell death: intrinsic and extrinsic cell death pathways. In the intrinsic pathway, elevated intracellular Ca^{2+} activates calpain which

cleaves the pro-apoptotic enzyme Bid. Cleaved Bid will activate other pro-apoptotic enzymes such as Bad and Bax and tip the balance against Bcl-2 and Bcl-xl favoring apoptosis. A cascade of events then occur leading to the release of Cytochrome C (Cyt-c) and AIF from the mitochondria. Cyt-c eventually causes the activation of caspase-3 which cleaves DNA related repair proteins leading to DNA damage and cell death. AIF, on the other hand, mediates DNA fragmentation in a caspase-independent fashion. In the extrinsic pathway, Fas ligand (FasL) binds to the extracellular Fas death receptors triggering a cascade of intracellular events that activate caspase-8. Activation of caspase-8 either directly cleaves caspase-3 or recruits mitochondrial mechanisms (Cyt-c) to eventually cause cell death [20]. Morphologically, cells undergoing apoptosis looks different from those undergoing necrosis. This causes a marked difference in the appearance of the necrotic core as compared to the apoptotic penumbra. In necrosis, intracellular organelles, most notably mitochondria and vesicles, swell and eventually lyse. This is associated with whole cell swelling, loss of membrane integrity and lysis as well. DNA is fragmented in a random fashion. Necrosis is associated with an intense inflammatory reaction. In apoptosis, cells show characteristic morphological features including membrane blebbing, cell shrinkage, chromatin condensation and nuclear fragmentation and formation of apoptotic bodies. Unlike necrosis, apoptosis is not associated with inflammation. While necrosis is a passive process, apoptosis is active and requires energy [21]. Recent evidence, however, has shown that a new type of cell death, termed hybrid cell death, occurs in the penumbra. Hybrid cell death is neither typical necrosis nor apoptosis and it has its unique morphological and biochemical features [22].

While excitotoxicity is a major cause of ischemic injury and cell death in the peri-infarct area in the very first few days after ischemic stroke, tonic neuronal inhibition mediated by extra-

synaptic $GABA_A$ receptors is increased after day 3 in the penumbra of rodents and contributes to functional deficits after stroke [23]. This increased GABAergic tonic inhibition is thought to be mediated by impairment in the GABA transporter (GAT3/4) in the peri-infarct area after stroke. Consistent with these data, there was an increase in functional recovery in animals administered with benzodiazepine inverse agonist specific for the α5-subunit containing extra-synaptic $GABA_A$ receptors starting at day 3 after stroke or in α5- or δ- GABA containing subunit knock-out animals. Indeed, post-stroke brain stimulation has proven to be an effective method in the rehabilitation of stroke patients and the enhancement of functional recovery [24].

2. Inflammation: A growing body of scientific evidence from animal models of stroke as well as from human patients shows that inflammatory modulators play a larger and more complex role in ischemic injury and recovery after stroke than previously known. Classically, animal studies have shown that antagonizing various inflammatory components could decrease the area of brain infarction, suggesting that inflammation may have primary neurotoxic effects following stroke injury. However, more recent studies have shown that the role of inflammation in stroke is actually more complicated. Inhibition of inflammation is not always beneficial as inflammatory molecules are involved in neurovascular repair after stroke. Cells of the neurovascular unit (neurons, astrocytes, pericytes and endothelial cells), microglia, and white blood cells recruited from the systemic circulation, as well as hundreds of cytokines, adhesion molecules and components of the coagulation pathway and the immune system are all involved in a tremendous cascade of events that regulate ischemic injury and contribute to repair and recovery after stroke [25].

Within few minutes after stroke onset, there is an upregulation of pro-inflammatory cytokines interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-

α) accompanied with the activation of NF-κB, a downstream signal in the TNF-α pathway. The role of these pro-inflammatory cytokines, in addition to other anti-inflammatory factors IL-10 and IL-4, is time- and context-dependent. Shortly after, brain capillary endothelial cells start to express adhesion molecules such as ICAM which facilitates white blood cell infiltration into brain parenchyma. Neutrophils are the first to respond to ischemia followed by monocytes and macrophages. T lymphocytes and microglia play different roles in the modulation of the inflammatory response. This activity can either be harmful to regenerating neurons through the acute release of pro-inflammatory cytokines and cytotoxins, or, later in the recovery phase, beneficial. Cellular adhesion molecules are important in maintaining the integrity of the BBB and for leukocyte trafficking. Matrix metalloproteases (MMPs) disrupt the BBB through enzymatic degradation and remodeling of the extracellular matrix [26, 27]. BBB damage and the associated vasogenic edema are directly proportional to infarct size. Damaged BBB can no longer maintain the integrity of the brain's privileged isolation and thus, components of the systemic circulation, cells and toxins as well, can easily reach the brain and aggravate the ischemic insult.

C. Endogenous repair and trophic support

1. Neurogenesis: Contrary to the traditional popular belief, adult animal brains have the ability to regenerate neurons. Neurogenesis in the adult mammalian brain was first demonstrated in 1961 [28]. It is now widely accepted that the adult brain has several neurogenic niches most notably the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus [29-31]. Under normal physiological conditions, neural progenitors migrate from the SVZ to the olfactory bulb [32] forming the rostral migratory stream

(RMS). These neural progenitors migrate in chains and differentiate into interneurons in the olfactory bulb where they functionally integrate themselves into the existing neural circuitry [29]. The reason for this migration and renewal in the olfactory bulb is not clear yet.

Several types of brain injuries including seizure, stroke and traumatic brain injury can up-regulate progenitor proliferation in the neurogenic niches [33-35]. Under ischemic conditions for example, proliferation of neural progenitors increases in the SGZ and the SVZ [36-38]. Neural progenitors from the SGZ do not seem to migrate to areas of injury after stroke. SVZ neural progenitors however are laterally diverted from the RMS to the peri-infarct area around the site of injury. Neurogenesis peaks 7 days (1 week) after the ischemic insult in the SVZ of rodents [39]. Migration of progenitors from the SVZ to the site of injury peaks few weeks after stroke and continues without decline for up to 4 months continuously supplying the injured brain with new neurons [40].

The process of neuroblast migration relies greatly on the upregulation of chemotactic factors at the injury site and the physical scaffolding provided by blood vessels. Cells of the neurovascular unit (neurons, astrocytes, and endothelial cells) and cells recruited from the systemic circulation produce chemotactic factors including but not limited to SDF-1α, BDNF, GDNF and VEGF [41-46]. Neuroblasts associate with vasculature to migrate to the site of ischemic damage guided by factors like SDF-1 and Angiopoietin 1 (Ang-1) released by capillary endothelial cells in the infarct area [47]. Under ischemic conditions, approximately half of the cells migrating to the penumbra are in close proximity and contact with vasculature [48]. SDF-1 in particular is a potent chemoattractant, which is upregulated in the stroke infarct in a gradient fashion to guide migrating cells [49].

Migration from the SVZ to the infarct occurs in chains and as individual cells [50] at a rate of 28.67±1.04 μm/h [50]. Doublecortin (DCX)-positive neural progenitors closely associate with and cluster around the vasculature. In all, the adult brain's endogenous neurogenic machinery requires a coordinated effort between the SVZ, several chemotactic factors, and the vasculature to replace dead or dying cells. Nonetheless, this endogenous system of repair does not suffice to fully repair tissue damage after brain injury. 80% of the newly born neurons die in the striatum stroke model and endogenous regeneration only replaces about 0.2% of dead striatal neurons [51]. Thus, the need for novel therapies to replace lost tissue is immense.

2. Angiogenesis: Angiogenesis, defined as the formation of new blood vessels from pre-existing ones, is an indispensible process for the development and maturation of organs during organogenesis and for further dynamic growth and development in adulthood [52]. Angiogenesis is also implicated in the growth and metastasis of tumors, wound healing, myocardial infarction and ischemic stroke. Shortly after cerebral infarction, there is an increased proliferation of brain capillary endothelial cells causing an increase in vessel density in the penumbra region surrounding the infarct core. Given the complex nature of angiogenesis and its relationship to tumor growth and metastasis, studies of angiogenesis in the context of cerebral ischemia are still exploring the potential effects of modifying the angiogenic response after stroke. It is generally accepted that within the right time frame, enhancing angiogenesis is beneficial after stroke [53-55]. Similar to neurogenesis, several trophic factors participate in the angiogenic response after ischemic stroke such as VEGF/VEGF-R, Ang-1 and Ang-2, Tie-1 and Tie-2 receptors and TNF-α. VEGF is up-regulated in all cells of the neurovascular unit (neurons, astrocytes and endothelial cells) in the penumbra region after stroke [56]. While VEGF can promote recovery following stroke [57, 58], it can also increase vascular permeability and promote brain edema

[59, 60]. Ang-1 and Ang-2 are also upregulated after ischemic stroke. Ang-1 is a ligand for the receptor Tie-2 on endothelial cells whereas Ang-2 acts as a biological antagonist to Ang-1. Ang-1 acts to stabilize blood vessels whereas Ang-2 acts in concert with VEGF and promotes vessel sprouting. Thus, the ratio of Ang-1/Ang-2 is very critical after stroke. Immediately after ischemia, there is a sharp increase in Ang-1 mRNA and a drop in Ang-2 mRNA which leads to vessel stabilization. Shortly after however, the ratio is reversed with an increase in Ang-2 mRNA and a drop in Ang-1 mRNA. This reversal allows sprouting of new vessels [61].

TNF-α is a pro-inflammatory cytokine that is implicated in a variety of inflammatory and neurodegenerative diseases. In the context of stroke, TNF-α acts as a pro-inflammatory cytokine and modulates several post-ischemic processes including angiogenesis. VEGF and other angiogenic and non-angiogenic trophic factors are downstream signals of TNF-α and the up-regulation of TNF-α after stroke leads to an increase in VEGF expression. Thus, the angiogenic response of TNF-α is primarily mediated by VEGF and other downstream signals [62]. Much of what is known about TNF-α comes from studies of animals where the p55 receptor has been deleted. $P55^{-/-}$ mice show increased infarct volume, reduced angiogenesis and reduced elevation in VEGF expression after ischemic stroke [63]. These results suggest important roles for the modulatory activity of TNF-α through the p55 receptor after stroke.

D. Stroke treatment

1. Prevention: Current treatment guidelines suggest that eating a diet high in fresh fruits, vegetables, and low-fat dairy products, combined with a high intake of dietary and soluble fibers, whole grains and protein from plant sources and decreasing consumption of saturated fat, cholesterol and sodium, can aid in the prevention of stroke [64]. Other lifestyle modifications

that have shown strong evidence for stroke prevention include participation in moderate exercise, maintaining a low-normal body mass index, smoking cessation, and decreasing alcohol consumption. Guidelines for secondary stroke prevention include intravenous tissue-type plasminogen activator (tPA), anti-hypertensives, anti-thrombotics, anticoagulants for atrial fibrillation, lipid-lowering medications, smoking cessation, and weight loss education.

2. Currently available therapies: Stroke is one of the leading causes of morbidity and mortality worldwide [4] and yet there is a paucity of acceptable treatments that improve neurological recovery. Hundreds of drugs that showed significant neuroprotection in animal models have failed clinical trials in humans [65] such as: peroxidase inhibitors [66], calcium channel blockers [67], NMDA receptor antagonists [68, 69], free-radical scavengers [70], potassium channel activators, GABA agonists, and many others. Other candidate drugs are still in ongoing clinical trials such as serotonin agonists and selective serotonin-reuptake inhibitors [71]. This paucity in effective therapies indicates that novel and more rigorous design and conduct of animal studies are required to choose the best candidate drugs for human clinical trials. This will increase the chances of successfully transitioning stroke medications.

Aside from supportive care, currently available treatment modalities for acute ischemic stroke primarily target tissue reperfusion through thrombolytics such as tPA in the immediate period following injury. The use of tPA is, however, limited by time, having a small window of administration of approximately 4.5 hours following stroke, and carries with it the risk of hemorrhage, since it works by clot dissolution. Newer generation thrombolytic agents have shorter durations of action and are more fibrin-specific, but of the recombinant tPAs available, only alteplase has been FDA approved [72].

After injury has occurred, the most promising method of neurological recovery remains prevention of further stroke or stroke-related complications (vasospasm, infection, hemorrhage, reperfusion injury) and rehabilitation. Peripheral stimulation and physical therapy (rehabilitation) can improve functional recovery and neurovascular plasticity in the central nervous system. This has been confirmed by animal studies using peripheral stimulation following focal ischemia in mice. Peripheral stimulation resulted in increased angiogenic factors such as VEGF, restoration of local cerebral blood flow, enhanced neuroblast migration through up-regulation of chemokines such as those in the SDF-1/CXCR4 signaling pathway, and enhanced neurogenesis [73]. After stroke, there is a limited period of neuroplasticity during which greatest recovery can occur. It is a challenging task to optimally engage drugs, cell transplantation and physical rehabilitation in stroke therapy [74]. In human clinical trials, the constraint-induced therapy, that encourages the use of the affected limbs, improved behavioral functions lost after stroke [75]. In our stroke model, the whisker barrel cortex shrinks and loses thalamic and cortical connections after stroke. Whisker stimulation during the critical recovery period after stroke [74] provides increasing afferent sensory input which enhances neurogenesis and angiogenesis, restores lost neuronal connection, guides axonal rewiring of endogenous and transplanted neural stem cells and, eventually, promotes functional recovery [55, 73].

Another available therapy to limit brain injury and promote neurological recovery following stroke involves mild to moderate hypothermia (reducing body/brain temperature by 3-5 ^{o}C). Both animal and human data show that hypothermia is protective against various brain insults [76-80]. Clinical evidence shows that a 3 to 5 ^{o}C reduction is generally safe and effective in reducing cell death and improving outcomes after brain ischemia [81]. One recent meta-analysis, however, showed that mild hypothermia to 33 °C does not significantly affect stroke

severity and has no impact on mortality [82]. However, therapeutic hypothermia continues to be studied in an attempt to elucidate the ideal degree of cooling and the settings in which it can be used, with the promise of randomized controlled trials underway [82, 83].

3. Cell-based therapies:

Successive failures in translating stroke drugs to the bedside prompted scientists to look for therapy in stem cells. Stem cell transplantation is a promising therapy for ischemic stroke to replace lost tissue, provide trophic support, limit inflammation and enhance endogenous repair mechanisms [84]. Regeneration of the brain after ischemia requires an active replacement of blood vessels, neurons and glial cells and the integration of the endogenously regenerated or transplanted cells in functional networks. Studies from our lab and others have shown that transplanted stem cells survive, differentiate, enhance functional recovery after ischemia [85-87] and suppress inflammation [88, 89]. Stem cells differentiate to neurons, glial and endothelial cells and provide trophic support that enhances endogenous repair mechanisms (angiogenesis and neurogenesis) [90]. Many types of stem cells have been used as therapy for stroke: embryonic [91], bone marrow [92, 93] and cord blood [94] cells. Studies have also shown that transplantation of human embryonic stem cells improves functional recovery after ischemic stroke [95, 96]. Recently, scientists, using a cocktail of transcription factors, reprogrammed mouse [97] and human [98] fibroblasts to pluripotent stem cells which are able to differentiate to all the three germ layers. Moreover, fibroblasts have been directly converted to neurons and neural progenitors [99-101]. The potential of these induced cells in the treatment of ischemic stroke is being investigated in animal models of stroke.

There are tens of clinical trials that are currently testing the potential of hematopoietic bone marrow mesenchymal or adult neural stem cells for the treatment of ischemic stroke. A full

list of these trials can be found on clinicaltrials.gov and will be discussed later. Most studies are either recruiting or in progress and no large randomized trials have been performed [102]. Whether stem cells will be effective in the treatment of human stroke patients is yet to be determined.

E. Experimental models of ischemic stroke

There are many *in vivo* and *in vitro* models used to study stroke. While successes in treating stroke in animal models have been unreliable when translated to human clinical trials, ischemic stroke models are necessary to build an understanding of the pathophysiology of stroke progression and develop appropriate therapeutics. There are many models for ischemic stroke and they vary by their induction methods, location, size, and duration [103]. *In vitro* investigations using cell culture models allow us to understand the basic cellular and biochemical mechanisms without the systemic influences of an *in vivo* model. *In vivo* models allow us to study stroke within the scope of a biological system. This allows for the study of both stroke pathophysiology and the optimal conditions for therapeutic interventions. We can divide ischemic stroke models into two broad categories: focal and global ischemia. Focal ischemia is more commonly used and is induced by an acute occlusion of specific vessels to cause localized damage. Focal ischemia can be modeled with transient or permanent occlusions. Global ischemia, on the other hand, is induced when all cerebral blood flow including that of the vertebral arteries ceases. Clinically, global ischemia is less common, commonly occurring during suffocation, cardiac arrest or after bilateral common carotid artery (CCA) occlusion. The middle cerebral artery (MCA) is the most commonly affected vessel in stroke patients. Accordingly, the

most common model of ischemic stroke in rodents is the middle cerebral artery occlusion (MCAO). This is our model of choice.

1. Focal Ischemic Stroke Models: The MCAO model is widely accepted because it closely resembles human stroke conditions. Originally, the proximal MCA in the rat is occluded causing ischemic damage in the cortex and basal ganglia [104, 105]. In the intraluminal method, on the other hand, a neck incision is made and a nylon intraluminal suture is introduced through the external carotid artery to occlude the MCA.

Another focal ischemia model is the whisker barrel ministroke, which is a variation of the MCAO model that targets the whisker barrel cortex. Wei et al. described the development of this model in the rat [106]. Here, the barrel cortex and its vasculature were visualized using intrinsic optical signal imaging, with stimulation of the contralateral whiskers revealing blood flow in the barrel cortex. Three to six branches of the MCA around the barrel cortex were identified and then ligated with 10-0 sutures.

Targeting an area of a known function lends itself well to the study of stroke pathology as well as functional recovery and rehabilitation after stroke. Because the affected area is well-defined with known function, specific tests to determine functional recovery and specific methods for rehabilitation have been devised. Functional recovery can be assessed by examining behavior associated with sensitivity of the whiskers after stroke. For example, the corner or the adhesive-removal tests can be used to test whisker sensitivity. This model is also useful for studying rehabilitation therapy models using whisker stimulation that provides afferent thalamocortical signals to the affected region of the cortex [49]. Studies have demonstrated that providing such signals to the stroke-injured area will improve functional recovery [49, 107].

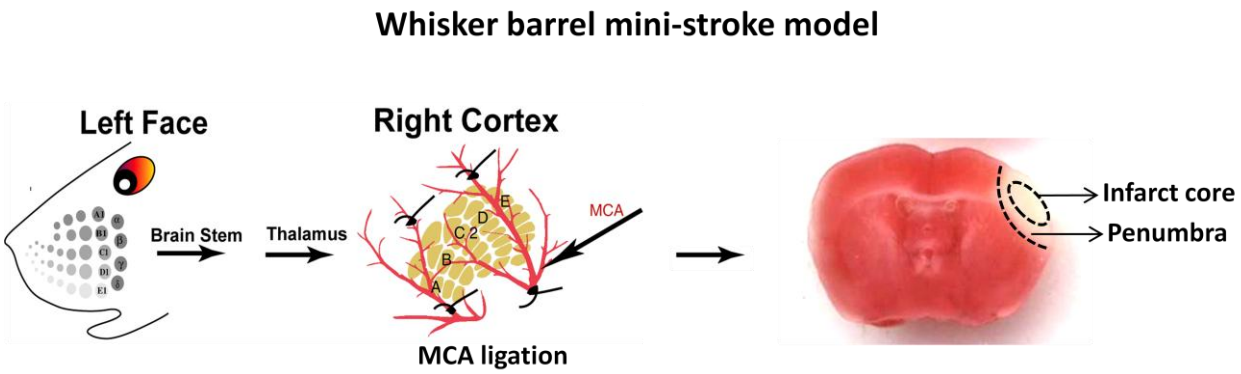

Figure 1.2. Whisker barrel mini-stroke models. Ligation of specific branches of the middle cerebral artery will cause an infarct in the barrel cortex controlling whisker functions of the opposite side of the face. The ischemic area comprises the infarct core and the ischemic penumbra.

There are several other ways to induce focal ischemia including the photothrombotic, autologous clot, and endothelin-1 methods. Discussion of these models can be found in [108-112].

2. Global Ischemia Models: Global ischemia can be induced by the occlusion of two or four vessels. In the two-vessel occlusion model, ischemic damage to the forebrain is induced by bilateral occlusion of the CCA in conjunction with systemic hypotension (down to 50 mm Hg) to attenuate forebrain blood flow in the animal [113]. Hypotension is induced by arterial or central venous exsanguinations along with CCA clamping. The hippocampus, caudo-putamen, and cerebral cortex can all be affected, depending on the duration of the ischemia [114].

In the four-vessel occlusion model, reversible clamps are loosely placed around the CCA and the vertebral arteries are electro-cauterized [113]. After 24 hours, the CCA clamps are tightened [113]. Within minutes after CCA clamping, isoelectric activity appears on electroencephalography (EEG). Reperfusion is allowed when the clamps on the CCA are removed, at which point animal activity is restored. Histopathology reveals that neuronal damage occurs within 20 to 30 minutes of ischemia. Damage usually appears in the hippocampus, striatum, and posterior neocortex [113].

3. Oxygen-glucose deprivation (*in vitro* ischemia): The oxygen-glucose deprivation (OGD) technique, applied to cultures of pure primary neurons or mixed cultures of glia and neurons, is the most commonly used *in vitro* model of stroke. Depriving neurons of oxygen and glucose simulates *in vivo* ischemic conditions. Briefly, growth medium is replaced with a physiological solution like Ringer's solution and cultures are placed in an airtight chamber with low oxygen (0.1 - 0.5%). The cells are then returned to their normal culture conditions after 2 or more hours depending on the degree of insult required. Cell death and survival are assessed after an appropriate "reperfusion" period.

F. Summary and conclusions

In summary, ischemic stroke is a world-wide devastating illness and more efforts are needed to establish protocols for its treatment. Despite their limitations, the currently available animal models are indispensible resources for studying the pathophysiology of ischemic stroke and have provided most of our knowledge about the mechanisms of cell death and recovery after ischemic insults. With the aforementioned introduction, the next two chapters will discuss novel approaches for the treatment of ischemic stroke. In chapters 2 and 3, we will introduce the topics of stem cells and optogenetics and will further explore how these new approaches can be utilized for the treatment of ischemic stroke.

Chapter II
Pluripotent Stem Cells

A. Stem cells: an introduction

1. Human ES and iPS cells: Stem cells are defined as cells which can both self-renew and differentiate into other cell types. Pluripotent stem cells, such as embryonic stem (ES) and induced pluripotent stem (iPS) cells, can differentiate into any cell type of the three germ layers, whereas multipotent stem cells, such as hematopoietic or neural stem cells, have a more lineage-restricted differentiation potential. Undifferentiated pluripotent stem cells can theoretically proliferate indefinitely *in vitro,* avoiding the senescence eventually observed in cultures of adult multipotent stem cells. However, it is easier to induce efficient differentiation of adult stem cells down a specific lineage compared to the less efficient and more time consuming differentiation protocols of pluripotent cells.

Since their first isolation in 1998 [115], storms of ethical disputes have arisen around human ES (hES) cells. Because they are derived from the inner cell mass of human blastocysts, research progress and funding for hES cells have been significantly slowed in the past few years. Alternatively, scientists have identified different cocktails of transcriptions factors and small molecules whose expression or addition can induce pluripotency in terminally differentiated somatic cells such as fibroblasts [97, 116]. These induced pluripotent cells are not equivalent to ES cells, but they share many similar properties such as their ability to differentiate into cell types from the three germ layers. In addition, protocols have been developed for creating neurons or neural precursors directly from fibroblasts or other terminally differentiated cells, avoiding the pluripotent cell state altogether [117-121]. Human induced pluripotent stem (hiPS) cells share similar genetic, epigenetic and morphologic characteristics with hES cells. iPS cell derivation has substantially developed over the past 3 years [122-128] and many protocols have been created to differentiate hiPS cells to terminally differentiated entities: neurons [129, 130], glial

[131], endothelial [132], cardiac [133] and pancreatic cells [134]. Initially, iPS cells were created using viral vectors to deliver pluripotency transcription factor genes. Given the tumor formation risk associated with viruses, the random integration of exogenous sequences and the intrinsic tumorigenicity of genes like c-myc, scientists have made hiPS cells that are free of any vector or transgene sequences [135]. iPS cells are setting the stage for personalized medicine where it is possible to replace any human tissue from one's own cells [136]. While studies have shown that hiPS cell transplantation improves symptoms of Parkinson's disease in rats [137], the potential of hiPS cell transplantation in the treatment of ischemic stroke is not explored yet.

2. Introduction to stem cell transplantation: Stem cell transplantation, primarily using mononucleated bone marrow cells, has proven to be beneficial in myocardial infarction and heart failure, with promising work in animal models translating to success in human clinical trials [138, 139]. The clinical use of bone marrow stem cells in neurologic injury models such as spinal cord injuries [140] and neurodegenerative diseases such as multiple sclerosis [141] has also demonstrated beneficial effects. However, it is not clear whether the observed improvements arise from cell engraftment and replacement of the damaged tissue. Instead, considerable evidence has shown that bone marrow derived stem cells release trophic factors providing protection for cells in ischemic regions and promoting endogenous repair mechanisms, particularly angiogenesis and neurogenesis. However, tissue engraftment in Parkinson's disease has provided a proof-of-concept for cell replacement [142], leading researchers to believe that stem cell therapies can eventually be used to replace damaged tissue in neurological injuries and disorders. Ethical concerns and the fear of rejection when using allogenic cells have prevented clinical trials using ES cells, but the introduction of iPS cells has spurred new research into the personalized use of pluripotent cells in neurodegenerative disorders.

In rodent models of ischemic stroke, endogenous neural stem cells in the sub-ventricular zone (SVZ) are recruited to the infarct site and form newborn neurons. However, the efficiency of this endogenous repair mechanism is very low and only a very small number of dead neurons are replaced. Transplanted stem cells, on the other hand, may produce better outcomes both by differentiating into neural or vascular cells and by secreting trophic factors that reduce cell death and increase endogenous neurogenesis and angiogenesis [143-145]. Cells derived from bone marrow [146] or umbilical cord blood [147, 148] have also been used in rodent models of stroke, most often injected intravenously (IV). As with other cell types, the improvements observed in such studies seems to have come primarily from trophic effects or enhancement of angiogenesis rather than replacement of damaged neuronal tissue. Improvement was observed too quickly for cell replacement to have occurred and cells did not need to cross the blood-brain barrier to provide beneficial effects [149].

Neural precursor cells that have been used in rodent models include conditionally immortalized cell lines derived from human fetal tissue [150, 151] or carcinomas [152, 153], fetal neuronal stem cells [154], mouse neural precursors derived from the post-stroke cortex [155], and precursors derived from mouse [156-158] or human [159, 160] embryonic stem cells. These are either injected into the circulation (intravenously or intra-arterially) or directly into the infarct core or penumbra regions and have been shown to improve functional measures of recovery in rodent models of stroke. In transplantation studies after stroke, survival of the engrafted cells is usually very low but has varied among studies. Many transplanted cells may die because of the hostile microenvironment of the ischemic brain. Surviving cells differentiate into neuronal or glial lineages and some express endothelial cell markers. While some cells may engraft in the host tissue, the number of transplanted cells generally drops over time, likely

because of apoptotic or necrotic cell death. Several strategies have been employed to improve survival and thus engraftment of stem cells *in vivo*, including genetic modification of implanted cells [85], hypoxic pre-conditioning [158], and the co-transplantation of supporting cells like endothelial cells with neural precursors [155].

B. Stem cells in stroke clinical trials: an overview

Human trials with stem cell transplantation for treating stroke are still in the early stages (Table 2.1). Full information can be acquired from clinicaltrials.gov. An early study demonstrated that hNT human neuronal cells can engraft into the stroke region and survive for more than two years in a human patient [161]. However, concerns about the cancerous origin and the possibility of tumor formation with hNT cells persist and no organization appears to have moved into larger scale trials with this cell type.

Table 2.1. List of clinical trials for stroke treatment with stem cells.

Identifier	Cell Type	Condition	Delivery	Location	Status	Phase
NCT01239602	Autologous CD34+ stem cells	Chronic ischemia	Intracerebral	Taiwan	Recruiting	Not listed
NCT01239602	Autologous CD34+ stem cells	Chronic ischemia	Intracerebral	Taiwan	Active, not recruiting	Not listed
NCT00473057	Autologous bone marrow cell	MCA infarction	IA (MCA)	Brazil	Completed	I
NCT01518231	Autologous peripheral hematopoietic stem cell	Ischemic stroke	IA (Cerebral Artery)	China	Recruiting	I
NCT01151124	CTX0E03 conditionally immortalized neural stem cells	Stable ischemic stroke	Intracranial	UK	Recruiting	I
NCT00859014	Autologous mononuclear bone marrow cells	Ischemic stroke	IV	USA	Recruiting	I
NCT01327768	Cultured olfactory Cells	Ischemic stroke	Intracerebral	Taiwan	Recruiting	I
NCT01438593	Allogenic CD34+ stem cell	Chronic ischemic	Intercerebral	Taiwan	Not yet open	I

NCT00761982	Autologous CD34+ stem cells	Acute stroke, MCA	IA (MCA)	Spain	Completed	I/II
NCT01468064	Autologous BMSCs and EPCs	Infarction, MCA	IV	China	Recruiting	I/II
NCT01287936	Modified stem cells	Chronic ischemic stroke	unspecified	USA	Recruiting	I/II
NCT01297413	Allogeneic adult mesenchymal bone marrow stem cells	Ischemic stroke	IV	USA	Recruiting	I/II
NCT01453829	Autologous adipose-derived stromal cells	Stroke	IA or IV	Mexico	Recruiting	I/II
NCT01091701	Ex vivo cultured adult allogenic MSCs	Ischemic cerebral stroke	IV	Malaysia	Not yet open	I/II
NCT00535197	Autologous CD34+ stem cells	Stroke, acute infarction, MCA	IA (MCA)	UK	Unknown	I/II
NCT00950521	Autologous peripheral blood stem cell(CD34+)	MCA infarction	Intercerebral implantation	Taiwan	Completed	II
NCT01501773	Autologous bone marrow stem cell	Acute stroke	IV	India	Completed	II
NCT00875654	Autologous mesenchymal stem cells	Ischemic stroke	IV	France	Recruiting	II
NCT01461720	Autologous bone marrow-derived mesenchymal stem cells	MCA Infarction	IV	Malaysia	Recruiting	II
NCT01389453	Umbilical cord mesenchymal stem cell	Cerebral hemorrhage and cerebral infarction	IV	China	Recruiting	II
NCT00875654	Autologous mesenchymal stem cells	Carotid ischemic stroke	IV	France	Recruiting	II
NCT01436487	Multi-Stem (bone marrow derived)	Ischemic stroke	unspecified	USA	Recruiting	II
NCT01273337	ALD-401 cells (bone marrow derived)	Ischemic stroke	IA (Carotid)	USA	Recruiting	II
NCT01310114	Human placenta-derived cells (PDA001)	Acute stroke - MCA or PCA	IV	USA	Not yet open	II

IA: intra-arterial, IV: intra-venous, MCA: middle cerebral artery, EPCs: endothelial progenitor cells

Bone marrow derived cells were shown to be promising in terms of safety and efficacy in 2009 [162] and a number of Phase I and II clinical trials using cell populations derived from

bone marrow have begun (See Table 2.1). Early results suggest that IV injection of mesenchymal stem cells (MSCs) does not cause any adverse affects and improves measures of function such as the Barthel Index (BI) [163], modified Rankin Scale (mRS) [164], and National Institutes of Health Stroke Score (NIHSS) [165]. These studies used autologous MSCs that were expanded in culture before use.

Stem cell transplantation in stroke provides a promising avenue of exploration, but further studies are needed before such therapy becomes a reality. The Stem cell Therapies as an Emerging Paradigm in Stroke (STEPS) meetings have brought together scientists, clinicians, regulators, and industry representatives to create guidelines for preclinical and clinical studies. The initial guidelines were published in 2009 [166], followed by an update in early 2011 [167].

C. Pluripotent stem cell differentiation

1. Murine ES (mES) and iPS (miPS) cell differentiation: In the absence of differentiation cues, pluripotent stem cells can remain in a state of continuous self-renewal in culture. mES and miPS cells have the potential to differentiate into any of the three primary germ layers including the ectodermal layer responsible for neural development [168]. The differentiation outcome greatly depends on the microenvironment in the cell culture [169]. Murine ES and iPS cells can be differentiated into neural progenitors which can further differentiate into any of the neural cell types, including glial cells and neurons [170]. Furthermore, specific neuronal subtypes (dopaminergic, GABAergic, etc.) can be cultivated in a controlled microenvironment with specific substrates and media components. There are many different ways to differentiate mouse pluripotent cells into neurons depending on the neuronal subtype of interest (i.e. midbrain dopaminergic [171, 172], serotonergic [172], forebrain [173], radial glia [174]).

Although there are several different protocols to differentiate murine pluripotent stem cells, differentiation using the vitamin A derivative, retinoic acid (RA), is the most commonly used technique [175-179]. RA plays a role in the developing mammalian embryo and specifies the anterior-posterior body plan [180]. Anteriorly, RA induces *Hox* gene expression [180-182] and it has specific effects on rhombomere development in the CNS [182]. RA interacts with Cellular RA-Binding Proteins (CRABPs) which bind to nuclear RA receptors. There are two families of RA receptors (RARs and RXRs) [177]. During neuroectodermal differentiation, RAR-α and -β mRNA are upregulated suggesting that neural differentiation requires these receptor subtypes [177]. In pluripotent cell differentiation, RA induction activates neural-specific transcription factors, signaling molecules, and RA-inducible genes resulting in the production of different neuron sub-types such as GABAergic and glutamatergic neurons [175]. The RA neural induction method produces both glial cells and neurons. Neurons derived in this method show mature neuronal morphology, express mature neuronal markers such neuronal nuclei (NeuN) and neurofilament and exhibit neuronal electrophysiology profiles that include mature sodium currents, potassium currents, and action potentials [179].

Undifferentiated mouse ES and iPS cells are regularly maintained in a self-renewing, proliferating state in the presence of leukemia inhibitory factor (LIF) which suppresses differentiation and is required for the maintenance of pluripotency [183]. Neural induction using the "4+/4-" RA protocol takes eight days. After LIF withdrawal, cells are grown in suspension to form tri-dimensional spherical aggregates of cells known as embryoid bodies (EB). RA is added in the last four days (termed "4+"). This treatment induces EBs to form neurospheres which are then dissociated and plated on an adherent substrate (laminin, matrigel or fibronectin) to allow terminal neuronal differentiation and neurite extension. These cells will express neuronal

markers such as the M subunit of neurofilament and class III β-tubulin protein within a few days. Most of the neurons derived in this fashion will express functional Na^{+}, K^{+}, and Ca^{2+} channels and fire action potentials [179].

There are also several other protocols for the neuronal differentiation of mouse ES and iPS cells. One particular protocol makes use of mouse stromal supportive cells for neuronal induction [184]. Murine bone marrow–derived stromal feeder MS5 cells [185] are mesenchymal stem cells that were originally derived to support the long-term growth and expansion of hematopoietic stem cells, but have also been found to induce a neuro-ectodermal lineage fate in mouse ES cells. In brief, undifferentiated murine ES cells are seeded at a very low density on MS5 cells in serum replacement and cultured for 7 days with bFGF added in the last two days. Co-culture of mouse ES with MS5 cells induces the expression of neural markers (Nestin and Musashi) as early as day 6. Although retinoic acid strongly induces neuronal formation in mouse ES cells, it was recently shown that neuronal induction with the MS5 cell line produces cortical pyramidal neurons that connect with their correct targets *in vivo* after transplantation [186]. These graft-to-host axonal connections did not occur when cells were differentiated using RA. The MS5 protocol may thus be more appropriate for use in cell replacement strategies using murine cells.

2. Human ES and iPS cell differentiation: hES and hiPS cells have the potential to differentiate into all human cell lineages including neurons, making them an attractive cell source for studying disease mechanisms and for drug screening as well as cell replacement therapy in neurodegenerative diseases such as stroke. As with mouse pluripotent cells, several approaches have been developed for the neural induction of human pluripotent cells. Protocols used with mouse cells do not necessarily produce the same results in human cells. The

neuralizing effect of RA, for example, is not exactly the same between human and mouse pluripotent cells. However, like their mouse counterparts, human cells can be differentiated either as EBs in suspension or in adherent culture. In this review, we will focus on two differentiation protocols because of their widespread use and proven efficiency.

After the first isolation of human ES cells in 1998, efforts focused on their neuronal differentiation based on the knowledge accumulated from the development of the human nervous system. One of the earliest efforts was lead by Zhang, S.C. and Thomson, J.A [187] and utilized EB formation. In this protocol, hES (and also hiPS) cell colonies are dissociated into smaller clusters of cells and grown in human ES cell media without bFGF in low adherence plates. When EBs form, they are grown for four days before being plated on tissue culture dishes in a neural induction media. After neural rosette formation (~8-10 days), rosettes are re-suspended and expanded as neurospheres in medium containing bFGF. Prior to neurosphere formation, cells in the rosette structures express neural markers such as Nestin and Musashi-1. After expansion, these cells are plated on laminin, with neuronal markers detectable within 7-10 days. Further differentiation will yield more neurons and, after longer periods in culture, glial cells. This protocol was one of the earliest methods used to generate neural cells from human ES cells and has successfully been applied to human iPS cells [188].

Despite its high efficiency, the EB protocol usually involves several weeks of expansion to obtain large numbers of neural precursors before differentiation into neurons and glial cells. In an attempt to overcome this time limitation and avoid the heterogeneous signals encountered by cells in aggregates, Chambers et. al. developed an adherent protocol for the neural differentiation of hES and hiPS cells to produce large numbers of neural precursors in approximately 15 days [189]. In brief, hES or hiPS cells are dissociated into single cells and grown on matrigel in the

presence of ROCK inhibitor, which reduces apoptosis and increases human pluripotent cell survival after dissociation [190]. When they become confluent, the cells are grown in a knock-out serum replacement media for five days. This medium is supplemented with two inhibitors of the SMAD signaling pathway, Noggin and SB431542. The media is then sequentially changed to neural induction media (increasing amounts of N2 media) for a total of six days. Consequently, neural rosette-like structures are formed and can be further differentiated into neurons of different subtypes. Unlike the EB based protocol, this adherent protocol yields Pax-6 positive neural precursors in more than 80% of the cells, with little need to separate rosettes from cells that have differentiated into other lineages. When used with hiPS cells, the EB protocol had a significantly reduced efficiency and increased variability as compared to hES cells [188]. On the contrary, the dual inhibition protocol significantly promoted neural differentiation from multiple hES and hiPS cell lines, [191] indicating more consistent results with this protocol. In our lab, we have shown that Noggin can be replaced with the small molecule dorsomorphin in this protocol, greatly reducing the cost of differentiation [192]. Dorsomorphin has been used to enhance neural differentiation in suspension cultures [191, 193] and has been shown to induce efficient neural differentiation alone in adherent culture [194].

D. Routes of stem cell transplantation

Once the appropriate animal model and the differentiation protocol have been chosen, the appropriate mode of stem cell transplantation must be selected for the study of interest. In addition to testing the safety and efficacy of cell therapy, one of the goals of preclinical cell transplantation studies is to optimize transplantation parameters so that the transplanted cells survive and differentiate appropriately *in vivo*. Two of the most important parameters to be

considered for transplanting any cell type are the administration route and the timing of delivery. These parameters vary by cell type and model of ischemic stroke. It is important to mention that before translating cell therapy into human patients, transplantation parameters need to be optimized in animal models with special emphasis on non-human primates. The STEPS committees have established guidelines for preclinical studies pointing out the need for a well-characterized cell population, dose-response studies, and testing in at least two species [166].

1. Local ipsilateral transplantation: Identifying a favorable microenvironment for stem cell transplantation allows for increased survival of the graft. One consideration is the transplantation location, which can greatly affect graft survival. Direct transplantation into the infarct core introduces the grafted stem cells into a harsh milieu of necrotic cells and inflammation. Researchers often aim to transplant cells into the penumbra rather than the core of the stroke. Cells transplanted into the necrotic core have lower survival rates than those in the apoptotic penumbra [195, 196]. Transplanting cells into the penumbra is a more successful approach because the penumbra provides a more favorable microenvironment for the graft [85, 158, 197]. For example, kidney cells injected into the penumbra resulted in a smaller infarct volume [197]. Consequentially, improved behavioral function is observed more in animals injected with cells into the penumbra.

2. Transplantation into the contralateral hemisphere: A viable alternative to avoid stem cell transplantation into the cytotoxic stroke core may be transplanting cells into the hemisphere contralateral to the infarct. In rats with MCA occlusion, a greater percentage of cells survived in the contralateral somatosensory cortex and striatum compared to ipsilateral or intraventricular injections [198]. The ipsilateral somatosensory cortex, which is the site of the damaged tissue, had the lowest numbers of surviving grafted cells.

This strategy may be useful in delivering cells to the infarct because of migration to the injured site. For example, MHP36 cells migrated from the contralateral hemisphere to the infarct area, improved functional recovery, and reduced stroke volume [199]. Using **G**adolinium-**R**hodam**I**ne **D**extran (GRID), which can be visualized by magnetic resonance imaging (MRI) and fluorescent microscopy, it was shown that cells migrate from the contralateral hemisphere to the stroke lesion [200] along the corpus callosum and partially repopulate the peri-infarct region in 14 days. Interestingly, stem cells transplanted into sham animals did not display trans-hemispheric migration suggesting that the stem cells are actively chemo-attracted to the stroke lesion.

3. Vascular administration: Stem cells may also be administered systemically through the vasculature. While this strategy is less invasive and does not necessitate craniotomy, vascular administration adds the requirement that cells must home to the brain injury site. Bone marrow mesenchymal cells systemically infused into ischemic rats have been reported to migrate to the site of injury [201]. Homing of systemically delivered cells to the infarcted brain has been extensively documented and some reports also showed minor neuronal differentiation of the transplanted cells [93]. Intravenous injection of human neural stem cells into an ischemic rat model led to incorporation of some cells into the ischemic infarct. However, transplanted stem cells were also trapped in other organs such as the kidney, lung, and spleen. Within the brain, neural stem cells were also found in the cortex, along the corpus callosum, and in the hippocampus [202].

This route of administration has been effective in preclinical and clinical trials. In one study, IV administration of cord blood stem cells in rat stroke animals resulted in improvement in motor functional recovery as assessed by the Rota-rod test [94]. Similarly, intra-arterial

administration of mesenchymal stem cells improved the NSS score and performance in the adhesive-removal test of stroke rats [203]. IV administration of autologous MSCs has already been shown to be safe and appears to be efficacious in small clinical trials in human stroke patients [162].

Vascular delivery of cells can be either intravenous or intra-arterial. There are advantages and disadvantages to both methods. While less invasive than intra-arterial delivery, the intravenous route delivers the cells through the systemic and pulmonary circulations where cells might get entrapped in other organs (spleen, liver, and lungs) and consequently fewer cells reach the brain. Intra-arterial delivery, on the other hand, circumvents the systemic circulation. 21% of cells enter the brain with intra-arterial administration compared to only 4% with intravenous administration [203].

Trophic factors may also increase the proportion of injected cells that reach the injured site. For example, intravascular injection of bone marrow stem cells coupled with subsequent injections of granulocyte-colony stimulating factor (G-CSF) and stem cell factor (SCF) increased bone marrow stem cell mobilization in the circulation, cell homing to the brain, and neuronal differentiation [204, 205] suggesting that bone marrow-derived stem cell therapy supplemented with trophic factors is a potential therapy for the repair of stroke.

4. Intranasal administration: Intranasal delivery of stem cells for therapeutic purposes is a relatively new avenue of investigation. Intranasal delivery offers two major advantages that are extremely important for the treatment of central nervous system related diseases. This treatment is not invasive and it can bypass the blood brain barrier. However, for intranasally delivered stem cells to cross to the brain, permeation enhancers (like hyaluronidase) might be needed [206]. Transplanted cells essentially move from the nasal mucosa into the CNS through the cribriform

plate and migrate into the brain parenchyma along the olfactory neural pathways, corpus callosum, and blood vessels. Complete reproducibility of cell distribution, however, still poses major difficulties in this delivery method.

Rat MSCs rapidly reach the brain only 1 hour after intranasal delivery through either the rostral migratory stream (RMS) or the cerebrospinal fluid (CSF) [207]. MSCs, delivered intranasally to hypoxic neonatal mice, remained in the brain 28 days after delivery and improved sensorimotor functional recovery [208]. Similarly, our group has shown that intranasally delivered bone marrow mesenchymal cells migrate to the brain immediately after injection and specifically distribute to the ischemic hemisphere (unpublished data). Nonetheless, despite the promises of intranasal delivery, more work still needs to be done before any clinical application in humans is tested.

E. Strategies to enhance cell survival in vivo

1. Cell number and timing of stem cell administration: The environment of the brain after stroke is constantly and dynamically changing. Timing of stem cell delivery is an essential parameter to be considered to increase cell survival and unfortunately, the optimal time for transplantation in animal models and consequently in patients is not yet known. Stem cell transplantation has been performed on the order of hours to weeks after stroke induction depending on the investigator's preference, cell type, mechanism of action, and the anticipated outcome. Immediate transplantation after stroke should be avoided due to the cytotoxic nature of the ischemic area soon after stroke and the overall neurologic condition of the animal. Transplanted cells are likely to be more viable once the cytotoxic and inflammatory factors in the core have diminished. However, relatively early transplantation may increase the ability of cells

to home to the infarct and to prevent ongoing cell death. In our lab, we typically transplant stem cells 7 days after stroke, at which point brain edema has subsided. Reported clinical trials with autologous MSCs have been limited by the time needed to expand cells to the point that an appropriate dose is available [163-165]

Darsalia and colleagues compared the results of transplanting neural stem cells 48 hours and 6 weeks after stroke damage [209]. Interestingly, there was greater cell survival when cells were transplanted at 48 hours, with about 58% of cells surviving as compared to 27% when transplantation occurred 6 weeks later. However, the later transplantation time at 6 weeks did not affect migration, neuronal differentiation, and cell proliferation. The dosages used were 3×10^5, 7.5×10^5, or 1.5×10^6 cells. An increase in the number of transplanted cells did not affect their survival or neuronal differentiation.

2. Genetic manipulation: Genetic manipulation of transplanted cells can enhance the cell survival of transplants. Pro-survival factors can play a significant role in enhancing graft viability after transplantation. For example, cells engineered to up-regulate B-cell lymphoma 2 (Bcl-2) survive better than control cells *in vivo* [85]. Bcl-2 is an anti-apoptotic factor of the intrinsic apoptotic pathway. It interacts with the pro-apoptotic factor, Bcl-2/Bcl-XL-associated death promoter (Bad), resulting in either cell death or cell survival. The ratio of these two proteins in a cell is correlated to the determination of whether or not the cell will undergo apoptosis. The human Bcl-2 gene was over-expressed in murine embryonic stem cell-derived neural precursors which were transplanted into the penumbra 7 days after stroke. Compared to controls, progenitors over-expressing the Bcl-2 gene exhibited greater cell survival and neuronal differentiation and improved neurological and behavioral outcomes after stroke.

Genetic engineering requires the alteration of DNA sequences in stem cells, generally by inserting the gene(s) of interest into the genome. While this method can be highly efficient, manipulating the genome of stem cells increases the risk of tumorigenesis in a population of cells with an already established risk of tumor formation. Thus, other techniques have been developed to increase survival of stem cell grafts after transplantation.

3. Preconditioning: Preconditioning is an experimental technique in which exposure to specific chemical or physical conditions produces resistance to ischemic damage. This idea was first put into practice in ischemic preconditioning, in which an early exposure of the heart to brief episodes of ischemia protects the myocardium against a later prolonged ischemic insult [210]. While the exact mechanistic details of how preconditioning works are still unknown, different studies have unraveled many potential mechanisms for preconditioning induced protection [211, 212].

Preconditioning has been successfully applied in cell therapy to protect the transplanted cells from apoptosis and increase their survival after transplantation *in vivo*. Hypoxic preconditioning (HP), defined by non-lethal exposure of cells to low oxygen tension (0.1-1%) before transplantation, has been shown to be very effective in increasing transplanted cell survival and improving overall functional recovery after stroke or myocardial infarction (MI) [87, 158, 213, 214]. HP has also been shown to enhance cell differentiation in culture [215] and after transplantation [158]. Non-lethal exposure to hypoxic conditions is believed to activate the hypoxia-inducible factor 1α (HIF-1α) pathway, increasing expression of HIF-1α-dependent genes. Expression of a number of trophic factors is also increased, including brain-derived and glial cell-derived neurotrophic factors (BDNF and GDNF), vascular endothelial growth factor

(VEGF) and its receptor Flk-1, erythropoietin (EPO) and its receptor EPOR, and stromal derived factor-1 (SDF-1) and CXC chemokine receptor 4 (CXCR4) [214].

The experimental success of HP led to increased interest in other potential modes of preconditioning, including exposure of stem cells to pharmacological agents or trophic/growth factors to increase their *in vivo* survival. Diazoxide [216], minocycline [217], and SDF-1 [218] can all enhance stem cell survival after transplantation in stroke or MI animal models. Preconditioning has thus far been very successful in preclinical models, but further studies are needed to fully elucidate the protective mechanisms induced by these treatments.

4. Co-transplantation: Another possible strategy to enhance survival in cell-based therapies is co-transplantation of MSCs with stem cells. In co-transplant studies where animal models of MS were injected with ES cell-derived oligodendrocyte progenitors (OPCs) with or without syngeneic MSCs [219], better survival of transplanted cells and greater levels of re-myelination were observed in the co-transplantation group. In another study, cotransplantation of endothelial cells and neural stem cells increased the survival, proliferation and neuronal differentiation of the transplanted neural stem cells compared with transplantation of neural precursors alone [155]. In unpublished data from our lab, we noted that bone marrow-derived MSCs increased the survival and differentiation of mouse ES cell-derived neural precursors in a mouse model of focal ischemia.

F. Mechanisms of stem cell induced benefit

1. Trophic support and attenuation of inflammation: Multipotent and pluripotent stem cells can enhance the survival of the surrounding tissue by providing trophic factor support. Trophic factors secreted by transplanted cells can support the injured parenchyma [197, 220]. As an

example, a large proportion of neural stem cells transplanted into the murine cochlea were found to express neurotrophins such as GDNF and BDNF. The release of these factors provided protection and support to the surrounding cells [221].

Transplanted adult marrow-derived MSCs release cytokines and trophic or growth factors that have autocrine and paracrine effects [222-224]. MSCs have been shown to secrete colony-stimulating factor-1 (CSF-1), stem cell factor (SCF), bFGF, nerve growth factor (NGF), and VEGF [225-229]. Such factors reduce host cell death and attenuate inflammatory processes [230]. Interestingly, injecting BMSC-conditioned media into the brain after stroke led to functional benefits, indicating that trophic support is a major mechanism by which MSCs contribute to functional recovery [223, 231, 232].

2. Cell differentiation and integration after transplantation: Cell differentiation and integration after transplantation is an important aspect of cell therapy if the intent of the therapy is to replace lost tissue. Cells have a greater potential to functionally integrate into the existing host circuitry if they differentiate into the appropriate neuronal phenotype. Several studies have examined the *in vivo* differentiation of transplanted stem cells. mES cell-derived neural precursors differentiated into different neuronal sub-types including cholinergic (1.4% of all grafted cells), serotonergic (1.8% of all grafted cells), GABAergic neurons, and striatal neurons (1.4% of all grafted cells) after transplantation into the infarcted region. At 12 weeks after transplantation, 25% of the remaining transplanted cells were NeuN-positive while 8% were positive for glial fibrillary acidic protein (GFAP), a marker of astrocytes. Transplanted cells also exhibited mature inward sodium and slow inactivating outward currents. While cells continued proliferation after transplantation, no tumors were detected at 4 or 12 weeks [233].

Another study reported that 30% of transplanted human fetal neural stem cells survived 1 month after stroke in the striatum [234]. Striatal-derived and cortical-derived neural stem cells from human fetal tissue were transplanted into the striatum of stroked rats. About 80% of the cells migrated from the original site of the transplant closer to the lesion. Unlike the aforementioned study with ES cell-derived progenitors, there was little proliferation (less than 1%) as measured by Ki67 staining. A high percentage of the cells differentiated into neurons expressing neural Hu protein D (HuD), calbindin, and parvalbumin, all markers of neuronal differentiation, but very few differentiated into astrocytes or oligodendrocytes [234].

3. Reduction of infarct volume: Both embryonic and adult neural stem cells led to a reduction in infarct volume when 100,000 cells were transplanted into the stroke-damaged brain [235]. This treatment led to a significant reduction in infarct volume observed 1 and 28 days after stroke induction. We have found that bone marrow-derived MSCs can also reduce infarct volume whether transplanted systemically or locally (accepted publication). It is important to mention that in order to reduce infarct size, stem cells have to be delivered acutely after stroke.

G. Immune responses in cell replacement therapy

1. Inflammation: In neurological injuries or disorders, inflammation may affect the ability of transplanted cells to replace damaged neurons. Suppression of inflammation is an important consideration in cell-based treatment of neurological disorders. Cell replacement therapies can modulate the host inflammatory response and establish a favorable microenvironment that prevents further endogenous cell death due to inflammation. By modulating the inflammatory processes, transplanted cells increase their own chances of survival. Otherwise, inflammation can

result in failure to survive or engraft and can reduce or even eliminate the therapeutic effects of transplantation.

Much of the research on the suppression of inflammation by stem cell therapy has focused on MSCs, which have been shown to suppress innate and adaptive immune responses. While suppression of the immune system may lead to infection or tumor cell proliferation, there is a consensus that human trials should move forward using MSCs in the treatment of multiple sclerosis (MS)[236]. MSCs can inhibit inflammation in multiple ways. *In vitro*, MSCs can reduce proliferation of peripheral blood mononuclear cells (PBMCs), [237] especially T- [238-240] and B-lymphocytes [239, 241]. MSCs also reduce tumor necrosis factor alpha (TNF-α) in dendritic cells (DCs) and interferon gamma (INF-γ) in differentiating T_H1 cells and natural killer cells [237]. They can also increase the anti-inflammatory interleukin IL-10 in DCs [237] and macrophages [242], as well as IL-4 in differentiating T_H2 cells [237]. While most human transplantation studies have used autologous MSCs [243], promising data with allogeneic MSCs suggests that these cells need not come from a genetically identical source. In fact, some clinical trials are using cells from allogenic sources (See Table 2.1).

Inflammation can affect the differentiation outcome of stem cells *in vivo* after transplantation. When mice were transplanted with neurospheres derived from mouse ES cells [244], no differences were observed in graft size or cell survival between cyclosporine A- and saline-injected animals, but fewer inflammatory cells were attracted to the graft in the cyclosporine A-treated mice. In addition, engrafted cells were more likely to be positive for NeuN and negative for GFAP in the cyclosporine A-treated animals, suggesting that the immune response creates an environment that favored glial over neuronal differentiation. *In vitro*, the addition of the inflammatory factor interleukin 6 (IL-6) to differentiating cells resulted in fewer

cells positive for the β-III tubulin and more GFAP-positive cells, further suggesting that inflammation can suppress neuronal differentiation.

2. Immune rejection: Another major hurdle in stem cell transplantation is that of rejection. Accumulating evidence suggests that culturing human ES cells on animal-derived substrates or with animal-derived media products increase their immunogenicity because of their increased expression of a non-human sialic acid residue, Neu5Gc [245]. This has pushed companies involved in the biotechnology of human ES cells to create media products and substrates that are fully defined and completely animal product free. While clinical trials have been carried out with cells expanded in xenogenic serum without any reported ill effects from the animals products [163, 164], the removal of animal products is still preferred.

As with tissue and organ transplants, allogenic stem cells can be rejected and destroyed by the host immune system. Rejection is modulated by the expression and detection of unmatched human leukocyte antigen (HLA) proteins, specifically major histocompatibility (MHC) proteins [246]. Host T-lymphocytes recognize these proteins, primarily MHC-I, on the donor cells and sensitize the immune system, causing it to react against the transplanted cells. The brain has historically been seen as an immune privileged location, but even with allogenic cells or cells directly injected into the brain [247, 248] rejection might still occur and may require the use of immune suppression for continued benefit [249]. However, immune suppression may be detrimental to endogenous healing, as some aspects of the immune response are actually neuroprotective and necessary to endogenous neurogenesis [250-253]. Immune suppression also may reduce the inflammatory signals that attract stem cells to the site of injury [254] or increase the risk of tumor formation [255, 256]. Stem cells such as BMSCs and adult neural precursors have also been shown to modulate the immune system, a functionality that

might be lost with systemic immune suppression [257, 258]. Given these issues and the health problems that can be caused by long-term immune suppression [259], it is clear that the use of such suppression should be avoided in stem cell transplantation whenever possible. In some cases, short-term measurements of the immune response to transplanted cells seem to suggest that immune suppression is not necessary and may even reduce graft survival [260]. Human ES cells express only very low levels of MHC-I in an undifferentiated state. However, MHC-I is upregulated when cells are differentiated and can be highly upregulated in undifferentiated or differentiated cells by interferons [261], which are expressed in endogenous immune responses such as inflammation. It is possible to reduce MHC expression in transplanted cells [262], but genetic changes and viral vectors can introduce their own problems.

iPS cells represent a very attractive solution to this problem, as patient-specific iPS cells would have matched HLA proteins and would be unlikely to face the threat of rejection. A recent study, however, has shown that iPS cells derived from B6 mouse embryonic fibroblasts formed teratomas that were immune-rejected after transplantation in B6 mice [263]. On the contrary, ES cells derived from B6 mice formed teratomas in B6 recipients without any evident immune rejection, suggesting a specific immune response to the iPS cells. Despite the significance of this study, there are criticisms that it may be largely inapplicable to clinical situations [264], both because they compared iPS cells to syngeneic ES cells, which cannot not be derived for human patients, and because of the use of pluripotent cells, which will not be used in clinical models. It is also important to note that the widespread use of autologous iPS cells may be economically infeasible, even if the cells are not immunogenic.

3. Tumor formation: One of the most prominent concerns in cell therapy is the propensity of many cell types to form tumors after transplantation. Because of their cancerous origin, there are

fears that hNT cells can produce tumors, but the retinoic acid treatment used to obtain hNT cells from N2N cells seems to suppress their tumorigenicity [265, 266]. Pluripotent cells, such as ES or iPS cells, can form teratomas or teratocarcinomas when implanted in a living host [115, 230]. In fact, as ES cells become karyotypically abnormal in cell culture, they acquire many mutations that make them more tumorigenic [267-269]. Human ES cells are routinely screened for karyotypic changes, but small additions and deletions within a chromosome, changes in mitochondrial DNA, and epigenetic changes that occur in culture can also select for populations of undifferentiated stem cells [270, 271]. Screening for such small mutations will likely be necessary if ES cells are to be used in clinical settings.

iPS cells have also been shown to have a risk of tumorigenicity when transplants are contaminated with undifferentiated cells. The two major concerns for tumorigenicity of iPS cells have been mutagenesis at viral insertion sites and continued expression of exogenous genes used for reprogramming [272]. c-Myc has been of particular concern as a possible oncogene [273]. In order to minimize these issues, transgene-free iPS cells have been created [124, 125, 274, 275] and some protocols have found ways to induce pluripotency without the use of c-myc [122, 276]. In addition, the tissue origin of iPS cells seems to play a role in tumorigenicity, with iPS cells derived from mouse embryonic fibroblasts or gastric epithelial cells having similar teratoma forming propensity like ES cells and iPS cells derived from hepatocytes or tail fibroblasts having higher risk of tumor formation [277]. As with teratocarcinomas, iPS cell tumorigenicity appears to be a result of cells that are resistant to differentiation, possibly because of incomplete reprogramming.

Interestingly, tumor formation from contamination of undifferentiated cells is dependent on the species of both the graft and host [278]. When undifferentiated murine cells were

xenotransplanted into a rat brain, tumors were small and relatively rare, occurring in 2 out of 22 animals. After transplantation to the contralateral hemisphere, these undifferentiated cells migrated to the ischemic infarct along the corpus callosum and differentiated into neurons. In mouse brain, however, undifferentiated mouse cells produced large malignant teratocarcinomas in the majority of animals, without migration or replacement of ischemic tissue. These tumors formed even after efficient neural differentiation in which a very small portion of cells remained pluripotent.

While xenotransplantation is unlikely as a viable cell therapy strategy in humans, these results do raise doubts about the applicability of xenotransplantation studies. Even if differentiation with a particular protocol is efficient enough to avoid tumor formation in xenotransplantation, a small number of contaminating cells may still induce tumorigenesis in homologous transplantation. Even very small numbers of contaminating undifferentiated ES cells caused tumors in nude mice [279]; further suggesting that any safe cell therapy using pluripotent cells will have to avoid such contamination.

We have empirically determined from work in our lab that if ES cells are efficiently differentiated into neural progenitors *in vitro*, their risk of tumor formation is drastically reduced. Many protocols have been tested to optimize the homogeneity of the transplant. While many transplantation studies reported tumor formation, there are also several successful examples of transplanting progenitors without tumorigenicity [85, 158, 170, 280, 281]. Nonetheless, it is extremely difficult to direct differentiation of pluripotent stem cells with 100% efficiency. Alternatively, methods to remove any undifferentiated cells from the transplant are being developed. In some cases, cells are sorted by staining for a pluripotency marker [282] or by adding GFP-conjugated proteins that allow for the removal of undifferentiated cells [283]. Other

strategies would cause apoptosis through the use of ceramide analogues [284] or survivin blockers [285], both of which selectively target pluripotent cells. Finally, the idea that a "suicide gene" should be included in transplanted cells so that they can be induced to undergo apoptosis if a tumor begins to form has been proposed [286].

H. Summary and conclusions

While a great deal of preclinical work has increased our knowledge of possible treatment options for both acute and chronic stroke, this area remains a largely unexplored. The promise of stem cell therapy offers hope for both protection of neural tissue in the acute phase of stroke and replacement of lost tissues (either through direct replacement or enhancement of endogenous mechanisms) in the chronic phase. As research moves forward, new technologies are incorporated into the field. Prior to the derivation of iPS cells, it was difficult to imagine a way to amass enough HLA-matched neural precursor cells for an effective cell therapy, but there are many laboratories using these cells in preclinical studies now. Optogenetics has also opened up new ways to gather information on cell incorporation into tissues and the effects of neural stimulation on stroke recovery. It is impossible to determine what studies new technology will allow for in the future.

A number of clinical trials investigating stem cell therapy in stroke are now ongoing, but these trials are still in Phase I and II, with a great deal of work ahead before cell therapy can become a standard stroke treatment. This is especially true in work intended to directly replace lost nervous tissue, as current trials typically do not expect long-term engraftment at the stroke site. These early treatments are using a number of modified and unmodified cell types and modes of transplantation, working through the safety concerns associated with each approach. The

STEPS guidelines provide a roadmap for moving forward with this emerging paradigm in stroke therapy.

The prospect of using stem cell therapy for stroke remains an exciting and promising avenue of exploration. It will be years before such treatments can be proven effective and eventually move into standard medical practice and emerging technologies will likely continue to alter the course of that path. With early preclinical and clinical successes, it seems likely that stem cell treatment will become a viable option for the treatment of stroke, offering an exciting possibility in a field with huge unmet needs.

Chapter III
Optogenetics

A. Historical overview and introduction to opsins

By definition, optogenetics refers to "the branch of biotechnology which combines genetic engineering with optics to observe and control the function of genetically targeted groups of cells with light, often in the intact animal" [287]. Unlike what the name implies, optogenetics is the use of light to control protein functions rather than genes. These functions include but are not limited to neuronal excitation, neuronal inhibition and modulation of biochemical signaling. This interdisciplinary exciting new manipulation technique has significantly advanced neuroscience as it provided molecular tools for the control of neuronal functions with high spatial and temporal specificity. For example, it is now possible to specifically excite or inhibit the dopaminergic cells of the substantia nigra without affecting the neighboring cells. To achieve this optogenetic control, researchers need vectors to deliver opsins to specific cells, technologies to deliver light to those cells, and methods to measure the read-outs after the light-induced perturbations.

The groundwork for optogenetics has been laid down in the twentieth century through pioneering work from different and maybe unrelated lines of research. From the discovery of bacteriorhodopsin in 1971 [288] to the breakthrough discoveries of both channelrhodopsin [289] and halorhodopsin [290], little incremental pieces of research led to the explosion of this new field in 2005 after a group of researchers succeeded to evoke action potentials with milli-second precision in mammalian neurons using channelrhodopsin-2 (ChR2) [291].

There are two arms for optogenetic manipulation: optogenetic sensors and actuators. Sensors translate a physiological cellular response such as a rise in intracellular calcium into light signals. Examples of sensors include detectors of changes in membrane potential (FlaSh, SPARC and voltage sensitive fluorescent proteins), detectors of changes in intracellular calcium

levels (Cameleon and Camgaroo) and detectors of synaptic transmission (synapto-pHluorin and sypHy). Actuators, on the other hand, translate light energy into a cellular physiological response. Most of the remaining discussion will focus on optogenetic actuators. The intrinsic properties of optogenetic actuators are essential for the proper application of optogenetics in neuroscience since electrical events in neuroscience such as action potentials and synaptic currents occur at a milli-second scale. Earlier attempts to control these micro-events failed because of many factors. Unlike earlier microbial opsins, newer actuators and all their derivatives are single subunit elements (compared to the multi-component character of earlier opsins), have low toxicity in mammalian tissue, have appropriate temporal kinetics for controlling neuronal events and are excited by low energy light in the visible spectrum [292, 293]. Actuators employ many tools but two classes of channels that transduce light of specific wavelengths into a biological response gain special attention. For the remaining of the discussion, we will focus on Channelrhodopsin (ChR) and halorhodopsin (HR) despite the presence of several other opsins such as the haloarchaeal proton pump bacteriorhodopsin.

When expressed in cells, these channels open in response to light of the proper wavelength and conduct ions altering the membrane potential. This leads to light-induced excitation (ChR; 470 nm) or inhibition (HR; 580 nm) of neurons with controlled frequency and timing. Both channels require the presence of all-*trans* retinal for proper functioning. All mammalian tissues, including the central nervous system, have sufficient retinal to operate these channels. ChR and HR can be genetically targeted to specific populations or sub-populations of cells (providing high spatial resolution), can control cell function on a millisecond scale (high temporal resolution down to a single action potential), and have proven to function in culture, acute brain slices, and living animals.

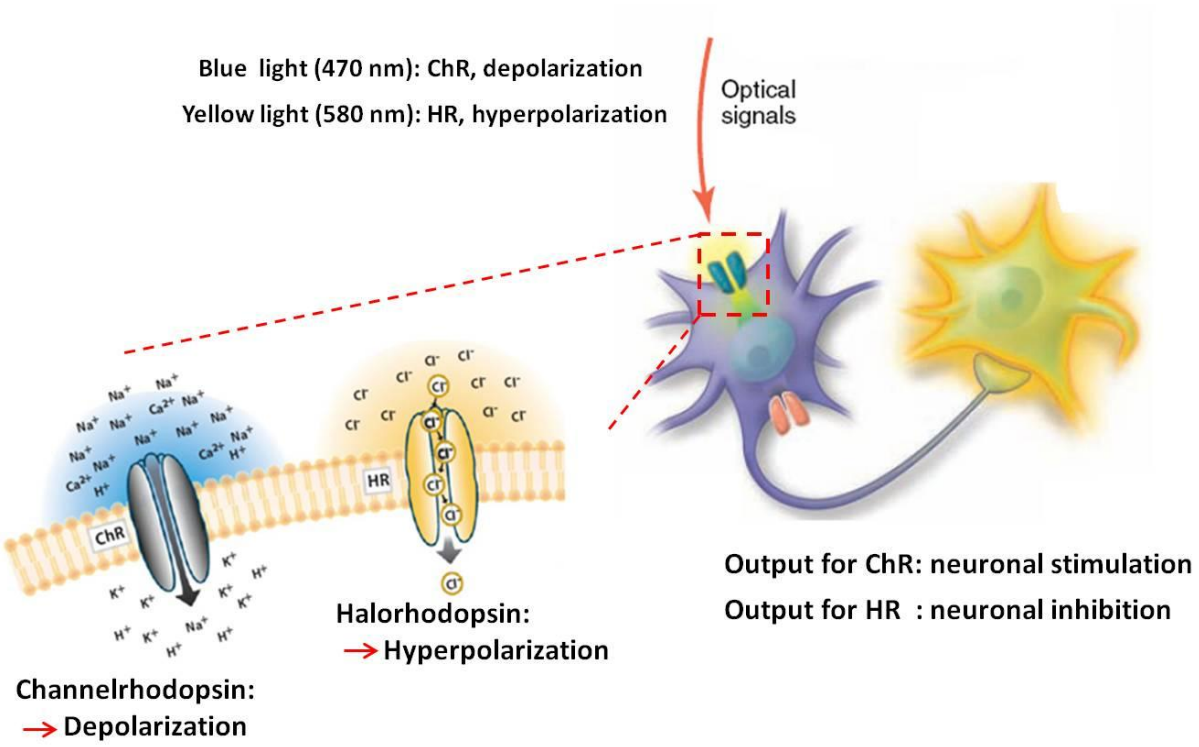

Fig. 3.1. Optogenetic actuators: Channelrhodopsin and halorhodopsin
Channelrhodopsin is a non-specific cation channel that opens in response to blue light of wavelength 473 nm. Opening of ChR causes membrane depolarization and subsequent neuronal stimulation. Halorhodopsin, on the other hand, is a chloride specific channel that opens in response to yellow light of wavelength 580 nm and causes hyperpolarization and subsequent neuronal inhibition.

Halorhodopsin is a light-activated chloride channels from archaebacteria [290]. When HR is opened by yellow light, chloride ions are pumped from the extracellular to the intracellular space leading to neuronal hyperpolarization. ChR, on the other hand, is light-activated non-specific cation channel [289]. When ChR is opened by blue light, cations (sodium, potassium, and protons) flow down their electrochemical gradients leading to neuronal depolarization. While these two channels represent the backbone for optogenetic control, amino acid sequence manipulation gave rise to new sets of channels with different kinetics, dynamics and biochemical properties.

In the previous few years, a wide variety of ChRs and HRs emerged through codon optimization and site-specific mutagenesis. In this respect, a humanized form of the ChR channel (hChR2) was created by substituting certain algae specific codons with mammalian codons [294]. Another major development was the design of a new ChR2 with a H134R mutation which had 2-fold increase in unitary conductance. Other variants of ChR2 have higher Ca^{2+} conductance [295]. Ca^{2+} gets to the intracellular space due to the activation of voltage-gated Ca^{2+} channels. These new calcium conducting ChR2 variants also increase intracellular Ca^{2+} via the calcium-induced calcium release mechanism and also have important implications for the Ca^{2+} activated potassium and chloride currents. Another major development came with the development of bistable ChRs. Step-function opsins (SFOs) can convert a brief blue light pulse into a stable step in membrane potential bringing the cell closer to the action potential threshold and increasing the chances of membrane spiking [296]. Despite these slow deactivation kinetics, this step-like membrane potential can be terminated with a brief pulse of yellow light (580 nm). These SFOs have a deactivation time-constant in the order of several seconds to several minutes [297] compared to the milli-second order of the regular ChR2 variants. Despite the fast kinetics of the traditional ChRs ($_T$ about 10ms), it was only until the ultra-fast ChR was developed when these channels could evoke neuronal spiking above 40 Hz with high fidelity [298]. This newly designed ChR variant is called ChETA (*Ch*R *E*123*T*/*A*) and can reduce extra spikes, eliminate plateau potentials generated with the regular ChRs and also allow sustained action potential trains up to 200 Hz, commensurate with many neuronal functions *in vivo*. Advances in design and conductance properties were not limited however to gain-of-function tools like ChRs. In fact, similar techniques of mutagenesis and codon replacement were used to modify and enhance loss-of-function tools such as HR. In contrast to the archaebacterial HR, a new and modified HR

was derived from *Natronomonas pharaonis* (NpHR) [299, 300]. While NpHR gave rise to an appropriate hyperpolarizing chloride current, it was shown that NpHR accumulate within the endoplasmic reticulum and had intracellular trafficking problems that made it difficult to use in mammalian tissue [301]. To circumvent this problem, scientists added an endoplasmic reticulum export motif from the Kir2.1 potassium channel and then a neurite trafficking sequence from the same channel to yield eNpHR2.0 and eNpHR3.0, respectively. Both eNpHR constructs had better extracellular membrane trafficking and enhanced photocurrents [301, 302]. All these modifications lead to the creation of ChRs and HRs that are suitable for exploring behaviors in freely moving animals [294, 303].

In addition to these loss- and gain-of-function tools, optogenetics have benefited from another fusion experiment where vertebrate rhodopsins and ligand-gated G-protein coupled receptors are fused to form what is referred to as OptoXRs. OptoXRs allow for the light controlled G-protein related signaling with high spatial and temporal resolution in freely moving animals [304].

All these modifications have revolutionized the application of optogenetics in different domains in neuroscience. Research that was recently considered impossible is now feasible and neuroscientists are steadily becoming able to untangle the secrets of neuronal circuits and gross behaviors.

B. Opsin delivery

Along with the continuous development in design of ChRs and HRs, modes of targeted delivery were also growing. To date, optogenetics has mostly benefited from using viral expressions systems as mean of delivery to mammalian cells *in vitro* and *in vivo*. Targeted

delivery with viral vectors is considered the most efficient delivery method of optogenetic actuators. In fact, lenti-viral [305] and adeno-associated viral [306] vectors (LV and AAV, respectively) gain special attention as delivery methods in the neurosciences because they are relatively easy to prepare (LV are much easier to prepare than AAV), flexible in use, have low toxicity and show a very high efficiency of transduction of high copy numbers of genes over a long period of time in the central nervous system. Both vectors have been extensively and successfully used in optogenetics research in mice [307], rats [308] and primates [309]. LV and AAV systems are not equivalent though. Although AAV are safer in a laboratory setting, have lower immunogenicity and can be produced in extremely high titers, LV are easier to prepare and offer the advantage of packaging larger insert genes. AAV can carry only up to 5 Kbps, while LV can hold up to 10 Kbps. In addition, LV are more restricted in their diffusion in vivo compared to AAV. AAV diffuse more widely in vivo but their diffusion depends on the specific serotype. Two AAV serotypes gained special attention in the central nervous system: AAV2 and AAV5 [310]. AAV5 has more viral spread than AAV2 in vivo. Thus, AAV2 and LV can be used where a more local transduction is needed (such as the subgranular zone of the hippocampus or the amygdale) whereas AAV5 can be injected where larger zones are to be transduced (such as the striatum) [311].

Another advantage for using these viral vectors is the ability to specifically target the region of interest. This specificity is conferred through two mechanisms: 1) the specific promoter upstream the opsin gene and 2) the viral tropism for different tissue types. Lentiviral vectors are more likely to infect excitatory neurons while AAV2 vehicles express more in inhibitory cells [312]. Certain promoters, on the other hand, are more likely to transfect certain groups of cells. For example, EF1α, while not neuron specific, has a very high expression in neurons and

CamKIIα is highly specific to excitatory glutamatergic neurons. While these rules hold for many applications, it is important to mention that in order to express a certain opsin gene, a given cell must express that promoter of interest and the receptors for the specific viral delivery method. To increase options for opsin delivery and capitalizing on the large number of Cre-recombinase expressing transgenic animals, scientists also designed AAV vectors where the opsin is double floxed with loxP sites (called Doublefloxed Inverse Open reading frame or DIO). These Cre-inducible DIO-AAV vectors ensure high opsin expression in regions where Cre-recombinase is expressed in the transgenic animals. This technique ensures a highly specific expression with high gene copy number and exceptional regional and temporal specificity. A full list of all available vector backbones can be found on www.stanford.edu/group/dlab/optogenetics. In summary, choosing the optimal delivery method for opsin expression *in vitro* or *in vivo* should take into consideration the opsin gene itself, the cells to be expressed into, the size of the tissue, the promoter used and the delivery vehicle.

An important consideration, however, is the potential toxicity of opsin expression. While generally safe and do not cause major cell death, high level expression of opsin especially under highly active promoters such as CMV might affect cellular membranes and eventually cell physiology and may cause toxicity. Toxicity might also arise with promoters of moderate activity if expression was maintained for a long time. Toxicity should be taken into consideration in long term studies and no-light controls are essential in such situations.

In addition to opsin expression using viral vectors, scientists have created two transgenic animals that express ChR2 and eNpHR under the control of the Thy1 promoter [313, 314]. In both these animals, opsin expression is largely restricted to projection neurons such as cortical

and hippocampal neurons. These animals have been and will continue to be largely used in exploring the mysteries of brain wiring and animal behavior [315-317].

C. Light delivery

The delivery of opsins to targeted cells is only the first step in any optogenetic investigation. After opsin delivery, appropriate light has to be delivered to activate ChRs or HRs for a proper readout. The process of delivering light is complicated and varies depending on the investigator's scientific inquiry, the specific properties of the opsin used, and the wavelength, intensity, duration and frequency of the light source. The wavelength is dictated by the characteristics of the opsins expressed whereas the intensity, duration and frequency of the light also depend on the particular experiment needed. For example, some opsins like ChR2 require brief pulses of blue light of 473 nm wavelength, whereas halorhodopsins may require continuous light of 580 nm for optogenetic inhibition. SFOs, on the other hand, require a brief pulse of blue light to achieve a long duration of depolarization that can be terminated with a brief pulse of yellow light [296]. Light power is also an important parameter in optogenetics and it is typically measured in mW/mm^2 (*in vitro*) or mW/mm^3 (*in vivo*). Two important thresholds have to be overcome to achieve a functional read-out independent of all other parameters. Both the expression level of the desired opsin and the light power have to be high enough for an appropriate response to be recorded. If either is insufficient, changes in membrane potential will not occur and a response cannot be read.

While *in vitro* light delivery to neuronal cultures or brain slices is more straight-forward, light delivery to *in vivo* targets in deep brain tissue is more complicated. Inside the brain, light is absorbed and scattered by brain tissue leading to a reduction in the delivered light intensity. In a

study of the properties of light propagation in brain tissue for *in vivo* experiments [318], it was found out that after 100 μm inside brain tissue, the transmitted light power was only about 50%, and at 1 mm depth, the total transmitted power was only about 10%. This study stresses the importance of considering a light source with enough intensity for *in vivo* optogenetic modulation of deep brain targets.

Just like the toxicities that potentially might arise with opsin expression, light might produce some undesired effects as well. Since brain tissue is very sensitive to temperature changes, light-generated heat might advertently affect brain physiology, animal behavior and also might cause toxic side effects. These effects might necessitate a no-opsin control to account for potential light toxicity.

D. Optogenetics and stem cells

Studying stem cell integration after transplantation is essential for a full and long-term characterization of stem cells *in vivo*. Earlier studies used electrophysiological approaches to investigate whether transplanted stem cell-derived neural cells functionally integrate within the established host brain neuronal circuits [319-321]. However, electrophysiological stimulation and recording paradigms are cumbersome, lack temporal and spatial resolution for studying information processing in living multi-cellular networks and may lead to spike generation and recording from unintended cells and passing axonal fibers. Luckily, the advent of optogenetic technologies has provided tools that allow *in vivo* probing and mapping of neuronal circuits with very high temporal and spatial resolution. These tools allow for very accurate control and observation of biological processes within target cells using light stimulation or detection [287, 322, 323].

The advent of optogenetic actuators has allowed scientists to accurately map brain circuits [313, 324-327], dissect pathways of learning and fear [328, 329], and understand the role of neural cells in pathology and physiology [330, 331]. Stem cell therapy has also benefited from the logarithmic growth in optogenetics. Integrating optogenetics into cell based therapies through the over-expression of ChR2 in stem cell-derived neural precursors, Weick et al. demonstrated that ChR2 expressing human ES-cell derived neurons receive excitatory and inhibitory post-synaptic current in acute brain slices from SCID mice [332]. In another study where ChR2 was over-expressed in mouse ES cells, optical excitation increased the expression of nestin and β–III-tubulin in ChR2-ES cell-derived neural precursors and neurons, respectively [333]. Tonnesen et al. used optogenetic methods to demonstrate that neural stem cell-derived dopaminergic neurons functionally integrate in the denervated striatum in an animal model of Parkinson's disease [334]. This technology offers an invaluable set of tools for studying stem cell differentiation and *in vivo* integration after transplantation.

E. Summary and conclusions

The advent of optogenetics will revolutionize neuroscience and logarithmically increase our knowledge about brain physiology, pathology and animal behavior. We aim to develop tools that could be used to study brain physiology, stroke pathology and stem cell biology building on the credentials of optogenetic tools *in vitro*, *in vivo* and *ex vivo*.

Chapter IV
Rationale, Aims, and Experimental Methods

A. Rationale and significance:

Although stroke is the fourth leading cause of death and the primary source of adult disability in the US, FDA approved medications for stroke treatment are extremely limited. Clinical trials testing hundreds of candidate drugs that were highly effective in animal models repeatedly failed. Successive failures in clinical trials prompted scientists to look for therapy in other avenues. The generation of neurons and glia from embryonic (ES) stem cells offers a promising cell based strategy to repair brain circuits and improve recovery of brain functions damaged by ischemia. However, the ethical debate around human ES cells has hindered ES cell-based therapies for human clinical conditions.

The formation of human iPS cells represents a great opportunity for the treatment of neurodegenerative diseases such as stroke. Moreover, vector-free human iPS cells are very promising to circumvent the risk of tumor formation associated with their virus-based counterparts. The development of cell-based therapeutic strategies to treat ischemia requires a greater understanding of the protective mechanisms through which cell based therapies provide benefit. The elucidation of the underlying mechanisms is critical to maximize therapeutic strategies, limit their negative side effects and facilitate their bench-to-bedside transition. This thesis focuses on testing the therapeutic efficacy of vector-free human iPS cells in the treatment of ischemic stroke in adult mice and studying the mechanisms of the induced benefit.

One challenge in stem cell therapies is to investigate whether the transplanted neural precursor cells functionally integrate within the established host brain neuronal circuits. Very few studies attempted to answer this question. No studies attempt to answer this question in a stroke model. Moreover, Current technologies do not provide clear-cut answers to the central question investigating functional integration of stem cells after brain transplantation. For

example, although electrophysiological recording techniques have answered many questions about the neuronal behavior of stem cells *in vitro*, the evidence of full neuronal phenotype *in vivo* is still lacking. Electrophysiological stimulation and recording paradigms lack temporal and spatial resolution for studying information processing in living multi-cellular networks. In testing the mechanisms of benefit, we established optogenetic tools and techniques for the *in vitro, ex vivo* and *in vivo* investigations. Our unique optogenetics experience will allow us to study stroke pathophysiology and novel therapeutic approaches in stroke animals while avoiding the short-comings of the traditional electrophysiological tools. In addition, these optogenetic tools will allow us to test stem cell integration after transplantation and enhance stem cell-based therapies in stroke models. To date, this is the first utilization of optogenetics in the stroke field. This will be the first attempt in which cell integration will be questioned *in vivo* in a disease model. This optogenetic-based strategy can be used to test functional integration of stem cells in other neurodegenerative diseases as well.

Along the same lines, this thesis investigated a novel technique to increase the efficiency of neuronal differentiation from mouse ES and iPS cells *in vitro*. By combining different techniques, we created a novel approach that resulted in 4-5 fold increase in functionally active neurons from mES cells. The application of this new differentiation protocol shall accelerate the *in vitro* investigation of stem cell-derived neurons and their transplantation in animal disease models.

This thesis hypothesized that: 1) hiPS cells can be differentiated *in vitro* to functional neurons, 2) hiPS cells enhance functional recovery after stroke, 3) optogenetics tools can be applied in stroke and stem cell research and 4) rotary cultures increase the yield of neuronal differentiation. In pursuing these questions, this dissertation not only contributes to the

understanding of a clinically relevant therapy for stroke, but also, to understanding the mechanism of such benefit and developing tools and protocols to optimize stroke research and stem cell therapies.

B. Specific aims:

Aim 1. To test the therapeutic efficacy of vector-free hiPS cells in treating ischemic stroke in adult mice

Hypothesis: hiPS cells differentiate to functional neurons *in vitro* and enhance functional recovery after stroke.

1.1. To differentiate human iPS cells to neurons and morphologically and functionally test hiPS cell-derived neurons *in vitro.*

1.2. To assess the survival, differentiation and functional recovery after hiPS cell transplantation in a mouse model of focal ischemic stroke.

1.3. To test mechanisms of stem cell induced benefit including measuring blood flow, neurogenesis and angiogenesis and trophic support.

Aim 2. To establish optogenetic tools in stroke research and stem cell therapies

Hypothesis: Optogenetic tools represent a unique opportunity for the *in vitro* and *in vivo* investigation of stroke pathophysiology and stem cell research.

2.1. To over-express ChR2 in cortical and miPS cell-derived neurons and test their responses to light stimulation.

2.2. To over-express ChR2 in cortical layer IV and assess field potential in brain slices in response to light stimulation.

2.3 To test light stimulation as a therapeutic approach in stroke mice expressing ChR2 in the barrel cortex.

Aim 3. **To increase neuronal yield from mouse ES and iPS cell differentiation using rotary cultures**

Hypothesis: Rotary cultures increase the yield of neuronal differentiation from mouse ES and iPS cells.

3.1. To assess neurosphere morphology, neural precursor yield and relevant gene expression in neurospheres using rotary cultures.

3.2 To test the morphology and functional response of mES cell-derived neurons from rotary cultures.

3.3 To test the yield and functional response of miPS cell-derived neurons from rotary cultures.

D. Materials and Methods:

Chapter V:

hiPS cell cultures

Vector-free transgene-free hiPS cells (iPS-DF19-9/7T) were purchased from the WISC stem cell bank (WiCell Research Institute, Madison, WI). The cells used for differentiation and transplantation were no older than passages 30 - 40. hiPS cells were maintained in feeder- and serum-free media (mTeSR1, Stem Cell Technologies, Vancouver, BC, Canada) on hES-qualified Matrigel (BD Biosciences, Sparks, MD). mTeSR1 media was changed every day and cells were passaged using dispase every 5-7 days after manual removal of differentiated colonies. For more information on the maintenance of hiPS cells with mTeSR1, please refer to the guidelines published by Stem Cell Technologies.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cell cultures (hiPS and hiPS-NPs) using the TRIzol reagent (Invitrogen Inc, Carlsbad, CA). Reverse transcription was performed with 1 μg total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA). SYBR green qRT-PCR was used to assess the relative levels of our target genes using the Applied Biosystems StepOnePlus machine. The primers used were:

GAPDH F: GTGGACCTGACCTGCCGTCT, R: GGAGGAGTGGGTGTCGCTGT

Oct-4 F: GGAGGAGTGGGTGTCGCTGT, R: ACTTCACCTTCCCTCCAACC

Nanog F: TTTGGAAGCTGCTGGGGAAG, R: GATGGGAGGAGGGGAGAGGA

Pax-6 F: TGTCCAACGGATGTGTGAGT, R: TTTCCCAAGCAAAGATGGAC

Fold change was calculated by the delta (delta Ct) method using the GAPDH amplification as the internal control.

Neural induction protocol

To obtain neural precursors, we used a modification of the adherent differentiation protocol described previously [189]. Cells were dissociated using accutase (Invitrogen, Carlsbad, CA) for 15 min and then plated on Matrigel (BD Biosciences) coated plates at a density of 18,000 – 20,000 cells/cm^2 in mouse embryonic fibroblast (MEF) conditioned medium [plus 10 ng/ml basic fibroblast growth factor (human recombinant bFGF, R&D, Minneapolis, MN) and 10 μM ROCK inhibitor (Y27632, Sigma, St. Louis, MO)]. After the cells had reached confluence (3-5 days later), the medium was changed to KSR medium (Knockout DMEM, 15% knockout serum replacement, 1x L-glutamine, 1x non-essential amino acids, 50 mM β-mercaptoethanol) with the addition of 3 μM dorsomorphin (Tocris, Ellisville, MO) and 10 μM SB431542 (Stemgent, Cambridge, MA). Cells were grown in this media for five days, with daily

media changes. On day 5, media was changed to a 1:4 mixture of N2 : KSR media (N2 media has DMEM/F12, N2 supplement, 1x L-glutamine, penicillin/streptomycin; Invitrogen) without the TGF-β inhibitor (SB431542). On days 7 and 9, the media was changed to 1:1 and 4:1 N2 : KSR media, respectively. On day 11, neural precursors were collected for Western blotting or RT-PCR, fixed for staining or dissociated with accutase for transplantation or for terminal differentiation. For terminal neuronal differentiation, neural precursors were dissociated into a single cell suspension using accutase and then filtered through a 200 μm mesh. 100,000 – 150,000 cells were plated on Matrigel in a 1:1 mixture of N2 and B27 medium (B27 media has Neurobasal media, B27 supplement, 1x L-glutamine, penicillin/streptomycin; Invitrogen) with 10 ng/ml bFGF. Media was changed every third day for 4 weeks. 4 weeks later, cells were clamped for electrophysiological recording and then fixed with 4% PFA for staining.

Immunocytochemistry

For immunocytochemictry, cells were fixed with 4% paraformaldehyde, post-fixed with a 2:1 mixture of ethanol : acetic acid, permeabalized with 0.2% Triton-X-100, and blocked with 1% fish gelatin. Primary antibodies [Nanog (1:400), OCT4 (1:400), SOX2 (1:400), β-III-Tubulin (1:200) - Cell Signaling, Danvers, MA; Tuj-1 (1:400), PAX6 (1:100) - Covance, Princeton, NJ; Nestin (1:200), NeuN (1:400), Neurofilament (1:400), MAP2 (1:400) - Millipore; Synapsin 1 (1:400) - Calbiochem] were applied overnight at 4^{o}C and Alexafluor or Cy-3 conjugated secondary anti-rabbit or anti-mouse antibodies (1:300) were used. Hoechst 33342 (Molecular Probes, Invitrogen) was used to counter-stain cell nuclei. All images were taken using an upright Olympus fluorescence microscope.

Western blot

Western blot analysis was performed to analyze protein expression in hiPS cell-derived neural precursors and from brain tissue after transplantation following previous procedures [335]. In brief, cells were scraped from dishes using lysis buffer. Brain tissue was also lysed in lysis buffer containing 25 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 2 mM sodium orthovanadate, 100 mM NaF, 1% triton, leupeptin, aprotinin, and pepstatin with continuous manual homogenization. Lysate was then spun at 13,000 rpm for 15 minutes and supernatant was collected. Protein concentration was determined using BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein were resolved on SDS-PAGE using gradient gels (6-18%) and gels were blotted onto PVDF membranes (Amersham, Buckinghamshire, UK), blocked with 5% BSA in TBST buffer (20 mM Tris, 137 mM NaCl and 0.1% tween) and incubated overnight with primary antibodies against erythropoietin EPO (1:50, Santa Cruz), EPO-receptor (1:50, Santa Cruz), vascular endothelial growth factor VEGF (1:100, Novus Biologicals), Flt-1 or VEGF-receptor 1 (1:50, Santa Cruz), VEGF-receptor 3 (1:500, Chemicon), brain-derived neurotrophic factor BDNF (1:50, Santa Cruz), CXCR4 (1:500, R&D), Tie-1 and Tie-2 (1:50, Santa Cruz), Angiopoietin-1 (1:500, Abcam), and Angiopoietin-3 (1:400, Oncogene). After 3 washes with TBST, blots were incubated with HRP-conjugated secondary antibodies (anti-rabbit or anti-mouse, 1:2000, Bio-Rad, CA) in 5% BSA for 1 hr. Blots were developed using Pierce ECL Western blotting substrate (Thermo Scientific, IL). The level of protein expression was normalized to β-actin controls.

Electrophysiology

Whole-cell patch clamp recording was obtained from hiPS cell-derived neurons 4 weeks (28 days) after terminal differentiation using an EPC9 amplifier (HEKA Elektronik, Lambrecht,

Germany) at 21–23°C. The external solution contained 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10mM HEPES, and 10 mM glucose at a pH of 7.4. Internal solution consisted of 120 mM KCl, 2 mM $MgCl_2$ 2, 1 mM $CaCl_2$, 2 mM Na_2ATP, 10 mM EGTA, and 10 mM HEPES at a pH of 7.2. Recording electrodes pulled from borosilicate glass pipettes (Sutter Instrument, USA) had a tip resistance between 5 and 7 MΩ when filled with the internal solution. Series resistance was compensated by 75-85%. Linear leak and residual capacitance currents were subtracted on-line using a P/6 protocol. Action potentials were recorded under current-clamp mode using Pulse software (HEKA Elektronik). Data were filtered at 3 KHz and digitized at sampling rates of 20 KHz.

Transient focal ischemia animal model

All experimental and surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University. Middle cerebral artery occlusion (MCAo) was performed according to a modified version of [106]. In brief, 8-10 weeks old C57BL6 mice (National Cancer Institute) were anesthetized using an intraperitoneal (IP) injection of 4% chloral hydrate. The right middle cerebral artery (MCA) was permanently ligated by a 10-0 suture (Surgical Specialties Co., Reading, PA) accompanied by a bilateral 7-min ligation of the common carotid arteries (CCA). During CCA occlusion, Barrel cortex blood flow was reduced to less than 20% as measured by laser Doppler scanning (Fig. 4B and 7C) ($p<0.05$, Student's *t-test*). Laser Doppler blood flow analysis was performed as described in [336] with the laser tip pointing to the area supplied by the MCA. Local cerebral blood flow was measured before stroke induction, during stroke (during MCAo), and 3, 7 and 14 days after transplantation. Stroke was visualized using the 2,3,5-triphenyltetrazolium chloride (TTC) staining procedure detailed in [337]. Body temperature was monitored during surgery and maintained at 37.0 °C using a

temperature control unit and heating pads. Animals were euthanized by decapitation at different time points after ischemic stroke. Brains were immediately removed, mounted in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA) and stored at −80 °C for further processing.

Cell transplantation after ischemic stroke

Seven days after stroke, mice were anesthetized for the transplantation of hiPS cell-derived neural precursors or media vehicle. On day 11 after neural induction, neural precursors were treated with Hoechst 33342 (1:10000) for one hour and then dissociated into a single cell suspension using accutase, and filtered through a 200 μm mesh. They were re-suspended in N2 media. Each animal received a total injection of 4 μl transplantation solution (100,000 cells/μl or media vehicle) at 2 different sites (the ischemic core and the penumbra region). The solution was injected using a 5 μl Hamilton syringe (Hamilton Company). Cells were injected very slowly (total time of injection 10 min at each location) and the needle was allowed to stay for 5 min after the injection to allow for diffusion of the transplanted cells.

Histological and immunohistochemical assessment and cell counting

Animals were sacrificed 28 days after transplantation and their brains immediately frozen on dry ice. Brain coronal sections were cut at 10 μm thickness using a cryostat (Leica CM 1950). Staining for NeuN and Collagen IV was performed following previously described protocols [338, 339]. In brief, sections were dried on a slide warmer for 30 min and fixed with 10% buffered formalin for 10 min. Brain sections were washed with −20°C pre-cooled ethanol : acetic acid (2:1) solution for 10 min and finally permeabilized with 0.2% Triton-X 100 (in PBS) for 5 min. Sections were then blocked with 1% donkey serum (Sigma) in PBS for 1 hr at room temperature, and incubated with the primary antibodies collagen IV (CoIV, 1:400, Millipore,

CA, USA) and NeuN (1:400, Millipore, MA, USA) overnight at 4°C. Slides were incubated with anti-mouse and anti-goat secondary antibodies for 1 hr at room temperature. Vectashield mounting media for fluorescence (Vector Laboratory, Burlingame, CA) was used to cover-slip slides for microscopy and imaging analysis. BrdU (Bromodeoxyuridine) was diluted in sterile saline. BrdU was injected intra-peritoneally (0.05 mg/kg; i.p.) beginning 72h after MCAO and then daily until sacrifice. For staining BrdU (1:200; AbD Serotec; Raleigh, NC), sections were incubated in cold methanol (-20 °C) for 15 min after the formalin step, incubated in 2N HCl at 37 °C for 1 hour and finally neutralized by washing in borate buffer three times. The staining was completed as described earlier starting at the Triton-X-100 step.

Nissl staining was performed by first fixing the sections in a 1:1 mixture of formalin and acetic acid for 10 min. Sections were then placed in a working solution of Cresyl violet, acetic acid and sodium acetate for 20 minutes, rinsed with 70% ethanol and finally dried overnight.

Cell counting was performed following a modification of the principles of design-based stereology. Systematic random sampling was employed to ensure accurate and non-redundant cell counting [340]. Every section under analysis was at least 100 μm away from the next. For each animal, six 20-μm thick sections spanning the entire region of interest were randomly selected for cell counting. Counting was performed on 6 randomly selected non over-lapping fields per section. Sections from different animals represent the same area in the anterior-posterior direction.

Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick end (TUNEL) staining of cell death

TUNEL staining was performed using a commercial kit (DeadEnd™ Fluorometric TUNEL system, Promega, Madison, WI, USA) to label dying and dead cells in the brain 48 hours after transplantation. The instructions were followed as dictated in the instructions manual. In brief, brain sections were placed in equilibration buffer and incubated with nucleotide mix and rTdT enzyme at 37 °C for 1 h. The reaction was stopped with 2× SSC.

Adhesive removal test

The adhesive removal test was performed according to [341]. In brief, a piece of adhesive tape was placed on each (right and left) forepaw and the time-to-contact (latency) and the time-to-remove (removal) the tape was recorded. Animals were trained for three days before stroke induction to get a basal level of performance. The test was performed before stroke (training), just before transplantation, and 7, 14, 21 and 28 days after transplantation. The final result (fold change compared to baseline) is the ratio of the time-to-contact (or time-to-remove) at each time point to the time-to-contact (or time-to-remove) immediately after training and is the average of 3 trials separated by at least 15 minutes at each time point.

Intrinsic Optical Signal (IOS) imaging

IOS imaging was used to assess the local neuronal activity [106]. Sham, media control and hiPS cell-derived neural precursor transplantation (30 days after transplantation) animals were anesthetized and the exposed cortex was rinsed with sterile buffered saline at 37 ^{o}C and cover-slipped before imaging under green light (570 nm) with a CCD camera. The green light is transmitted to the penumbra region through the same cranial window used for stroke induction.

We detected the signal reflected by blood hemoglobin after absorbing the green light. Imaging was performed with and without whisker stimulation for sham animals, media injection controls, and transplantation animals. The resulting images were processed using ImageJ. Five animals were imaged in each group.

Whisker stimulation protocol

The whisker stimulation (WS) protocol was followed as previously described [73]. Mice were divided into 4 groups: 1) ischemic stroke + media injection group, 2) ischemic stroke + WS, 3) ischemic stroke + hiPS and 4) ischemic stroke + WS + hiPS cell transplantation. For whisker stimulation, whiskers on the left side of the mice's face were swiped in a rostro-caudal direction with the wood end of a cotton swab for 15 min at about 140 strokes per minute, three times per day. During and after the stimulation, animals were allowed to move freely in the cage and no anxious behavior was observed. WS started immediately after transplantation and the adhesive removal test was performed 1, 2 and 3 weeks after transplantation.

Statistical analysis

All results are expressed as mean ± S.E.M. Statistical comparisons were made with Student's *t-test*, one- or two-way analysis of variance (ANOVA) with Bonferroni's *post-hoc* analysis to identify significant differences. $p<0.05$ was considered significant for all comparisons.

Chapter VI:

Primary Cortical Neuron Culture

As previously described, primary neuronal cultures were isolated from E15 Swiss Webster fetal mice by dissecting their cerebral cortex [342]. In brief, the mantle of the

embryonic cortical brain tissue was isolated in dissection media containing Hank's balanced salt solution and glucose and then incubated in 0.1% trypsin-EDTA at 37 °C for 30 minutes. Tissue was dissociated by pipetting with a glass pipette in Minimal essential media (Gibco) containing 10% horse serum (Gibco). 800,000 cells were plated in each 3.5 cm Laminin coated dish. Cells were maintained in Neurobasal media with B-27 supplement (50X) and L-glutamine (400X) (Invitrogen).

Mouse iPS cell culture maintenance and neuronal differentiation (4-/4+ protocol)

Please see next Methods section of Chapter VII.

Lenti-virus preparation

The lenti-viral DNA was acquired from Addgene. The envelope plasmid (VSV-G) and the packaging plasmid (delta8.9) were generously donated by the Gross lab. Lenti-viruses were prepared using the HEK 293FT cells cultured in 10% FBS and 1 % penicillin-streptomycin in DMEM.

For 5 15-cm dishes, we prepared: 125 µg vector plasmid, 63 µg VSV-G plasmid, and 94 µg delta8.9 plasmid. All plasmids were mixed in a 50 ml tube to which we added 3.3 ml 0.1X TE (Tris 1 mM and EDTA 0.1 mM at pH 8.8), 1.75 ml ddH_2O and 565 µl of 2.5 M $CaCl_2$ (in ddH_2O). After mixing briefly, we added 5.7 ml of HBS2X (280 mM NaCl, 100 mM Hepes, and 1.5 mM Na_2HPO_4 with $7.11<pH<7.13$) drop wise under agitation by mild vortexing. A whitish precipitate was formed in the solution of which we added 2.25 ml to every 15-cm dish of 70% confluent HEK 293FT cells. Media was replaced 14-16 hours later with fresh HEK cell media. We collected the supernatant every 24 hours for the next three days. The supernatant was pooled, centrifuged at 1500 rpm for 5 min and filtered to remove cell debris. It was then concentrated by

ultracentrifugation using 20% sucrose solution in a SW32 Ti rotor at 28000 rpm at 4 °C for 2 hours. Virus pellet was resuspended in PBS.

Viral transduction of cortical and miPS cell-derived neurons

Viruses were stored at -80 °C. Lentiviruses had a titer of 10^9-10^{10} viral particles/ml. AAV5 had a titer of 10^{12} viral particle/ml. For all transductions, viral particles were added 1-2 days after plating. Viral expression started at least 6 days after infection.

Staining of cortical neurons and miPS cell-derived neurons

Please refer to ***Immunocytochemistry*** in the Methods section of Chapter V. We also used the Millipore mouse monoclonal antibody (# 05-532) against CaMKII-α (1:100).

Electrophysiology and light stimulation of cortical neurons and miPS cell-derived neurons

Please refer to the ***Electrophysiology*** in the Methods section of Chapter V. For light stimulation, we used a DPSS blue (473 nm) laser (Shanghai Laser & Optics Century). Each light pulse duration was 10 ms at 2, 5, 10 or 20 Hz.

Ex-vivo optogenetics experiments

For virus injection, C57BL/6 mice were anesthetized with isoflurane on a stereotaxic frame. Skull was exposed and a burr hole was drilled over the center of the barrel cortex (AP: -1mm, ML: ± 3mm from the Bregma and 500 μm deep).

Both ChR2 transgenic mice and C57BL/6 animals were anaesthetized, decapitated, and their brains were removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) containing: 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 26 mM $NaHCO_3$, 2.0 mM $CaCl_2$, and 10 mM glucose, saturated with 95% O_2 and 5% CO_2, at pH 7.4. Thalamocortical slices were prepared following the description in [343]. 400-μm thick slices

were obtained and collected with a soft brush, then incubated at room temperature 23-24 °C in aCSF for 60-90 min before recording.

Thalamocortical slices were transferred to a small recording chamber (RC-22C, Warner Instruments, Co., Hamden, CT) on a fixed-stage microscope (Olympus CX-31, Olympus Co., Tokyo, Japan) and superfused at the rate of 3 ml per min with aCSF at 23 °C. Under the microscope, the barrel field was clearly visible. To record excitatory activity in layer IV, a glass microelectrode filled with aCSF with resistance of 3-4 MΩ was placed 500 μm below the surface of cortex. Recording electrodes were placed 200 μm (located by Narishige Hydraulic, MO-103 Micromanipulator) below the slice surface. The stimulation output (Master-8; AMPI, Jerusalem, Israel) was controlled by the trigger function of EPC9 amplifier (HEKA Elektronik, Lambrecht, Germany). The evoked field response was recorded in current-clamp mode of EPC9 amplifier. Data was filtered at 3 KHz and digitized at sampling rates of 20 KHz using Pulse software (HEKA Elektronik).

For the *ex vivo* experiments, blue laser light (473 nm) was illuminated over areas of mcherry or YFP expression. The duration of each light pulse was 15 ms.

Stroke induction

Please refer to ***Transient focal ischemia model*** in the Methods section of Chapter V.

Adhesive removal test

Please refer to ***Adhesive removal test*** in the Methods section of Chapter V.

Assessment of ischemic infarct volume

For measuring infarct volume, brains were sliced into 1-mm coronal sections in a mouse brain matrix (Harvard Bioscience) and incubated in 2% 2,3,5-Triphenyl Tetrazolium Chloride (TTC) at 37^{o}C for 5 min. Brain sections were scanned and infarct volume was determined using

the NIH ImageJ program using six equivalent brain slices from each animal. Infarct volume (in mm^3) was measured by multiplying the area of staining in each slice by slice thickness (1 mm) and summing the volume of all slices for each animal. The indirect infarct volume ratio takes into account brain swelling after stroke and includes the contralateral hemisphere volume into the calculations.

Chapter VII:

Mouse ES and iPS cell culture and differentiation

Mouse D3 ES cells were prepared from stocks and mouse WP5 iPS cells were purchased from Stemgent (Stemgent Inc, USA). Cells were grown and differentiated in rotary and static conditions as previously described [179, 344]. Briefly, undifferentiated cells were maintained in T25 flasks in ES cell growth media (ESGM) consisting of Dulbecco's modified eagle media (with L-glutamine, without pyruvate, Gibco), supplemented with 10% fetal bovine serum, 10% newborn calf serum, 8 µg/ml adenosine, 8.5 µg/ml guanosine, 7.3 µg/ml cytidine, 7.3 µg/ml uridine, 2.4 µg/ml thymidine, leukemia inhibitory factor (LIF, Gibco) at 1000 units/ml and β-mercapto-ethanol.

For neuronal differentiation, cells were harvested from the growth flasks by trypsinization with 0.25% trypsin and EDTA for 5 min. Cells were seeded onto standard 10 cm bacterial Petri dishes in ESGM lacking LIF and β-mercapto-ethanol (ESIM). After 2 days, the media was replaced and cells were then returned to the culture dish for an additional 2 days. The culture media was then replaced with ESIM containing $5x10^{-7}$ M retinoic acid (RA, all-trans retinoic acid, Sigma). Media was replaced again 2 days later to ESIM plus RA. Cells treated by this method, designated the "4-/4+" protocol, were ready for further *in vitro* differentiation. For

the rotary experiments, the Petri dishes were placed all the time on a Stovall Belly Button Shaker.

At day 8, cells were harvested by dissociating the neurospheres (using trypsin/EDTA) and plated onto Laminin coated dishes in SATO media (containing 100 mM MEM pyruvate, 1x SATO, N-acetyl cysteine, 1M HEPES stock, 5% fetal bovine serum and 5% newborn calf serum in DMEM) for five days. At different time-points, EBs or neurospheres were precipitated by centrifugation (1000 rpm, 3 min), embedded in O.C.T compound (Sakura, Finetek, CA, USA) and then stored at -80 °C. Frozen tissue was cut with a cryostat Vibratome (Leica CM 1950) into 10 µm thick sections for staining.

5-bromo-2′-deoxyuridine (BrdU, 10 µM, Sigma, St. Louis, MO, USA) was added to rotary or static cultures 24 hours before collecting EBs or neurospheres at each respective time-point.

Quantitative Real-Time PCR (qRT-PCR)

Please refer to the ***Quantitative real-time Polymerase Chain Reaction (qRT-PCR)*** in the Methods section of Chapter V. We used the following primers:

Oct-4 F: CCGTGTGAGGTGGAGTCTGGAG, R: GCGATGTGAGTGATCTGCTGTAGG

Nestin F: GGAGAAGCAGGGTCTACAG, R: AGCCACTTCCAGACTAAGG

GAPDH F: GCCTTCCGTGTTCCTACC, R: GCCTGCTTCACCACCTTC

Staining of EBs, neurospheres and ES and iPS cell-derived neurons

Please refer to ***Immunocytochemistry*** and ***Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick end (TUNEL) staining of cell death*** in the Methods section of Chapter V. ImageJ was used to determine neurite length in mouse ES cell-derived neurons of both static and

rotary conditions. ImageJ was also used to determine areas of expression in EBs and neurospheres in both rotary and static cultures.

For staining BrdU (1:200; AbD Serotec; Raleigh, NC), sections of EBs and neurospheres were incubated in cold methanol (-20 °C) for 15 min after the formalin step, incubated in 2N HCl at 37 °C for 1 hour and finally neutralized by washing in borate buffer three times. The staining was completed as described earlier in ***Immunocytochemistry*** starting at the Triton-X-100 step.

Electrophysiology of mouse ES and iPS cell-derived neurons

Please refer to ***Electrophysiology*** in the Methods section of Chapter V.

Chapter V
Vector-free human iPS cells differentiate to functional neurons and improve functional and behavioral recovery after ischemic stroke

A. Introduction:

In the United States, stroke is the fourth leading cause of death and the number one cause of disability in the adult population. With an average of one victim every 40 seconds, almost 795,000 individuals experience a stroke every year [4]. Despite the substantial health and economic burden of stroke, clinical trials have failed to establish the therapeutic benefit of hundreds of candidate drugs that were effective in animal models [345, 346]. Alternatively, cell-based therapies using a variety of embryonic and adult stem cells are currently being investigated as a potential treatment for ischemic stroke.

Stem cell transplantation may be used in cell replacement therapy to repair damaged tissues and enhance endogenous repair mechanisms after ischemic stroke [84]. Previous studies from our lab and others have shown that transplantation of embryonic stem (ES) cells improves functional recovery after ischemic stroke [85, 96]. The use of human ES cells represents a unique opportunity to test cells that can potentially be used in human patients. However, the derivation and application of human ES cells raise ethical concerns that hinder their basic and clinical research [347]. Recently, a cocktail of transcription factors has been shown to be able to reprogram mouse [97] and human [98, 348] fibroblasts to pluripotent stem cells that have the differentiation potential of becoming all the three germ layer cells. These induced pluripotent stem (iPS) cells are genetically, epigenetically [97, 349], and morphologically similar (but not the same) to ES cells, with similar differentiation capacities [188, 191]. Moreover, many protocols have been established to differentiate iPS cells to neurons and use them to treat or model neurodegenerative diseases [129, 130, 137, 166, 189, 350-352]. iPS cell derivation has substantially developed over the past 5 years [122-128]. Initially, iPS cells were produced by lenti-viral constructs carrying transcription factors that include c-myc. However, both c-myc and

the lenti-viral DNA contribute to a potential cancerous transformation of iPS cells after transplantation. More recently, iPS cells were produced by using non-integrating episomal vectors which circumvent the continuous presence of both lentiviral DNA and c-myc [135]. The production of vector-free and transgene sequence-free iPS cells has removed a major obstacle for translating the therapeutic potential of hiPS cell to the clinic. Despite the great potential of iPS cells in treating neurodegenerative diseases, the use of vector-free hiPS cells as a transplantation therapy for ischemic stroke has not yet been explored.

After stroke, there is a limited period of neuroplasticity during which greatest recovery can occur. In human clinical trials, the constraint-induced therapy, i.e. encouraging the use of the affected limbs, improved behavioral functions lost after stroke [75]. In our model, the whisker barrel cortex shrinks and loses thalamic and cortical connections after stroke. Peripheral stimulation and physical therapy can improve functional recovery and neurovascular plasticity after stroke. This has been confirmed in animal studies using peripheral whisker stimulation (equivalent to rehabilitation therapy in our mini-stroke model) following focal ischemia in mice. Peripheral whisker stimulation increased angiogenic factors such as VEGF, restored local cerebral blood flow, enhanced neuroblast migration through up-regulation of chemokines such as those in the SDF-1/CXCR4 signaling pathway, and enhanced neurogenesis [73]. Whisker stimulation during the critical recovery period after stroke [74] provides increasing afferent sensory input which enhances neurogenesis and angiogenesis, restores lost neuronal connection, guides axonal rewiring of endogenous and transplanted neural stem cells and, eventually, promotes functional recovery [55, 73].

In this section, we report the first attempt of using vector-free hiPS cell-derived neural precursors in animal models of ischemic stroke. We demonstrate successful hiPS cell cultures in

feeder- and serum-free conditions and high-efficiency *in vitro* differentiation to functional neurons. We also demonstrate *in vivo* neuronal differentiation and enhanced functional recovery and trophic support after transplantation in stroke animals. Whisker stimulation alone improved functional recovery but did not have synergistic effects when combined with hiPS cell transplantation. In summary, vector-free hiPS cells appear to be a plausible alternative to human ES cells for the treatment of neurodegenerative diseases.

B. Results:

Characterization of hiPS cells cultured in serum-free and feeder-free media

hES and hiPS cells have typically been cultured on mitotically-inactivated MEFs that support their undifferentiated growth. However, stem cells cultured under these conditions start to express the non-human sialic acid residue Neu5Gc, which is immunogenic in humans [245]. Thus, a serum- and feeder-free condition is essential in human stem cell cultures for transplantation therapy. We cultured hiPS cells in mTeSR1 media on hES-qualified matrigel [353]. Under this condition, cells showed morphology typical of hES and hiPS cells growing in monolayered colonies (Fig. 5.1A, B). These hiPS cells stained positive for pluripotency markers Oct4A (Fig. 5.1C, D, E), Nanog (Fig. 5.1F, G, H) and Sox-2 (Fig. 5.1I, J, K). They were also negative for SSEA-1, a marker for differentiating hiPS cells. A full characterization of these hiPS cells can be found in [135] and on www.wicell.org.

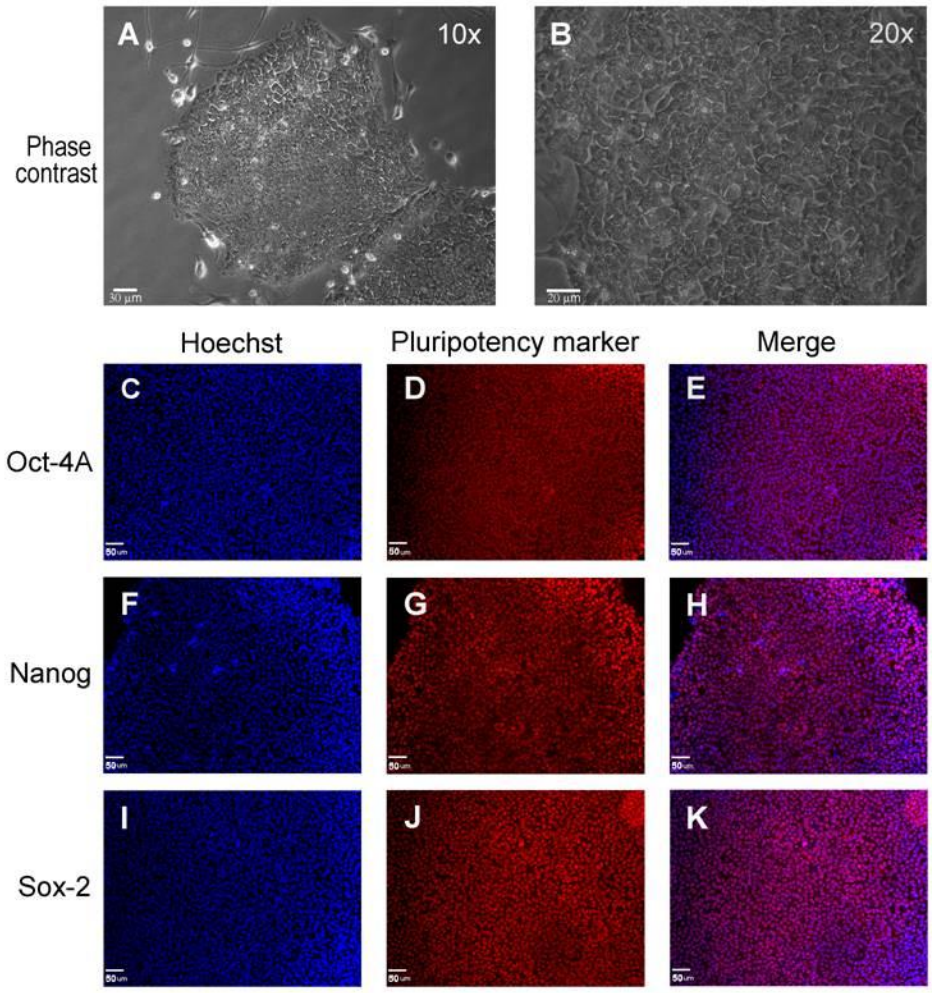

Figure 5.1. In vitro culture of vector-free human iPS cells in mTeSR1

(A, B) Vector-free hiPS cells cultured in mTeSR1 media on human ES qualified matrigel under serum- and feeder-free conditions, show a typical pluripotent cell morphology growing in colonies as monolayers. (B) is a magnified image (20x) of (A). (C to K) hiPS cells express pluripotency markers Oct-4A (C, D and E), Nanog (F, G and H) and Sox-2 (I, J and K). Nuclei were labeled with the nucleic acid counter-stain Hoechst. Bar = 30 μm for A, 20 μm for B and 50 μm for C to K.

Neural differentiation of hiPS cells

Neural differentiation protocols used with human ES cells have traditionally been long and laborious processes [187, 354]. These protocols often depend on embryoid body formation or co-culture with other cell lines, as well as expensive recombinant factors such as Noggin [355]. Recently however, a new differentiation protocol that depends on dual inhibition of the SMAD pathway has been developed [189]. In this protocol, neural induction was achieved in 11 days using Noggin, a bone morphogenetic protein (BMP) inhibitor, and SB431542, a transforming growth factor beta (TGF-β) inhibitor. We developed a similar protocol replacing the expensive recombinant factor Noggin with Dorsomorphin, a relatively inexpensive small molecule BMP antagonist [356]. We have characterized human ES cell neuronal differentiation and provided evidence that hiPS cells can be similarly differentiated to neurons using Dorsomorphin and SB431542. Using this protocol, hiPS cells were grown on Matrigel and allowed to reach confluence in MEF-conditioned media (Fig. 5.2A, B). Following 11 days of neural induction, tri-dimensional rosette-like structures (Fig. 5.2C) and Pax6-positive neural precursors (Fig. 5.2D) were formed. After induction, we obtained 46±5% Pax6- positive cells (n = 5). In addition, nestin, another marker of neuronal precursors, was positive in over 60% (n = 5) of the cells (Fig. 5.2E, F). Gene expression levels of key pluripotency genes (Oct-4 and Nanog) and the neural precursor marker (Pax-6) were also analyzed in hiPS cells and hiPS-NPs using qRT-PCR. There was a significant drop in gene expression level of Oct-4 and Nanog after hiPS cell differentiation to hiPS-NPs (Fig. 5.2G) concomitant with a significant increase in Pax-6 gene expression level (Fig. 5.2H) indicating a loss of pluripotency phenotype and gain of neural characteristics by the end of the differentiation protocol.

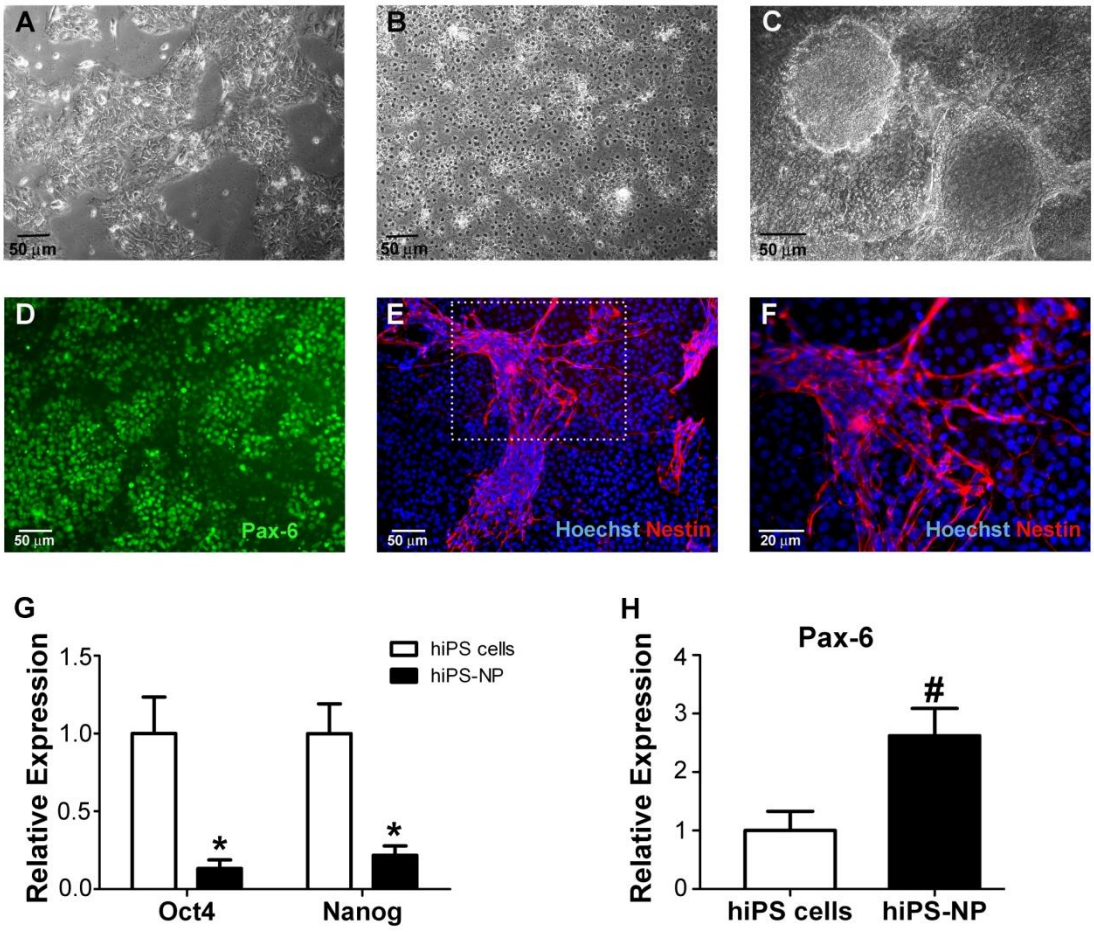

Figure 5.2. Differentiation of hiPS cells to Pax6- and Nestin-positive neural precursors
(A, B) After dissociation and plating on Matrigel, hiPS cells start to grow to confluence as monolayers. (A) shows hiPS cells 2 days after plating and (B) shows confluent hiPS cells 4 days after plating. (C - F) Eleven days after neural induction, hiPS cells form 3-dimensional neural rosette-like structures (C) that are immunoreactive to the neural precursor markers Pax-6 (D) and Nestin (E). (F) is a magnification of the area designated in (E). Green is Pax-6, blue is Hoechst and red is Nestin. Bar = 50 µm for A to E and 20 µm for F. (G and H) qRT-PCR analysis of pluripotency (G; Oct-4 and Nanog) and neural (H; Pax-6) markers showing a significant drop in Oct-4 and Nanog and significant increase in Pax-6 in hiPS-NPs compared to hiPS cells. GAPDH was used as internal control. Expression level in hiPS-NPs is showed relative to expression level in hiPS cells (n=5 in all groups; *$p < 0.05$, Two-way ANOVA with Bonferroni's correction; #$p < 0.05$, Student's *t-test*).

hiPS cell-derived neurons exhibit functional neuronal characteristics

During terminal differentiation 4 weeks after the 11-day neuronal induction protocol, hiPS cell-derived neurons formed extensive networks and stained positively for NeuN and neurofilament L (Fig. 5.3A-D), Tuj1 (β-tubulin III) and synapsin 1 (Fig. 5.3E-H) and the mature neuronal marker MAP2 (Fig. 5.3I, J). NeuN was positive in 23±7% (n = 3) of all the Hoechst-positive cells. MAP2 was positive in 20±1 of the cells. Most NeuN positive cells were also positive for Neurofilament. Over 80% of the NeuN positive cells were also positive for Tuj1 and MAP2. Nestin was negative in all cells. These images were chosen from areas dense with cells showing neuronal morphology.

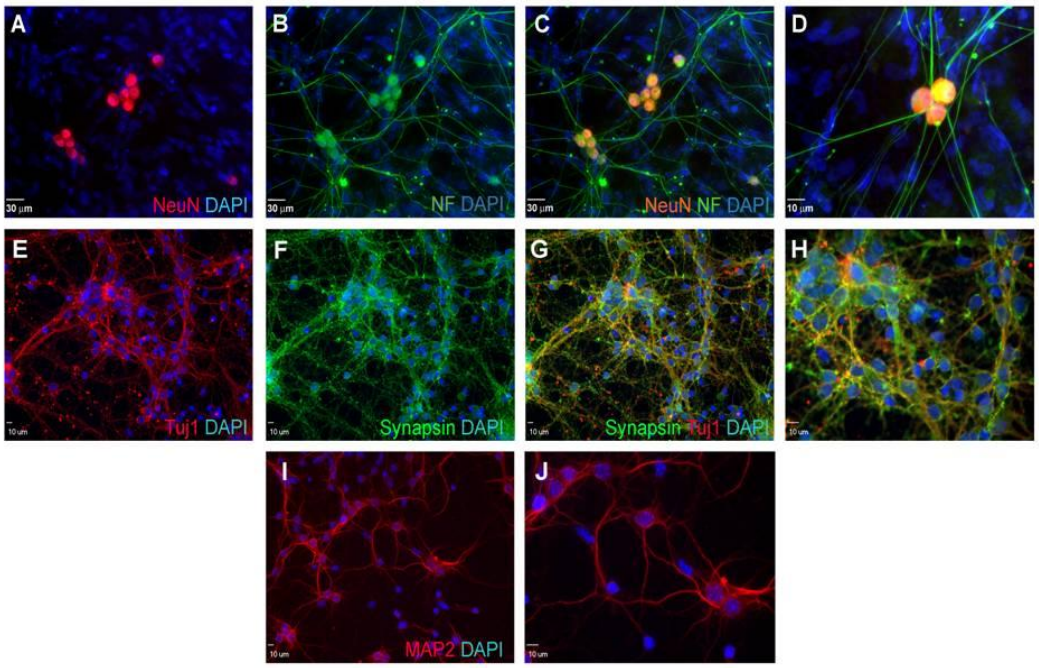

Figure 5.3. hiPS cell-derived neurons exhibit functional neuronal characteristics
(A - J) hiPS cells differentiate to mature neurons that express the neuronal markers NeuN (A), Neurofilament L (B), Tuj1 (E), Synapsin1 (F), and MAP2 (I) 4 weeks after terminal differentiation on Matrigel. (C) and (G) are merged images from (A-B) and (E-F), respectively. (D) is a magnified image of hiPS cell-derived neurons. (H) and (J) are magnifications of (G) and (I), respectively. Green is NF-L and synapsin; blue is DAPI; and red is NeuN, Tuj1 and MAP2. Bar = 30 μm for A to C, 10 μm for D-J.

The resting membrane potential of hiPS cell-derived neurons matured to 72.0±2.0 mV (n=7) 4 weeks after differentiation (Fig. 5.4A). Likewise, we noticed a gradual increase in the number of action potential spikes with time (Fig. 5.4B). In current clamp recording mode, a depolarizing pulse of 1,000 ms duration triggered either a single action potential (70%) or repetitive spikes (30%) 4 weeks after differentiation (Fig. 5.4B) suggesting functional neurons (n=10). TTX-sensitive sodium currents were elicited in all patched iPS cell-derived neurons (with average amplitude of 5.4±1.1 nA) (Fig. 5.4C). The selective Na^+ channel blocker TTX (0.5 µM) completely abolished the fast inward currents (Fig. 5.4C, middle panel) whereas the potassium channel blocker TEA blocked the delayed current (Fig. 5.4C, lower panel).

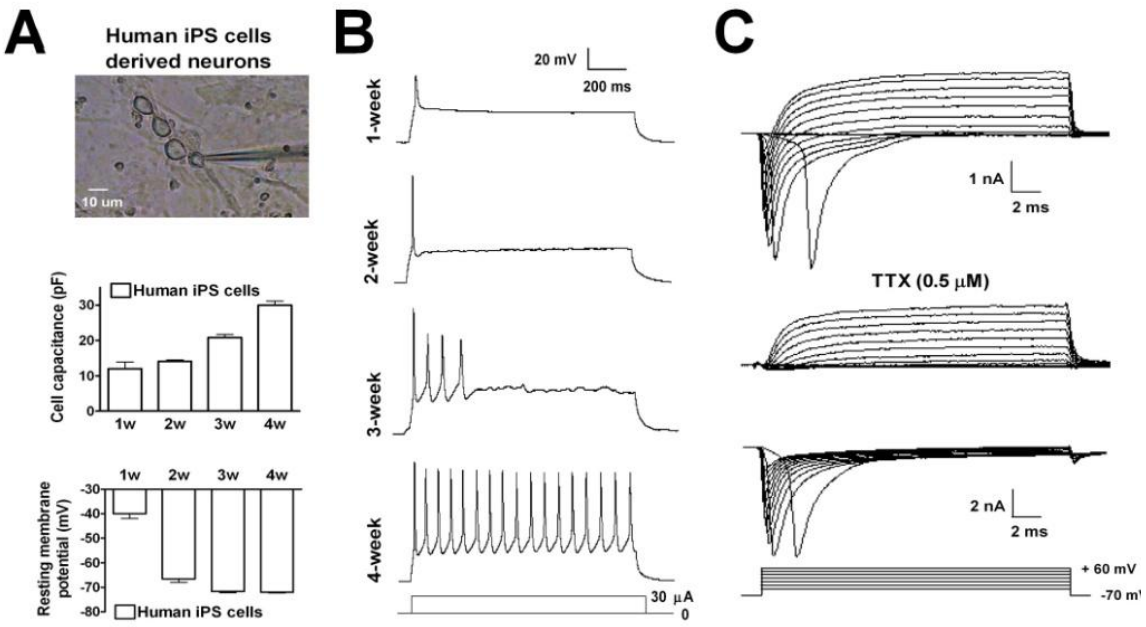

Figure 5.4. Whole cell recordings of human iPS cells after neuronal differentiation.
(A) Upper panel: a glass pipette forming a tight seal on a hiPS cell-derived neuron. Middle panel: Cell capacitance progressively increased over the 4-week differentiation period. Bottom panel: resting membrane potential shifted from relatively positive to more negative levels. (B) Action potentials observed in differentiating neurons derived from hiPS cells at different time points. At 4 weeks, most cells showed a single action potential spike while some exhibited a train of action potentials. The recorded action potentials are in response to 30 µA current injections under current clamp mode. n = 10 neurons. (C) Upper panel: Depolarization steps from a holding potential of -70 mV triggered fast inward and slow outward current in cells of 14 days differentiation. Middle panel: The selective Na^+ channel blocker TTX (0.5 µM) completely blocked the fast inward currents. Lower panel: The fast inward I_{Na} current recorded in the presence of 140 mM Cs^+, 10 mM TEA, and 100 µM Cd^{2+} (n=10 neurons).

Transplantation of hiPS cell-derived neural precursors after focal cerebral ischemia in mice

The effect of transplantation therapy using our hiPS cell-derived neural progenitor cells was tested in the focal ischemia mouse model of barrel cortex stroke [106]. A timeline for the *in vivo* experiments is shown in Fig. 5.5A. Dissociated neural precursors (labeled with Hoechst 33342) were transplanted into the ischemic core and the penumbra region 7 days after stroke induction (Fig. 5.5C). TUNEL staining for cell death was performed 48 hrs after transplantation. Approximately 15±3% of the Hoechst 33342-positive cells were positive for TUNEL staining (Fig. 5.5D).

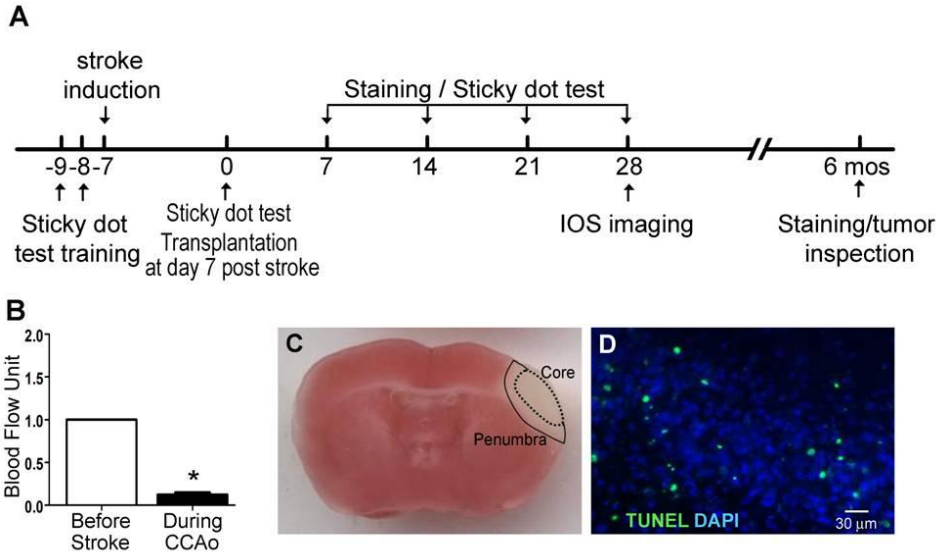

Figure 5.5. Experimental design and stroke model

(A) The *in vivo* experimental design for hiPS cell-derived neural precursor transplantation and post-stroke experiments. (B) During stroke induction, blood flow drops to less than 20% compared to that before MCA and CCA occlusion (n=6, *p<0.05, Student's *t-test*). (C) Focal ischemic barrel cortex stroke as shown by the TTC stain. The stroke core (dotted area) and the ischemic penumbra (filled area) are marked and represent the areas of cell transplantation. (D) TUNEL staining of the transplanted hiPS cells indicating that most hiPS cells survive 2 days after transplantation. Green is TUNEL and blue is Hoechst. Bar = 30 μm for D.

In vivo neuronal differentiation was assessed by staining brain slices for NeuN 28 days after transplantation. At 28 days after transplantation, the percentage of NeuN/Hoechst 33342 double positive cells was 26 ± 5 % among all Hoechst 33342-positive cells in the penumbra region (Fig. 5.6A-F). Six and twelve months after transplantation, we inspected six animals (at each time point) for signs of tumor growth using Nissl staining. No signs of tumor or malignant growth could be identified at the site of transplantation in the penumbra, in the core, or in the surrounding regions (Fig. 5.6G, H).

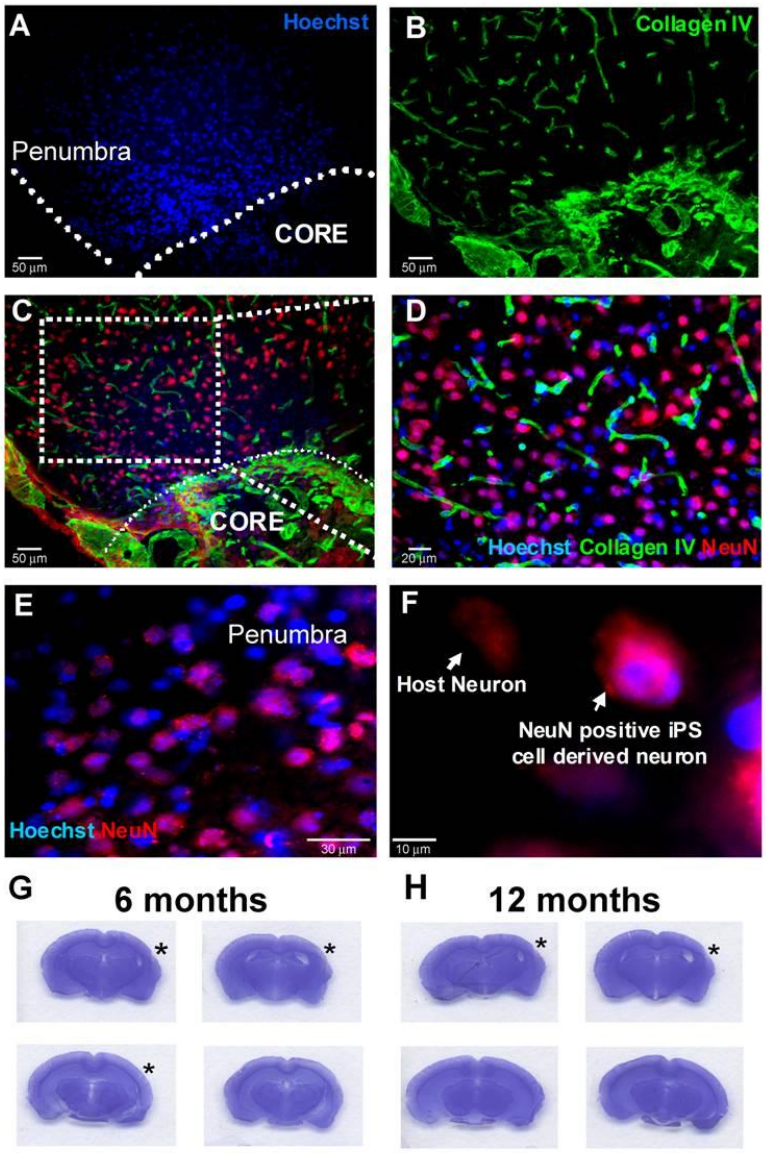

Figure 5.6. In vivo survival and differentiation of human iPS cell-derived neural precursors in stroke animal models
(A – D) hiPS cells survive and differentiate to neurons 28 days after transplantation in the penumbra region of stroke animals. The core region is delineated in (A) with the Hoechst-positive hiPS cell-derived neurons residing in the penumbra. hiPS cell-derived neurons are identified by co-labeling Hoechst and NeuN. (D) is a magnified image of the marked area in (C). (E, F) are further magnifications to show the co-labeled Hoechst-positive NeuN-positive hiPS cell-derived neurons. A Hoechst-negative NeuN-positive host neuron is also shown in (F). Bar = 50 µm for A to C, 20 µm for D, 30 µm for E and 10 µm for F. (G – H) Nissl staining of sections representing brains of hiPS cell-derived neural precursor transplanted animals 6 and 12 months after transplantation showing no indication of tumor formation. Asterisks indicate stroke location.

hiPS cell-derived neural precursor transplantation enhances functional behavioral recovery after stroke

The adhesive-removal test for sensorimotor deficits in rodents was performed 7, 14, 21, and 28 days after transplantation in stroke mice [341]. While the focal ischemic stroke model mostly affects the barrel sensory cortical area, the ischemic core also extends into the sensorimotor cortex representing the upper limbs [341]. This makes the adhesive removal test a valuable tool in assessing sensorimotor recovery in our study. Indeed, the distal right MCA ligation caused sensorimotor deficit on the left side of the body.

Although focal ischemia did not affect the sensorimotor function in testing the right paws (Fig. 5.7A, C), the test of left paws revealed that the time-to-contact and time-to-remove the adhesive tape increased in stroke control group at all time points tested (Fig. 5.7B, D). On the other hand, mice in the cell transplanted group maintained almost nearly unaltered time-to-contact and time-to-remove. In these mice, there was no significant difference between the right and left side. In comparison to stroke control group, mice transplanted with iPS cell-derived neural precursors performed better and significantly better function was detected at day 14 and 21. At day 14, there was a significant difference between the experimental treated and stroke control groups in the time-to-contact measurement ($p=0.0364$, $n=9$, student's *t-test*). Even more difference was seen at day 21. The time-to-contact was 5.09±1.46 sec vs. 0.90±0.24 sec in the control and transplantation group, respectively (Fig 5.7B), while time-to-remove was 2.16±0.75 sec vs. 0.63±0.07 (Fig. 5.7D; $p<0.05$ in both comparisons; two-way ANOVA; $n = 9$ in both groups). These data indicate that hiPS transplantation can accelerate the rate of sensorimotor recovery after ischemic stroke.

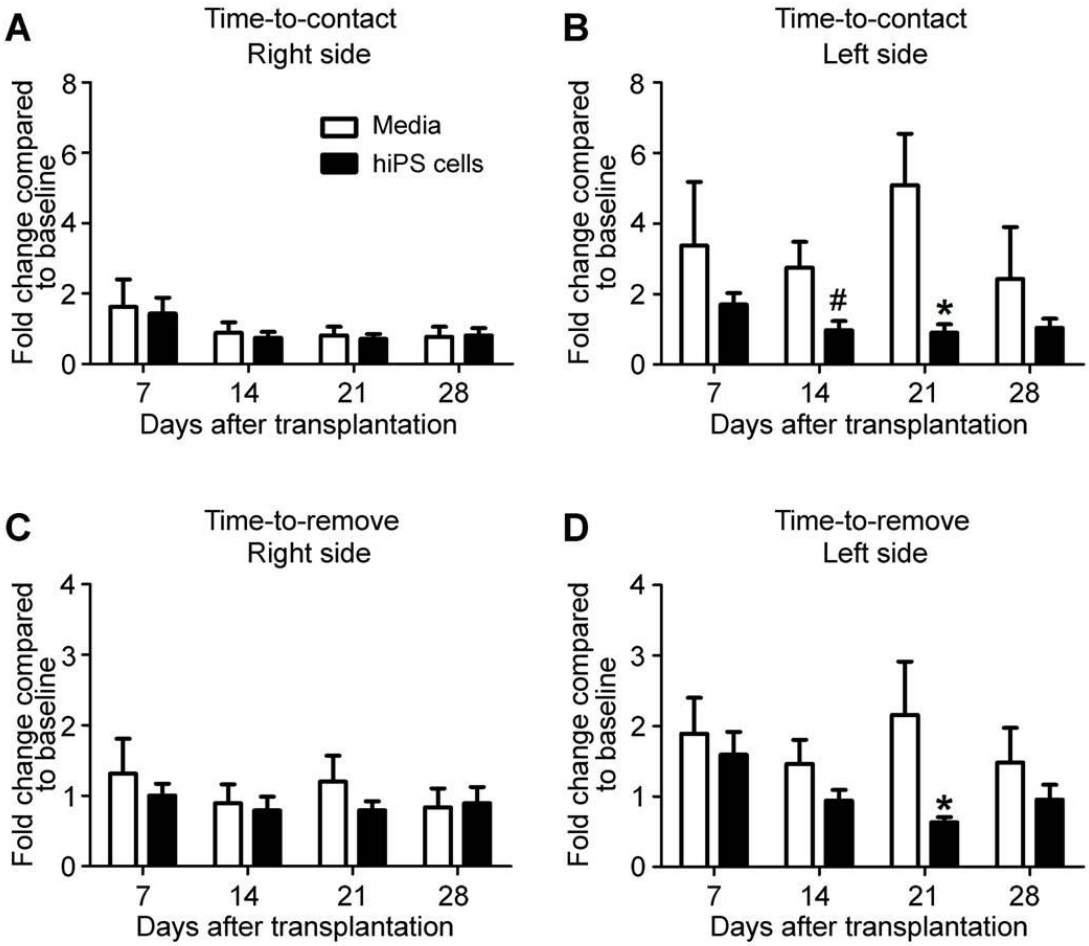

Figure 5.7. hiPS cell-derived neural precursor transplantation enhances sensorimotor functional recovery

The adhesive-removal test was performed 7, 14, 21 and 28 days after transplantation. The values represent the ratio of the time-to-contact or time-to-remove the adhesive tape at each test date to the average time spent by the same animal immediately after training. (A and C) On the right side of the body, there was no difference in the time-to-contact or time-to-remove the adhesive tape between the media injection and cell transplantation groups and all values were centered on 1 indicating no difference before and after stroke induction. (B and D) On the affected (left) side, there was a significant difference in the time-to-contact (B) and time-to-remove (D) the adhesive tape between the media injection and cell transplantation groups at days 14 and 21, indicating a faster sensorimotor recovery with hiPS cell-derived neural precursor transplantation. (n = 9 for both groups at all time points, *$p < 0.05$, Two-way ANOVA with Bonferroni's correction; #$p < 0.05$, Student's *t-test*).

Local cerebral blood flow after transplantation

We used laser Doppler blood flow imaging to determine local cerebral flow before and after transplantation in the control and the transplantation groups. The mean perfusion values were imaged from six adjacent areas around the medial border of the infarct area before and during stroke and 3, 7, and 14 days post-stroke. The area where imaging was analyzed is shown in Fig. 5.8B (arrow). The recorded values are the mean values normalized to the blood flow before MCAo for each mouse. As shown in Fig. 5.8A and 5.8C, blood flow dropped to about 20% of the initial perfusion after MCAo. At each time point after transplantation (3, 7 and 14 days), local cerebral blood flow was was not statistically different between the transplantation and media injected group at any given time ($n=10$ in each group; $p>0.05$; Two-way ANOVA with Bonferroni's analysis).

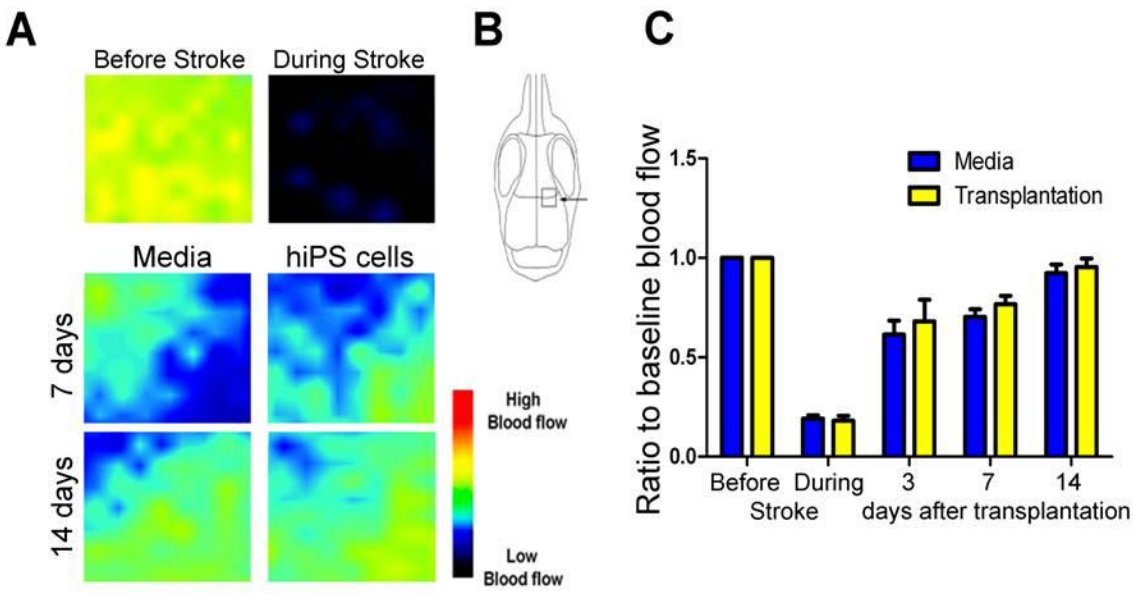

Figure 5.8. hiPS cell-derived neural precursor slightly increase blood flow after stroke
(A) Local cerebral blood flow is shown as pseudo-colored images of flow intensity before and after stroke and 7 and 14 days post transplantation in the media or cell injected groups. A color scale for blood flow is shown in the lower right corner. (B) Blood flow was measured in areas around the medial border of the infarct (arrow). (C) Quantification of the mean intensity values normalized to before stroke mean values showing no statistical difference in flow between the transplantation and media injected groups (n=10 in each group; $p>0.05$; two-way ANOVA with Bonferroni's post-hoc analysis).

Intrinsic optical signal imaging after transplantation

Intrinsic optical signals (IOS) evoked by whisker stimulation at the barrel cortex were assessed 30 days after transplantation. IOS imaging is a method to measure changes in neurovascular coupling after stroke. The detected signals indicate an increase in blood flow in the activated barrel corresponding to stimulations of specific whiskers [106]. IOS imaging was performed on normal sham control, stroke plus media injection, and stroke plus iPS cell-derived precursors transplanted mice (Fig. 5.9). Functional imaging of IOS showed normal evoked barrel activity in sham animals after whisker stimulation. This evoked activity disappeared in stroke animals that received only media injection indicating a damage to the whisker-barrel pathway and a defect in neurovascular coupling after ischemic stroke. While gross local cerebral blood flow was not significantly increased with cell transplantation (Fig. 5.8), hiPS cell-derived neural precursor transplantation significantly enhanced the evoked IOS response in the barrel cortex (Fig. 5.9). This robust response in the transplantation group indicates that transplantation of hiPS-derived neural progenitor cells is capable of restoring the damaged neuronal pathway and neurovascular function in the ischemic brain.

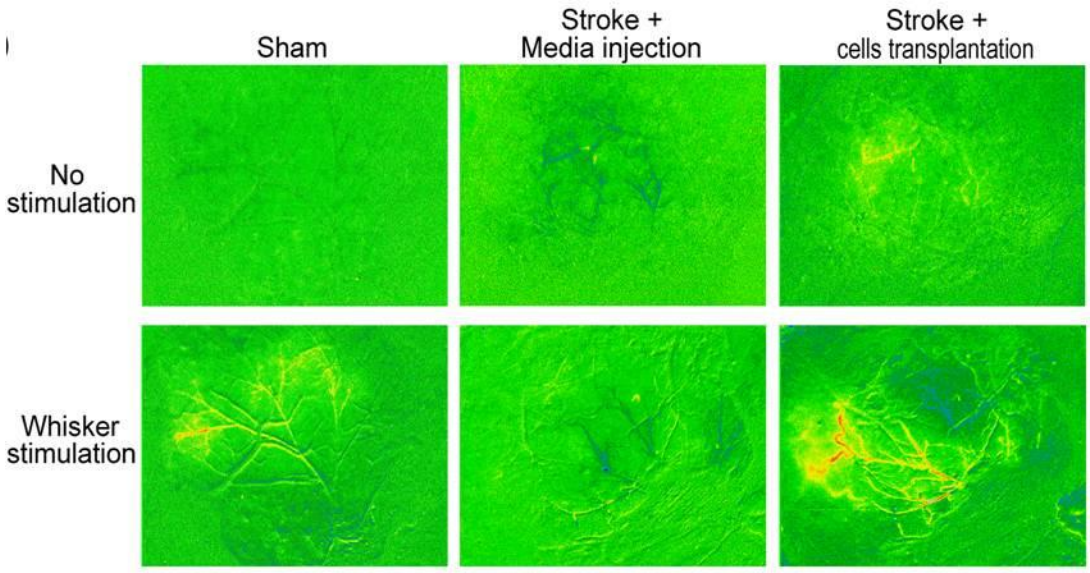

Figure 5.9. hiPS cell-derived neural precursor transplantation enhances neurovascular coupling after stroke

Intrinsic optical signals imaging in control and transplantation animals 30 days after transplantation. Normal evoked barrel cortex responses are induced in sham animals after whisker stimulation. Stroke induction with only media injection resulted in the destruction of the neurovascular infrastructure and the disappearance of IOS in the barrel cortex after whisker stimulation. hiPS cell transplantation after ischemic stroke restored the neurovascular architecture resulting in robust evoked responses after whisker stimulation. n = 5 in each group. Representative images are shown.

Trophic factor expression in hiPS cell-derived neural precursors and after transplantation

To understand the mechanisms of stem cell induced benefit after transplantation in stroke animals, we qualitatively detected the protein expression of some trophic factors and some of their receptors in hiPS cell-derived neural precursor *in vitro* just before transplantation (Fig. 5.10A). hiPS cell-derived neural precursors expressed EPO, EPO-R, VEGF, VEGF-R1 or Flt-1, VEGF-R3, BDNF, GDNF, CXCR4, Tie-1, Tie-2, Ang-1 and Ang-3. All these trophic factors play important role in cell survival, proliferation and migration and can potentially enhance endogenous repair mechanisms in the brain. We also compared protein expression of these factors in the penumbra of the media injected and the cell transplanted animals 14 days after transplantation (Fig. 5.10B). While most of the factors did not show any difference between the two groups, there was a significant increase in BDNF expression levels in the transplanted group compared to the media injected animals. VEGF, VEGF-R and EPO-R were also increased but the difference was not significant between the two groups.

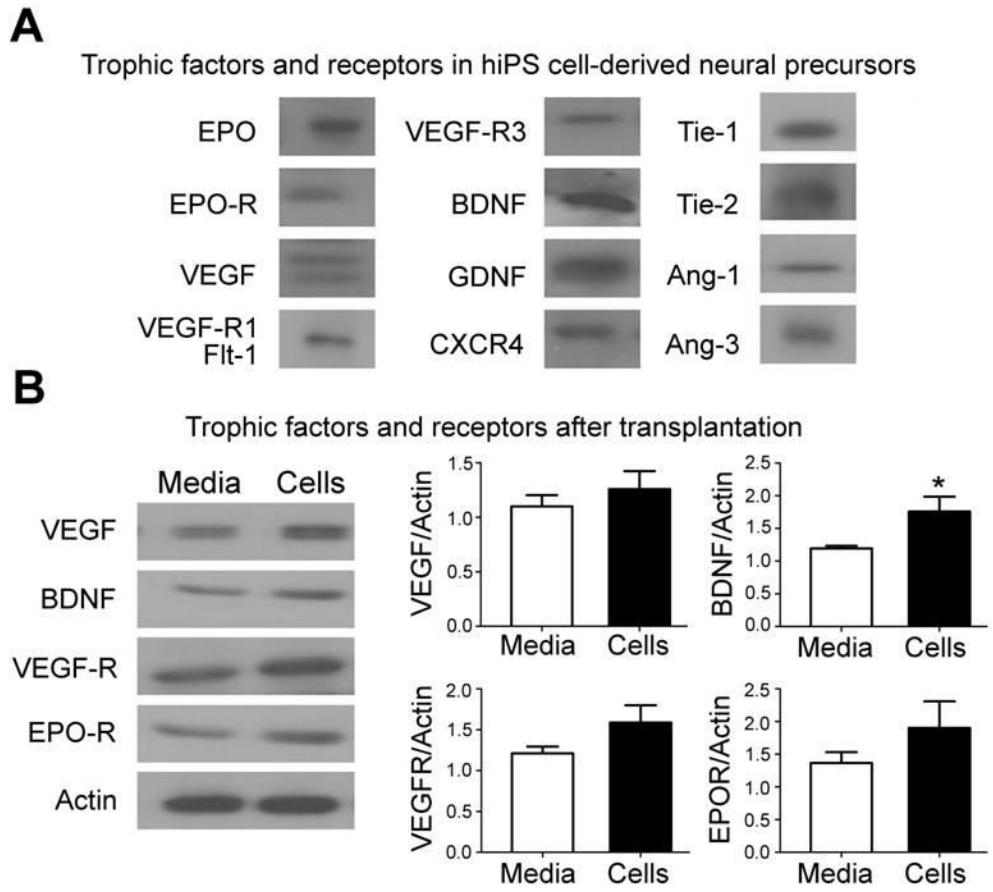

Figure 5.10. Trophic factor expression in hiPS cell-derived precursors before and after transplantation

(A) Qualitative assessment of trophic factor expression in hiPS cell-derived neural precursors. hiPS cell-derived neural precursors expressed EPO, EPO-R, VEGF, VEGF-R1 or Flt-1, VEGF-R3, BDNF, GDNF, CXCR4, Tie-1, Tie-2, Ang-1 and Ang-3. (B) BDNF expression level was significantly increased in the transplanted group compared to the media injected animals (*$p<0.05$; Student's *t-test;* n=6 for both groups). VEGF, VEGF-R and EPO-R were also increased but the difference was not significant between the two groups ($p>0.05$; Student's *t-test*; n=6 for both groups).

Neurogenesis and angiogenesis after hiPS cell-derived neural precursor transplantation

Attempting to further understand the mechanisms of the enhanced functional recovery after transplantation, we sought to measure the levels of angiogenesis and neurogenesis in the penumbra region after transplantation. As discussed earlier in the chapter I, both angiogenesis and neurogenesis are tightly linked and contribute to recovery after stroke. We hypothesized that the trophic factors secreted by the hiPS cell-derived neural precursors could potentially increase angiogenesis and neurogenesis in the penumbra which will eventually promote functional recovery. To determine whether newly generated neuroblasts differentiated into mature neurons in the peri-infarct region where they are needed for tissue repair, NeuN and BrdU were co-stained 7 and 14 days after transplantation. To explore angiogenesis, Collagen IV (CoIV) and BrdU were co-stained 7 and 14 days after transplantation.

There was no difference, however, in the number of NeuN/BrdU- or CoIV/BrdU-positive cells in ischemic mice that received media or neural precursors indicating that hiPS cell-derived neural precursors did not increase angiogenesis and neurogenesis after stroke (Fig. 5.11). Moreover, a quick glance at the structure of blood vessels after transplantation did not reveal any difference in the tortuosity of blood vessels with transplantation (Fig. 5.11A, B).

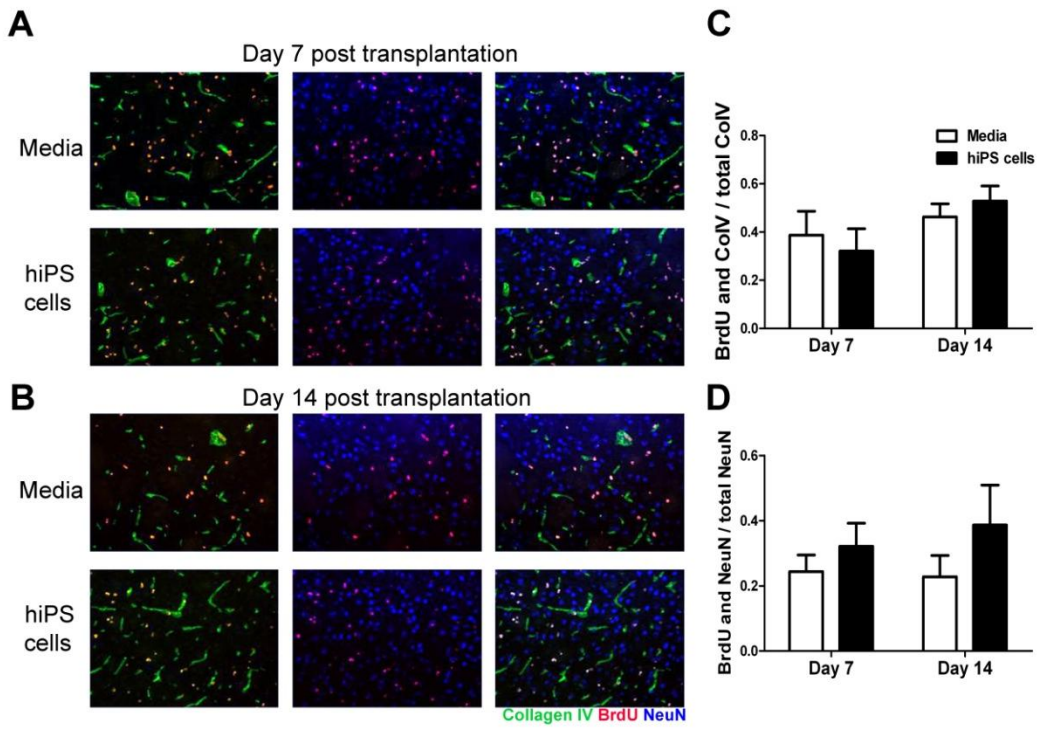

Figure 5.11. hiPS cell-derived neural precursor transplantation does not affect angiogenesis and slightly increases neurogenesis in the penumbra after stroke

Angiogenesis in the penumbra region was examined by co-localization of the capillary basement membrane marker Collagen IV (green) and the proliferation marker BrdU (red) 7 (A; column 1) and 14 (B; column1) days after transplantation. (C) Quantification of double labeled vessels with BrdU showing no changes in angiogenesis. Neurogenesis in the penumbra region was examined by the co-localization of the neuronal marker NeuN (blue) and the proliferation marker BrdU (red) 7 (A; column 2) and 14 (B; column 2) days after transplantation. (D) Quantification of double labeled neurons with BrdU showing slight increase in neurogenesis (but not statistically significant). Column 3 in (A) and (B) contains the merged images of columns 1 and 2. N=8 animals in each group. Data are expressed as mean ± SEM. $P>0.05$ using two-way ANOVA.

Whisker stimulation enhances behavioral recovery after stroke but not synergistically with neural precursor transplantation

As discussed earlier, whisker stimulation, analogous to rehabilitation in humans, is a proven therapy for alleviating worsening stroke symptoms in mice after focal barrel stroke. We hypothesized that whisker stimulation would improve behavioral outcomes after stroke injury and the combination of whisker stimulation and neural precursor transplantation will further enhance recovery. We tested the adhesive removal test after stroke transplantation and whisker stimulation 7, 14, and 21 days after transplantation. Mice were divided into 4 groups: 1) ischemic stroke + media injection group, 2) ischemic stroke + WS, 3) ischemic stroke + hiPS and 4) ischemic stroke + WS + hiPS cell transplantation. Whisker stimulation, cell transplantation and the combination therapy all improved performance (time-to-contact and time-to-remove) 7 days after transplantation. There was no significant improvement in the combination therapy compared to each treatment alone. While any of the treatments did not significantly further improve recovery with time, the media injection group showed significant improvement with time as expected from the natural recovery of animal models with small infarcts. All therapies showed improvement in behavior 14 and 21 days after transplantation but none of the differences, except for the combination group at 14 days, was statistically significant. In summary, whisker stimulation enhanced behavioral recovery after stroke but the effect is not synergistic with cell transplantation (Fig. 5.12).

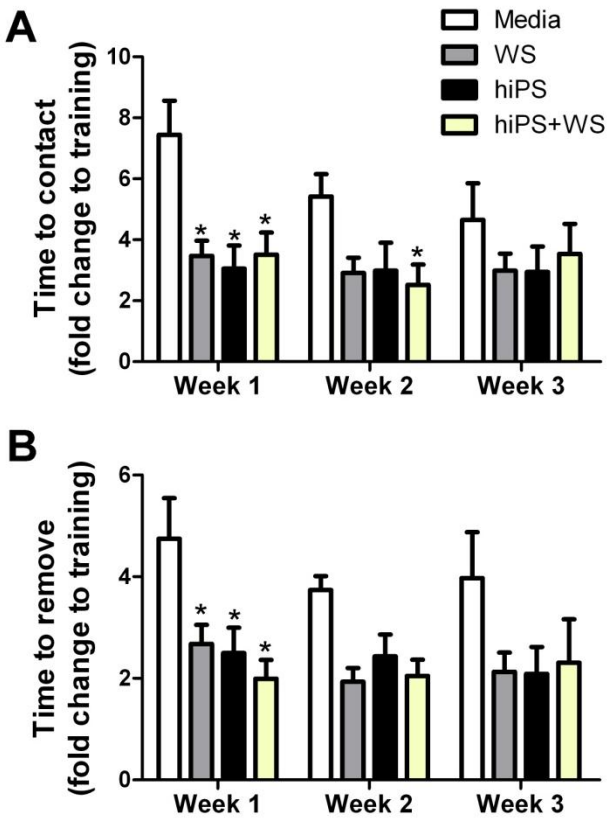

Figure 5.12. hiPS cell-derived neural precursor transplantation and WS enhance sensorimotor functional recovery but not synergistically

The adhesive-removal test was performed 7, 14, and 21 days after transplantation. The values represent the ratio of the time-to-contact or time-to-remove the adhesive tape at each test date to the average time spent by the same animal immediately after training. These values are for the left side of the body. (A) WS, hiPS cell transplantation and the combined WS+hiPS cell transplantation all equally improved the time-to-contact (A) and time-to-remove (B) 1 week after transplantation on the left side of the body. While performance was also enhanced on week 2 and 3, it was not statistically significant. WS+hiPS transplantation, however, significantly improved time to contact at week 2 as well (A). ($n = 8$ for all groups at all time points, $*p < 0.05$, Two-way ANOVA with Bonferroni's correction).

C. Discussion:

In this study, we investigated the therapeutic potential of vector-free and transgene-free hiPS cell-derived neural precursors in the treatment of focal ischemic stroke. *In vitro* experiments demonstrated that hiPS can be cultured in serum-free and feeder-free conditions in mTeSR1 and that hiPS cells differentiate to neural precursors and fully mature neurons with functional electrophysiological properties. *In vivo* experiments showed that, after transplantation, hiPS cell-derived neural precursors survive and differentiate to neurons with mature neuronal markers without any evidence of tumor formation. Behavioral tests demonstrated that hiPS cell transplantation enhances sensorimotor outcomes and restores functional networks within the whisker-barrel pathway in the barrel stroke model. Western blot analysis showed hiPS cell-derived precursors express a variety of trophic factors and increase trophic support after transplantation. This report is the first in demonstrating efficacy of vector-free hiPS cells in transplantation therapy showing clear repair capability of these cells after ischemic stroke.

iPS cell derivation has substantially developed over the past 5 years. Lenti-viruses were first used to induce pluripotency using a cocktail of transcription factors that included c-myc. Both c-myc and the lenti-viral DNA, however, contribute to a potential cancerous transformation of the iPS cells after transplantation. Lenti-viral DNA can potentially integrate in the iPS cell genome in a random fashion increasing the risk of tumor formation and malignant transformation and c-myc is a transcription factor involved in cell cycle regulation and proliferation and is aberrantly expressed in a variety of tumors [357]. Recent techniques using non-integrating episomal vectors circumvents the continuous presence of both lentiviral DNA and c-myc [135]. The production of vector-free and transgene sequence-free iPS cells will likely accelerate the clinical application of cell-based therapy using human iPS cells.

Since their derivation, human ES and iPS cells have been routinely derived and cultured on MEFs [115, 116]. Accumulating evidence, however, shows that contamination of human pluripotent stem cells with animal products increases their immunogenicity due to the increased expression of a non-human sialic acid residue, Neu5Gc. Because of this concern and the fear of graft rejection with human transplantation studies, it will be essential to use a culture system that is independent of feeders and of other animal products like bovine serum [353]. Acknowledging the fact that we used MEF-conditioned media in the differentiation process, our study is the first effort to show the effectiveness of feeder- and serum-free maintenance conditions in experimental transplantation therapy utilizing hiPS cells.

While human ES and iPS cells have great potential for neuro-regenerative medicine, current protocols for their neural differentiation are highly heterogeneous with low yield of neuronal formation. This heterogeneity arises primarily from the inherent nature of the differentiation protocols. Whether using embryoid bodies (EBs) [187] or stromal supportive cells [358, 359], these protocols are non-reliable for human use because of the structural heterogeneity of the EBs and the continued presence of animal products with feeder cells. Given that, we utilized, with modifications, a recently developed adherent neural differentiation protocol avoiding EB formation and stromal cells [189]. This protocol relies on the dual inhibition of the SMAD pathway for efficient neural induction. While the original protocol relies on the use of Noggin and SB431542, we replaced the expensive Noggin with a relatively inexpensive small molecule inhibitor of the bone morphogenic proteins (BMPs). Neuronal differentiation of hiPS cells has also been shown to be less efficient and more variable when compared to hES cells [360]. Other reports however, have shown that the neural differentiation propensity of human pluripotent stem cells (ES or iPS cells) could be overcome using different protocols [189, 361].

In our work, we obtained Pax-6-positive neural precursors in 46% of the cells at the end of neural induction which is less than the 80% reported with the dual SMAD inhibition protocol [189]. Moreover, with RT-PCR, we only saw 3-fold increase in Pax-6 mRNA expression compared to the 10-folds reported earlier in the same study. In a previous study, we have shown that using the same protocol with H1 human ES cells, we got 80% pax-6- and 90% Nestin-positive cells (compare to 46% and 60%, respectively, with hiPS cells) [356]. These data indicate that while relatively efficient, the neural differentiation protocol of hiPS cells can still be optimized.

While several studies have reported low survival of neural precursors after transplantation [362, 363], our high survival rate could be a reflection of a better *in vitro* culture (mTeSR) and differentiation (no EBs, no stromal cells) conditions. Moreover, control of transplantation parameters (timing, cell number, etc.) also increases survival. We transplanted hiPS cell-derived neural precursors 7 days after transplantation when brain edema has subsided and conditions are thus better for cell survival. After local transplantation, cells appear in the core and ischemic penumbra without significant distribution to other brain areas. While we understand the complications of Hoechst labeling of the transplanted cells including interference with DNA replication and potential leakage into neighboring cells, Hoechst has been successfully used in other transplantation studies [91].

It is important to note that we did not use immunosuppression despite the immunohistocompatibility issues between the host (mouse) and the graft (human). In comparison tests, we did not observe any difference in human neural precursor survival after transplantation into mice with and without administration of an immunosuppressant. A recent report, however, has shown that while ES cells form teratomas and do not trigger an immune reaction when

transplanted in a host of the same strain, iPS cells do [263]. We did not, however, see an immune rejection in our transplantation experiments despite the major graft-to-host immune difference.

A previous study showed that undifferentiated iPS cells could form more tumors in the ischemic compared to the intact brain [364]. Six and twelve months after transplantation using hiPS cell-derived neural progenitor cells, we did not detect any sign of tumorigenesis. These results may indicate that the use of vector-free cells and feeder-free conditions, cell differentiation into a committed cell lineage before transplantation, and the control of culture and differentiation conditions are critical for eliminating generation of cancer stem cells and tumor formation.

Several studies have explored the use of iPS cells in various models of stroke [365-370]. All of these studies however used viruses in the process of iPS cell induction and one of them did not show any functional recovery with the transplantation. In conjunction with several reports about stem cell transplantation in stroke models [85, 148, 159], we have seen improved behavioral performance after neural precursors transplantation using the adhesive removal test. Given the small stroke size in this model and the high brain plasticity after stroke, even media injected animals usually recover 1 or 2 months later. However, transplantation of human iPS cell-derived neural precursors significantly accelerated the rate of recovery. Moreover, IOS imaging, an indicator of neurovascular coupling, showed significantly better neurovascular architecture after stroke and neural precursor transplantation although gross cerebral blood flow was not significantly increased. It could be argued, however, that laser Doppler analysis is a gross measurement and cannot detect the minute enhancement in blood flow after transplantation.

We also detected expression of trophic factors in the transplanted cells before transplantation and an increase in trophic support especially BDNF after transplantation indicating that the transplanted cells provide trophic support to the infarcted brain. BDNF has been previously associated with neural stem cell-induced benefit in transgenic model of Alzheimer disease [371] and has a critical role in recovery after ischemic stroke [372, 373]. In addition, we did not detect any significant increase in neurogenesis or angiogenesis after transplantation. However, another mechanism of stem cell induced benefit is a possible integration of these cells in brain circuits. These experiments are ongoing in our lab using complex electrophysiological recording paradigms and novel approaches such as optogenetics (See next chapter).

As discussed earlier, whisker stimulation is analogous to rehabilitation in humans and has been shown to improve functional recovery after stroke. We hypothesized that whisker stimulation would improve behavioral outcomes after stroke injury and the combination of whisker stimulation and neural precursor transplantation will further enhance recovery. Whisker stimulation, cell transplantation and the combination therapy all improved performance after transplantation but there was no synergistic effect with the combination treatment. We are aware that the absence of synergism in this case could be due to saturation in the recovery response because of the small infarct size in our model. These experiments should be repeated in a larger stroke model to draw more accurate conclusions about the synergism between whisker stimulation and stem cell transplantation.

In this chapter, we have shown that vector-free and transgene-free human iPS cell-derived neural precursors can restore functional outcomes and neurovascular coupling after stroke. This is the first study to report that vector-free human iPS cells can be successfully

maintained and differentiated in animal product-free environments and effectively used in transplantation studies of neurodegenerative diseases.

Chapter VI
Establishing Optogenetics Tools for Studying and treating ischemic stroke

A. Introduction:

Stroke research has significantly developed in the past decades. News tools and techniques have been added to an already huge repertoire of available approaches. No FDA approved drugs, however, have made the transition from the bench to the bedside except tissue plasminogen activator (t-PA) which only benefits <5% of stroke patients. Studying the pathophysiology of stroke as well as the mechanisms of different treatments remains a cornerstone in ischemic stroke research. Given the lack of therapeutic breakthroughs in clinical stroke research, the field benefits from new and innovative approaches to study the pathophysiology and mechanisms of ischemic stroke. In this chapter, we introduce the application of optogenetic tools to studying stroke and stem cell research for stroke treatment.

Optogenetics is a new technology that allowed the *in vivo* probing and mapping of neuronal circuits with very high temporal and spatial resolution. Optogenetics employ two classes of channels that transduce light of specific wavelength into a biological response. These tools created a field in which light stimulation or detection allow very accurate control of biological process within target cells [322]. When expressed in the corresponding cells, these channels open in response to light of specific wavelength and conduct ions altering the membrane potential and allowing light induced neuronal excitation (ChR-2; 470 nm) or inhibition (eNpHR; 580nm) of neurons with controlled frequency and timing [301, 322, 323]. ChR2 and eNpHR can be genetically targeted to specific population or sub-population of cells (providing high spatial resolution), control cell function on a millisecond scale (high temporal resolution down to a single action potential) and have been proven to function in culture, acute brain slices and in living animals. The advent of optogenetic actuators allowed scientists to accurately map brain circuits [313, 324-327], dissect pathways of learning and fear [328, 329],

and understand the role of neural cells in pathology and physiology [330, 331]. Moreover, Zhang et al. demonstrated that ChR2 expressing human ES-cell derived neurons receive excitatory and inhibitory post-synaptic current in acute brain slices from SCID mice [374].

One important challenge in stroke and stem cell research is to investigate whether the transplanted neural stem cells functionally integrate within the established host brain neuronal circuits after transplantation in stroke animals. Very few studies attempted to answer this question [375] [374]. Despite their significance, these studies did not investigate the question of integration neither *in vivo* nor in a disease model where the microenvironment is altered by the pathology. Moreover, Current technologies do not provide clear-cut answers to the central question investigating functional integration of stem cells after brain transplantation. For example, although electrophysiological recording techniques have answered many questions about the neuronal behavior of neural stem cells *in vitro* [376], the evidence of full neuronal phenotype *in vivo* is still lacking. Electrophysiological stimulation and recording paradigms lack temporal and spatial resolution for studying information processing in living multicellular networks.

In addition to their benefit in investigating stem cell transplantation, optogenetic tools can help better understand stroke pathophysiology. Using mice that over-express ChR2 or eNpHR (either transgenic or by virus injection) in the penumbra or neurogenic niches, it is possible to understand the effect of depolarization or hyperpolarization on a variety of post-stroke cell death and repair mechanisms. For example, it would be interesting to investigate the effect of stimulation or inhibition before or after stroke onset on cell death in the penumbra or on neuroblast migration from the SVZ. It would be also interesting to change the location of excitation/inhibition between the penumbra and other locations such as SVZ to study cell death

and/or migration or to explore the effect of pre-stroke stimulation/inhibition in the penumbra (pre-conditioning) on cell death. While stroke is usually not a predictable condition in human patients, the application of preconditioning will have minimal application clinically; however, elucidation of the underlying mechanisms of such neuroprotection is vital to further understand stroke pathophysiology and help advance therapies.

In this chapter, we will apply optogenetic tools to stroke and stem cell research. We aim to create an optogenetic tool box that could be used to understand mechanisms of stroke or stem cell integration *in vitro*, *ex vivo* in brain slices or *in vivo*.

B. Results:

Establishing optogenetic techniques for stroke and stem cell research

As discussed before, optogenetic tools offer great advantages for studying neuronal mapping and circuitry. The goal of this part of the thesis is to establish tools and techniques to help us incorporate optogenetics in our stroke model and in stem cells to: 1) study stroke pathology with special focus on circuit repair, 2) study stem cell integration after transplantation in stroke mice and 3) enhance stroke recovery, endogenous and exogenous stem cell treatment after stroke. To attain this goal, we first have to obtain tools and establish basic techniques for optogenetics. To this end, we have acquired the following:

- Different viral vector that drive ChR2 over expression in different cell lines focusing on neuronal expression (Fig. 6.1A). Lentiviruses were either purchased from the Emory viral vector core or made in the lab. Adeno-associated viruses (AAVs) were all purchased from the University of North Carolina – Chapel Hill viral core. We specifically used the promoters CaMKII-α or Synapsin-1 to drive expression in cortical neurons or stem cell-derived neurons *in vitro* or *in vivo.* Synapsin-1 is a protein that associates with synaptic vesicles and the synapsin-1 promoter confers high neuron-specific expression whereas CaMKII-α promoter generally drives expression specifically in glutamatergic excitatory neurons. The "double-floxed inverse open reading frame" (DIO) drives the expression of the downstream inserts only in cells expressing Cre-recombinase. Different types of ChR2 were purchased. Typically, we are using the humanized ChR2-(H134R) for all neuronal expression except for hChR2(C128S/D156A) which is also called stabilized step function opsin (SSFO). This ChR converts a brief pulse of blue light (473 nm) into a stable step in membrane potential that slowly repolarizes to normal membrane

potential (half-life about 30 min) and can be deactivated with a brief pulse of yellow light (580nm).

- Temperature stabilized diode-pumped solid state blue laser (Fig. 6.1B). This DPSS blue laser has output wavelength of 473 nm and an output power of 150 mW. ChR2 is activated with 473 nm blue laser stimulation and 150 mW is essentially enough power to drive *in vitro* and *in vivo* ChR2 activation.

- Doric™ lenses fiber-optic cannulas (Fig 6.1C). These special cannulas consist of a fiber-optic ferrule with a light source connector on one side and an implantable optic fiber on the other side. This relatively new cannula system offers significant advantages over the traditional cannulas. It is easier to use and implant and significantly reduces the risks of infection at the implantation site.

- Thy1-ChR2-YFP transgenic mice (Fig. 6.1 D). These mice express ChR2 fused to EYFP under the control of the thymus cell antigen 1 (*Thy1*) promoter. Mice are viable, with normal fertility, normal size and without any gross phenotypic or behavioral abnormalities. This promoter is primarily expressed in projection neurons in the brain. Expression of the transgenic ChR2-YFP fusion protein is detected throughout the brain in the cortex, including the whisker barrel cortex, hippocampus, midbrain and cerebellum and does not affect neuron viability or function. This mouse will be used to study the repair of the cortico-thalamic pathway after stroke, stem cell integration after transplantation and to explore new avenues for stroke treatment and cell based therapies.

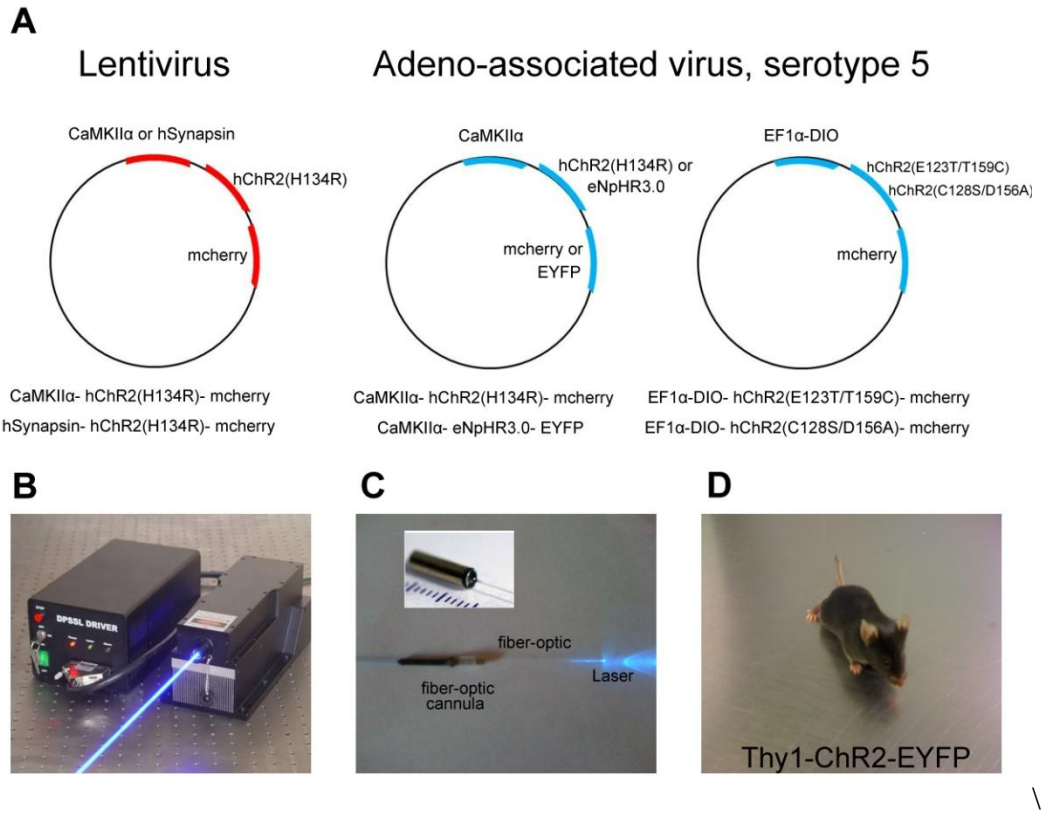

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Fig. 6.1. Various optogenetics tools for stroke and stem cell research
(A) Different viral vector tools have been invested for ChR2 or eNpHR over expression in different cell lines. Lentiviruses as well as adeno-associated viruses (AAVs) were either purchased from the Emory viral vector core or the University of North Carolina –Chapel Hill viral core or made in the lab. We specifically used the promoters CaMKII-α or Synapsin-1 to drive expression in cortical neurons or stem cell-derived neurons *in vitro* or *in vivo.* One particular construct uses what is called the "double-floxed inverse open reading frame" which drives expression of the downstream insert only in cells expressing Cre-recombinase. Different types of ChR2 were purchased. One particular ChR2 is hChR2(C128S/D156A) which is also called stabilized step function opsin (SSFO) and it converts a brief pulse of blue light (473 nm) into a stable step in membrane potential that slowly repolarizes to normal membrane potential and can be deactivated with a brief pulse of yellow light (580nm). (B) Temperature stabilized diode-pumped solid state blue laser (output wavelength: 473 nm; output power: 150 mW) and its power supply. (C) Doric™ lenses fiber-optic cannulas (shown enlarged in the inset) consist of a fiber-optic ferrule with a connector on one side and an implantable fiber on the other side. The connector is shown here connected to a laser source and laser light is emerging from the fiber optic on the other side. When implanted into the tissue, this is a great light delivery system that is easier to use than the typical cannula and significantly reduces risks of infection. (D) Thy1-ChR2-EYFP transgenic mice that express ChR2 fused to EYFP under the control of the thymus cell antigen 1 (*Thy1*) promoter. Mice are viable, with normal fertility, normal size and without any gross physical or behavioral abnormalities. Expression of the transgenic ChR2-YFP fusion protein is detected throughout the brain, including the whisker barrel cortex and does not affect neuron viability or function. This mouse will be useful for studying repair of the cortico-thalamic pathway and stem cell transplantation after stroke.

Testing optogenetic tools in vitro: expression and function of hChR2 in mouse cortical neurons

Next, we determined to test whether we can over-express hChR2 in mouse cortical neurons. Cortical neurons were prepared from E15-16 mouse embryos and plated on laminin substrate. We tested both the lenti-viruses and the AAV5 and both synapsin-1 and the CaMKII promoters. Mouse cortical neurons actively express both promoters (synapsin 1 and CaMKII) as shown in Fig. 6.2C. Viruses were added 1-2 days after plating and expression was followed for the following 10-12 days. No expression was noticed until 6 days after the addition of the virus. ChR2 increased significantly afterwards till reaching an efficiency of 60-80% with either virus (Fig. 6.2A, B). We noticed however better survival, overall neuronal morphology and efficiency with the use of lentiviruses.

Before testing the functional activity of ChR2 in cortical neurons, we tested whether they exhibit normal inward sodium current and normal action potential firing (Fig. 6.2D). Using patch clamp recording, we recorded significant inward sodium current in the voltage clamp mode and action potentials in the current clamp mode. These data indicate that ChR2 expressing cortical neurons still retain normal electrophysiological properties. To test for ChR2 activity, we used 10 ms pulses of blue laser light (473 nm) (Fig. 6.2E). We noticed high fidelity action potential spiking in response to low frequency light stimulation and low fidelity at slightly higher frequencies (10Hz). While it is expected that fidelity of responses drops at higher frequencies due to intrinsic ChR2 properties, we think that the parameters of this experiment can be optimized to get better recordings.

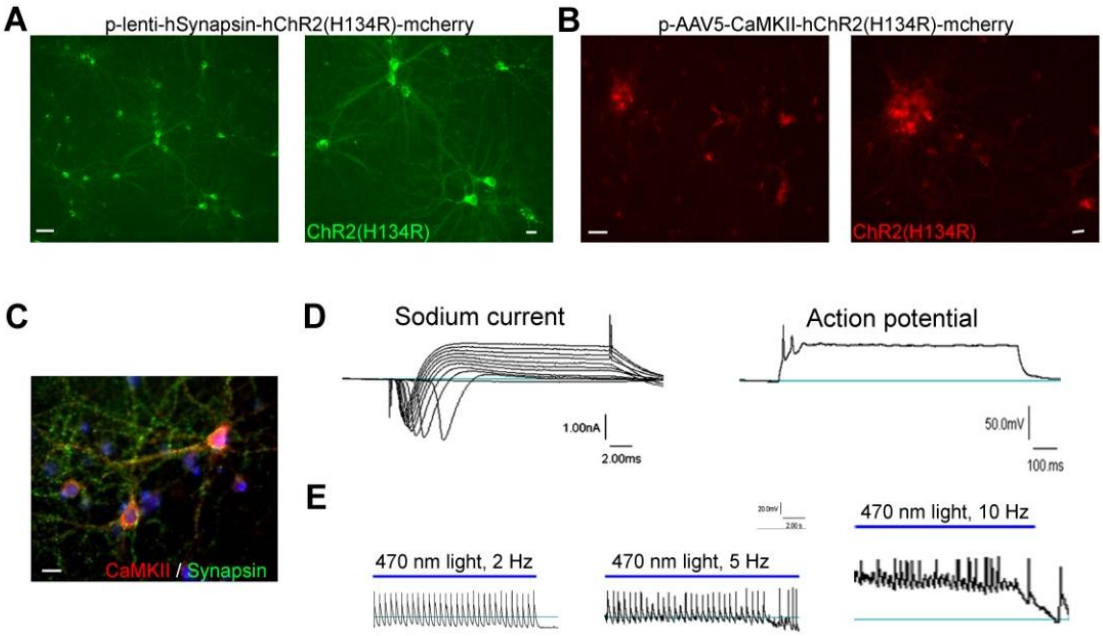

Fig. 6.2. Functional expression of ChR2 in mouse cortical neurons in vitro
(A and B) Expression of hChR2(H134R) in mouse cortical neurons using lenti-virus (A) or AAV5 (B). Expression is driven by the synapsin-1 promoter in the lentivirus and by CaMKII in the AAV. While viruses were added 1 day after plating, expression started 6 days later and displayed an efficiency of 60-80%. (C) Cortical neurons express both Synapsin-1 and CaMKII. (D) ChR2 cortical neurons display normal fast inward sodium current and action potentials indicating neuronal functional activity. (E) Light driven action potentials from cortical neurons expressing ChR2 at 2, 5 and 10 Hz. It is clear that spike fidelity (ratio of light pulses to neuronal spiking) is highest at low light stimulation frequency.

Testing optogenetic tools in vitro: expression and function of hChR2 in miPS cells

Since our goal is test stem cell integration *in vivo* after transplantation and to optimize stem cell therapy with light activation of stem cell-derived neurons after transplantation, we first tested over-expressing ChR2 in mouse iPS cells. To achieve this goal, we tested both lenti-viruses and AAVs. Using AAV5, we could not achieve any expression of ChR2 *in vitro* even with prolonged cultures. Using lenti-viruses, expression of ChR2 was only successful under the CaMKII promoter. All attempts using the synapsin-1 promoter failed. Using the p-lenti-CaMKII-hChR2(H134R)-mcherry, we successfully over-expressed ChR2 in mouse iPS cell-derived neurons (Fig. 6.3A). Expression started 8 days after transduction and imaging and electrophysiological recordings were performed 10 days after the addition of the virus. These miPS cell-derived neurons expressed markers of mature neuronal phenotypes like NeuN and neurofilament. They also express CaMKII. Despite their expression of synapsin-1, we could not detect ChR2 expression using the p-lenti-synapsin-hChR2(H134R)-mcherry. miPS cell-derived neurons over-expressing ChR2 also exhibited evoked action potentials in response to 30 and 60 pA current injection. To test for ChR2 activity, we used 10 ms pulses of blue laser light (473 nm) at 10 and 20 Hz and we recorded high fidelity action potentials in response to light stimulation (Fig. 6.3B). Neurons that did not express ChR2 showed evoked action potentials in response to current injections but no responses to blue light (Fig. 6.3C).

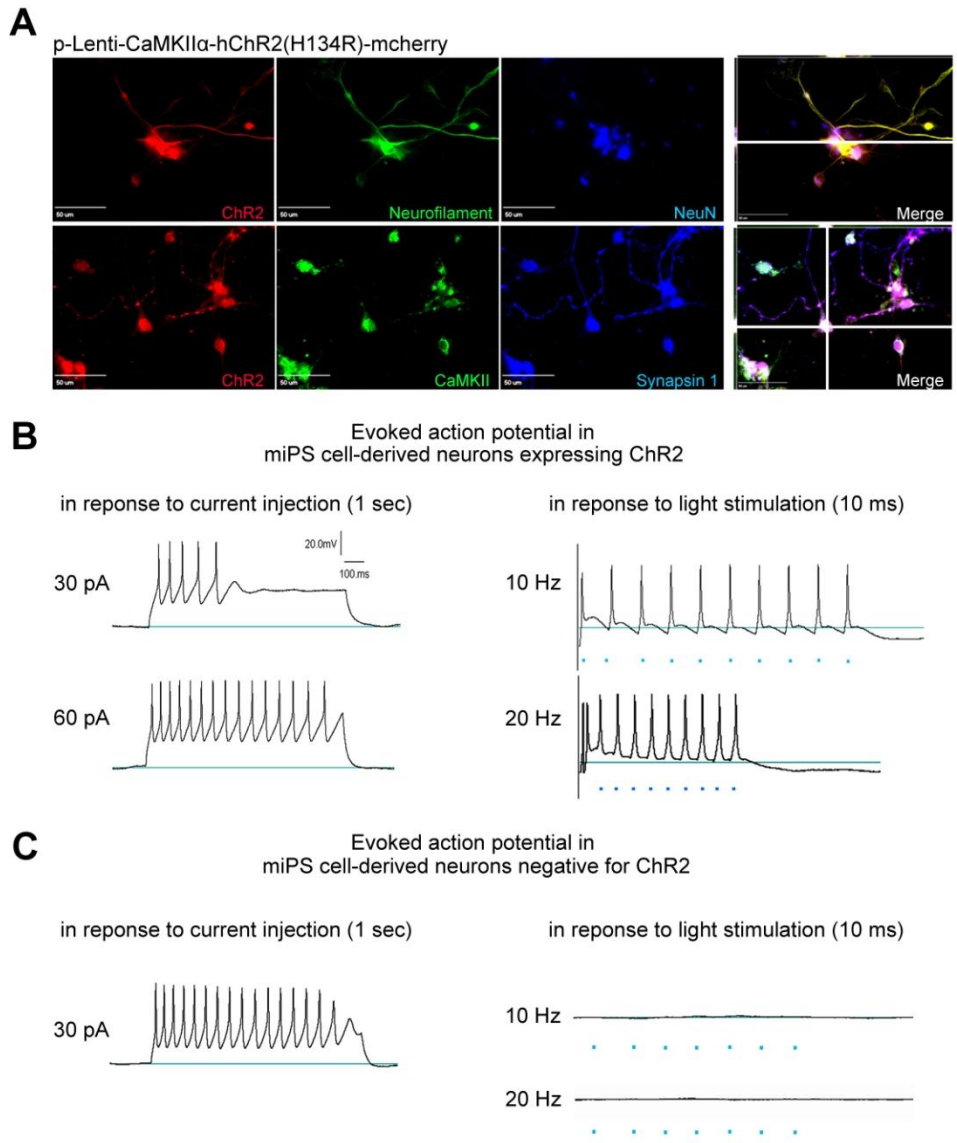

Fig. 6.3. Functional expression of ChR2 in miPS cell-derived neurons in vitro
(A) Expression of hChR2(H134R) in miPS cell-derived neurons using lenti-viruses under the CaMKII promoter. The lenti-viruses were added 1 day after harvest and images were taken 10-12 days later. Transfection efficiency is >80%. Staining for CaMKII and synapsin-1 and other mature neuronal markers (NeuN, NF) is also shown. (B) miPS cell-derived neurons exhibit trains of evoked action potentials in response to 30 and 60 pA current injections indicating mature neuronal function and in response to 10 and 20 Hz 473 nm light stimulation indicating functional ChR2 expression. Note the high fidelity of response to light pulses. (C) Control miPS cell-derived neurons without ChR2 expression show trains of action potential in response to current injection but no response to 10 and 20 Hz light stimulation.

Testing optogenetics responses in ex vivo brain slices

After confirming functional activity of ChR2 *in vitro*, we set to determine whether ChR2 expression in *ex vivo* brain slices can induce field potential responses to light stimulation. To achieve this, we used both AAV virus injection and ChR2 transgenic mice. For virus injections, we injected AAV5-synapsin-ChR2-mcherry in cortical layer IV (400-500 µm deep) of the barrel cortex (AP: -1mm, ML: ± 3mm). ChR2-mcherry expression was detected 2 weeks after viral injections. Light (473 nm blue light) illumination induced a reproducible field potential response when the recording electrode was placed in area of mcherry expression (Fig. 6.4A). In ChR2 transgenic mice, ChR2 expression was also detected in the barrel cortical layer IV and field potential was also recorded with blue laser illumination (Fig. 6.5B).

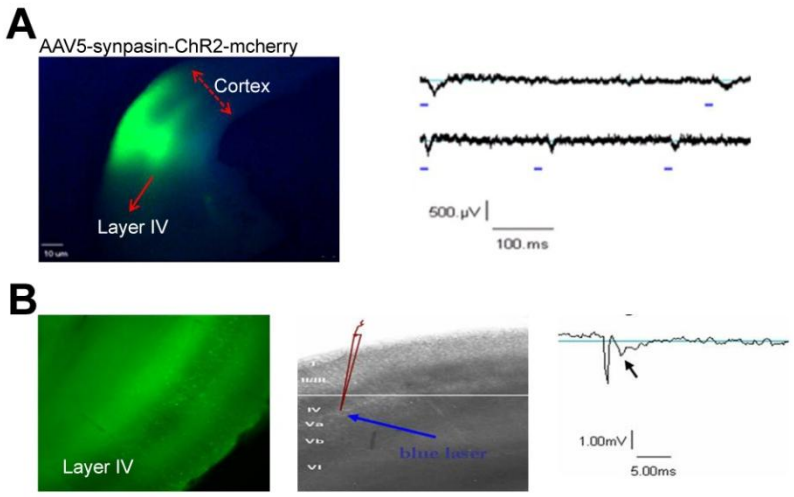

Fig. 6.4. ChR2 expression and functional activity in brain slices
(A) Image of ChR2 expression in layer IV of the barrel cortex after injecting AAV5 carrying ChR2-mcherry under the control of synapsin promoter. Recording electrode was placed in layer IV and blue laser light was illuminated on areas of mcherry expression. Field potential recordings (right) after light stimulation (continuous light for 15 ms). (B) Image of ChR2 expression in cortical layers of the barrel cortex in ChR2 expressing transgenic mice (under thy-1 promoter). Recording electrode was placed in layer IV and blue laser light was illuminated on areas of EYFP expression. Field potential recordings (right) after light stimulation.

Using in vivo optogenetic tools to study stroke mechanisms

Another goal for this work was to establish *in vivo* techniques for studying stroke pathology. To this end, we acquired transgenic mice that express ChR2 in the whisker barrel cortex. We aimed to show a proof-of-principle that optogenetics can be successfully applied in stroke research. We wanted to test whether light induced stimulation of the barrel cortex before stroke can precondition neurons to injury and thus protect them from ischemic cell death. We also wanted to test whether light stimulation post-stroke can help improve recovery. To achieve the first goal, we delivered light to the barrel cortex of ChR2 mice 3 days before stroke (Fig. 6.5A). Light (473 nm) stimulation was delivered 3x per day, at 50 Hz. Twenty-five stimulations were delivered with 60 seconds interval between every 2 stimulations. Each stimulation lasted 1 second. This stimulation paradigm was empirically designed by trial and error using different parameters. Infarct volume was measured 24 hours after stroke. Light stimulation significantly reduced infarct volume (Fig. 6.5B) indicating that light can be used as a preconditioning stimulus to protect against cell death.

To achieve the second goal, light stimulation was started 24 hours after stroke and was delivered every day till day 14 (Fig. 6.6A). Light (473 nm) stimulation was delivered 3x per day, at 2.5 Hz. Twenty-five stimulations were delivered with 50 seconds interval between every 2 stimulations. Each stimulation lasted 1 second. Adhesive removal test was performed at days 3, 7 and 14 after stroke. The time-to-remove the adhesive tape was significantly reduced at day 3 in mice that received light stimulation compared to controls (Fig. 6.6B). There was also some reduction in the time-to-remove the tape at 7 and 14 days but it was not statistically significant. This data indicates that light stimulation of the penumbra after stroke accelerates functional behavioral recovery.

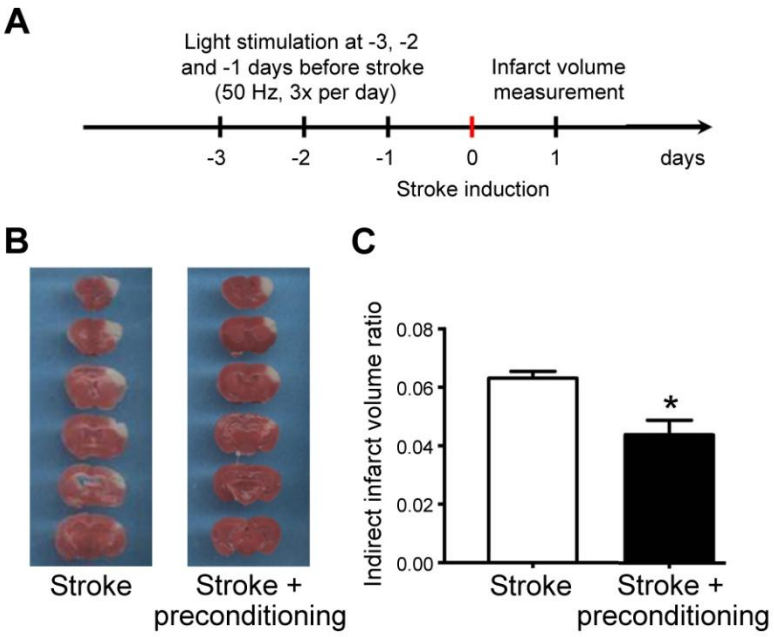

Fig. 6.5. Light preconditioning of ChR2 expressing barrel cortex reduces infarct volume after stroke

(A) Experimental paradigm for light preconditioning of the ChR2 transgenic mice. Light stimulation was started 3 days before stroke induction and was given for 3 days, 3x a day using a frequency of 50 Hz. Infarct volume was measured 24 hours after stroke. (B) Stroke volume measurements in both the stroke control and stroke plus light groups using TTC staining. (C) Light preconditioning significantly reduced infarct volume after stroke.

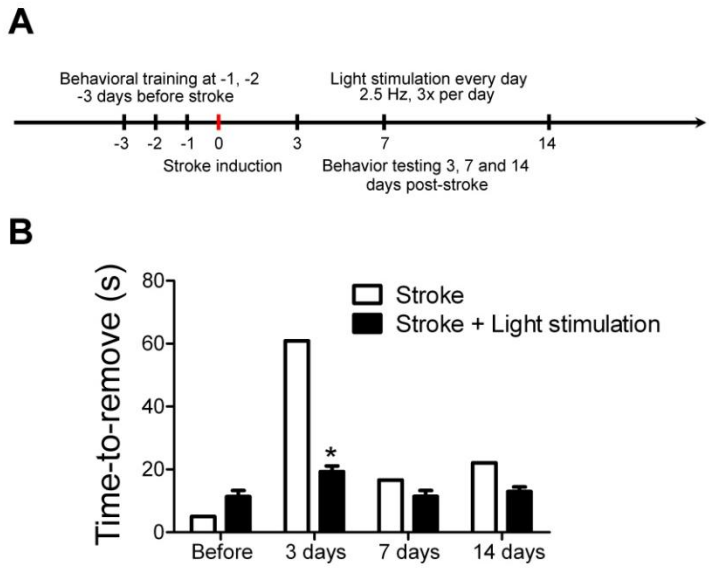

Fig. 6.6. Light treatment in the penumbra of ChR2 transgenic stroke mice improves behavioral outcome after stroke

(A) Experimental paradigm showing that mice were trained before stroke and light stimulation was started 1 day after stroke induction and was given daily for 13 days, 3x a day using a frequency of 2.5 Hz. Behavioral assessment was performed at 3, 7 and 14 days after stroke. (B) Time-to-remove the adhesive tape in the adhesive removal test was significantly reduced at day 3 in mice that received light stimulation compared to controls.

C. Discussion

In this section of the thesis, we developed *in vitro*, *ex vivo* and *in vivo* optogenetic tools for studying the pathophysiology of stroke, the biology of stem cells and their application in cell replacement therapies. Particularly, we are interested in studying how light stimulation affects stem cell neuronal differentiation *in vitro* and *in vivo* and exploring the question of stem cell integration after transplantation in stroke animals. We successfully over-expressed ChR2 in mouse cortical and iPS cell-derived neurons and recorded functional responses to light stimulation. We also recorded field potentials in brain slices infected with ChR2 expressing virus or slices from ChR2 transgenic mice. Lastly, we performed *in vivo* light induced stimulation in the penumbra of ChR2 mice and showed that optogenetic preconditioning can reduce infarct volume and post-stroke light stimulation of layer IV neurons can accelerate functional recovery. In total, this chapter proves the utility of optogenetic tools in stroke and stem cell research.

ChR2 expression in stem cells is a very effective tool to study stem cell physiology, differentiation and integration after transplantation. In a recent study, light stimulation increased neuronal differentiation of mouse ES cell-derived neural precursors [333]. In the setting of the proposed automated light induced stimulation, it would be possible to control stem cell differentiation *in vitro* and explore the contribution of the transplanted cells to tissue and network functions *in vivo*. That said, another study showed that stem cells integrate into functional neuronal networks after transplantation in SCID mice [332]. In our experiments, we will use ChR2-expressing iPS cell-derived neural precursor cells to study stem cell integration in our whisker barrel cortex stroke model. Our whisker barrel stroke is a very convenient model to study stem cell integration because of the structure-function relationship between the whiskers and barrel cortex through the thalamus. We expect stem cells transplanted to layer IV of the

barrel cortex to send and receive appropriate connections from the VPM nucleus of the thalamus. Light stimulation in the barrel should be recorded as a change in excitatory post synaptic potential in the VPM. Experiments should be easily conducted in brain slices and possibly *in vivo* in anesthetized animals.

In our *in vivo* experiments we wanted to test whether light induced stimulation of layer IV ChR2 expressing neurons can reduce stroke volume if administered before stroke (pre-conditioning) or accelerate behavioral recovery if delivered after stroke. Preconditioning is a paradoxical phenomenon where a short-lived exposure to a sub-lethal event induces tolerance to a subsequent potentially lethal episode of similar nature to the brief exposure. The priming event induces the expression of endogenous protective genes and other signaling mechanisms which protect the tissue from ischemic injury. There are many different kinds of preconditioning stimuli in the brain such as ischemia [377-379], hypoxia [380], hypothermia [381], hyperthermia [382], hyperbaric oxygen [106, 383], inflammatory mediators [384], and oxidative stress [74]. While preconditioning does not make a tissue completely invincible to ischemic damage, the amount of neuroprotection is directly linked to the nature, strength and duration of the preconditioning and ischemic insults. This thesis introduces a novel preconditioning stimulus. Light stimulation of ChR2 expressing neurons 3 days before stroke induction can protect from cell death and reduce infarct volume. In addition to preconditioning, we have shown that light induced stimulation of neurons in the penumbra after stroke can accelerate behavioral recovery. The exact mechanism of this benefit is not yet known but it probably involves mechanisms of excitotoxicity and/or post-stroke GABAergic tonic inhibition. In any case, this will be the subject of rigorous experimentation in the future.

In summary, this section aimed to generate different tools for use in stroke and stem cell research. Stem cell-derived or cortical neurons expressing ChR2 will prove to be useful in exploring stroke pathology and treatment modalities. In addition to ChR2, similar work can be done with eNpHR to understand the role of inhibition on stem cell-derived neurons and the role of neuronal inhibition in stroke physiology. Moreover, over-expressing ChR2 or eNpHR in the SVZ neural precursors will help us understand the role of light-induced stimulation or inhibition on post-stroke neuroblast migration for neuronal repair.

Chapter VII
Highly efficient production of neurons from mouse ES and iPS cells using rotary cultures

A. Introduction:

Mouse embryonic stem (ES) and induced pluripotent stem (iPS) cells have the ability to remain in a state of continuous proliferation and renewal in the absence of differentiation cues in cultures [97, 385, 386]. However, ES and iPS cells can potentially differentiate to any of the three germ layers (ectodermal, mesodermal and endodermal) and all somatic cell types in the presence of adequate and appropriate signals *in vitro*. For example, mouse ES and iPS cells can generate ectodermal cells such as neurons [137, 172], mesodermal cells such as cardiac cells [387, 388] and endodermal cells such as insulin-producing pancreatic cells [389, 390]. The differentiation outcome greatly depends on the microenvironment in cell culture [169]. Differentiation protocols usually provide microenvironments that recapitulate the *in vivo* differentiation milieu. This differentiation capacity made mouse ES and iPS cells an attractive cell source for studying different disease mechanisms and for drug screening as well as suitable models for cell replacement therapy research in a plethora of pathologies.

In vitro differentiation of mouse pluripotent stem cells is induced using various techniques. One popular and powerful approach to studying and optimizing pluripotent stem cell differentiation down a particular lineage is the use of embryoid bodies (EBs). When formed in the absence of signaling cues, EBs have cells of all the three germ layers. EB formation recapitulates a similar tri-dimensional structure to that of mouse embryo blastocysts and represents a great *in vitro* model system to study lineage specification and further organogenesis in animals [391].

There are several different protocols to differentiate murine pluripotent stem cells to neural cells. The vitamin A derivative, retinoic acid (RA), plays a role in the developing mammalian embryo. When it binds to its receptor, RA specifies the anterior-posterior body plan

[180] and activates the transcription of downstream target genes leading to neural lineage selection. RA is commonly used to induce neural differentiation from mouse pluripotent stem cells [175-179]. In pluripotent cell differentiation, RA activates neural-specific transcription factors, signaling molecules, and RA-inducible genes resulting in the production of different neuron sub-types such as GABAergic and glutamatergic neurons [175]. The RA neural differentiation method depends on the formation of EBs, induces neurosphere production from EBs and produces both glial cells and neurons. Since RA is added in the last 4 days in the 8-day protocol, this procedure is generally referred to as the "4-/4+" protocol. Neurons derived in this method show mature neuronal morphology, express mature neuronal markers such neuronal nuclei (NeuN) and neurofilament and exhibit neuronal electrophysiology profiles that include mature sodium currents, potassium currents, and action potentials [179]. Furthermore, specific neuronal subtypes (dopaminergic, GABAergic, etc.) can be cultivated in a controlled microenvironment with specific substrates and media components. Despite its widespread use for neuronal differentiation from mouse pluripotent stem cells, the efficiency of this 4-/4+ differentiation protocol is generally moderate to low.

EB and neurosphere formation during the 4-/4+ neural differentiation protocol is a critical step that determines the outcome and yield of neurons. It is essential thus to control the size and shape of EBs and neurospheres and to keep them in suspension and prevent their adherence. Currently, the suspension culture 4-/4+ protocol produces great numbers of neurosphere that, however, aggregate to form large irregular clusters with apparent cell death in the center and edges and strongly adhere to the bottom of the petri dishes. Cells at different locations within these irregular clusters perceive different gradients of oxygen, nutrients and differentiation cues making this process highly non-homogenous and consequently affecting the yield, quality and

uniformity of the produced neurons. Accordingly, it is essential to develop a technique to prevent aggregation and adhesion of neurosphere and also increase their uniformity. Recently, scientists have developed methods to circumvent these problems. Rotary suspension cultures enhanced the efficiency, yield and homogeneity of EBs [344]. Rotary cultures increase diffusion of nutrients and small molecules into EBs, facilitate waste removal and increase the efficiency of formation of the spheroidal EBs. Rotary cultures have been successfully applied to form spheroidal structures from tumor [392] and neural stem [393] cells. Rotary cultures, however, have not been applied to produce neurospheres and induce neural differentiation from mouse pluripotent (ES and iPS cells) stem cells yet.

The importance of *in vitro* expansion of a homogenous population of stem cells before transplantation cannot be over-emphasized. In this study, we hypothesized that using rotary cultures together with the 4-/4+ differentiation protocol will increase neurosphere homogeneity, neural precursor (NP) yield and produce neurons that are either of higher or similar quality as those produced with static suspension cultures. We, thus, compared the morphology of EBs and neurospheres, the yield of NPs after neurosphere dissociation and the quality of neurons after terminal differentiation in both rotary and static suspension neural differentiation protocols using RA. Rotary suspension cultures produced highly uniform neurospheres, and significantly more neural precursors and functional neurons compared to static cultures in both mES and miPS cells.

B. Results:

Rotary cultures increase neurosphere homogeneity and the number of neural precursors from mouse ES cells

Rotary and static D3 mES cell differentiation cultures were initiated with equal number of cells ranging from $1x10^6$ to $4x10^6$ cells per dish. We followed EB and neurosphere formation over the course of the 4-/4+ differentiation protocol. We called spheroids EBs before day 4 (when RA is added) and neurospheres after the addition of RA (days 4 to 8). At day 2, static cultures formed small EBs and occasionally large clusters of EB aggregates whereas rotary cultures formed predominantly small, uniformly spherical EBs without aggregation. By day 6, static culture neurospheres appear as large and highly irregular clusters with dense centers and loosely attached dead cells on their edges whereas rotary culture neurospheres were slightly larger than those at day 2 but still highly spherical and uniform. No dead cells were noticed to hang from neurosphere edges in rotary cultures (Fig. 7.1A). It was also noticed that static cultures had much greater numbers of floating single or small dead cell clusters and great proportion of adherent EBs and neurospheres. There were no adherent EBs or neurospheres in rotary cultures. These data indicate that rotary cultures produce higher quality and more uniform neurospheres compared to static cultures. To study the effect of initial seeding densities on final NP yield, we initiated rotary and static differentiation cultures starting from 1, 2 or 4 million cells (Fig. 7.1B). We counted the number of NPs after neurosphere dissociation at the end of the 8-day differentiation protocol. Independent of the initial densities, the number of neurospheres did not significantly change in the static cultures. Rotary cultures, however, always had higher numbers of NPs at all seeding densities. There was 2-fold increase in yield at 1 and 2 million cells initial densities and over 4-fold increase when starting with 4 million cells indicating that higher initial seeding densities produce higher yields in rotary cultures. These data show that

rotary cultures significantly increase the yield in the 4-/4+ neuronal differentiation of mES cells. To study the effect of rotation speeds on NP yield in rotary cultures, we tested 3 different speeds (20, 30 and 40 rpms) stating from 4 million ES cells (Fig. 7.1C). There were significantly higher numbers of NPs after neurosphere dissociation at 30 and 40 rpm compared to 20 rpm. There was no difference in yield or neurosphere morphology (Fig. 7.1C, see inset) at the two higher speeds. Because of this, we used 30 rpm for all our subsequent experiments.

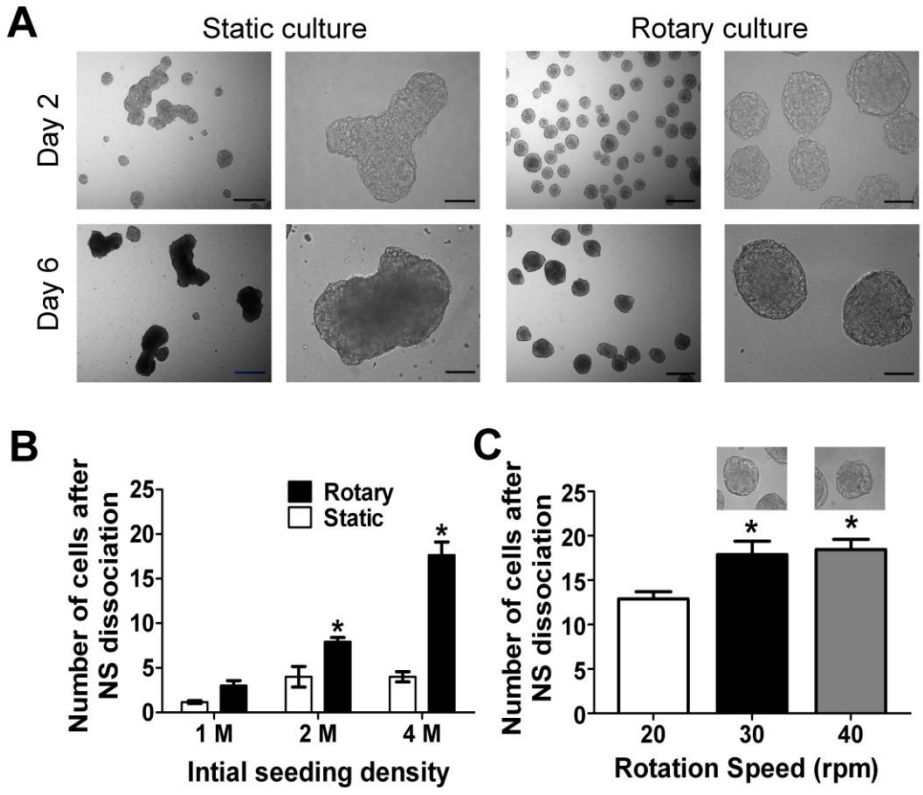

Fig. 7.1. Rotary cultures increase neurosphere homogeneity and the number of neural precursors from mouse ES cells

(A) By day 2, EBs formed in static cultures are small or large aggregates. Rotary EBs formed at day 2 are small, circular and uniform. By day 6, neurospheres formed in static cultures are large and highly irregular. Rotary culture EBs at day 6 are smaller, round and more uniform. (B) The numbers of NPs derived from rotary and static cultures after neurosphere dissociation were assessed after 8 days starting with different initial seeding densities. There were significantly more NPs in the rotary culture when we started from 2 or 4 million cells with about 4-5 fold increase in efficiency starting with 4 million cells (n=3; *.P<0.05 using two-way ANOVA with Bonferroni's post-hoc analysis). (C) The numbers of NPs derived from rotary cultures (starting from 4 million cells) at different rotation speeds. There were significantly more cells at higher speeds (30 and 40 rpm) compared to 20 rpms (n=3; *.P<0.05 using one-way ANOVA with Bonferroni's post-hoc analysis). There were no differences in neurosphere morphology at the two higher speeds (see inset).

Reduced cell death and increased proliferation in EBs and neurospheres in rotary cultures

We tried to investigate the mechanisms underlying the increased yield of NPs after neurosphere dissociation in the rotary cultures. We hypothesized that rotary culture EBs/neurospheres have either reduced cell death or increased proliferation or both. We thus stained for TUNEL in EBs at day 4 just before the addition of RA to detect cell death and for BrdU at days 4 before addition of RA and days 6 and 8 to detect proliferation. For cell death analysis, we counted the number of TUNEL-positive/DAPI-positive cells in day 4 EBs (Fig. 7.2A). There were significantly more TUNEL-positive cells in EBs from static cultures compared to rotary cultures (35.35 ± 2.48 vs. 22.26 ± 1.53 cells; $p<0.05$ using Student's *t-test*) indicating reduced cell death in rotary cultures. For cell proliferation, we measured the area of BrdU staining to DAPI staining in day 4 EBs and days 6 and 8 neurospheres (Fig 7.2B, C and D). BrdU staining was higher in day 4 rotary culture EBs compared to static cultures; the difference, however, was not statistically significant. There were significantly more BrdU-positive staining in day 6 and day 8 neurospheres from rotary compared to static cultures (for example 0.54 ± 0.03 vs. 0.72 ± 0.01 at day 8; $p<0.05$ using Student's *t-test)* indicating increased cell proliferation with rotary cultures. These data indicate that rotary cultures reduce cell death and increase proliferation in EBs/neurospheres during the 4-/4+ neuronal differentiation of mouse ES cells.

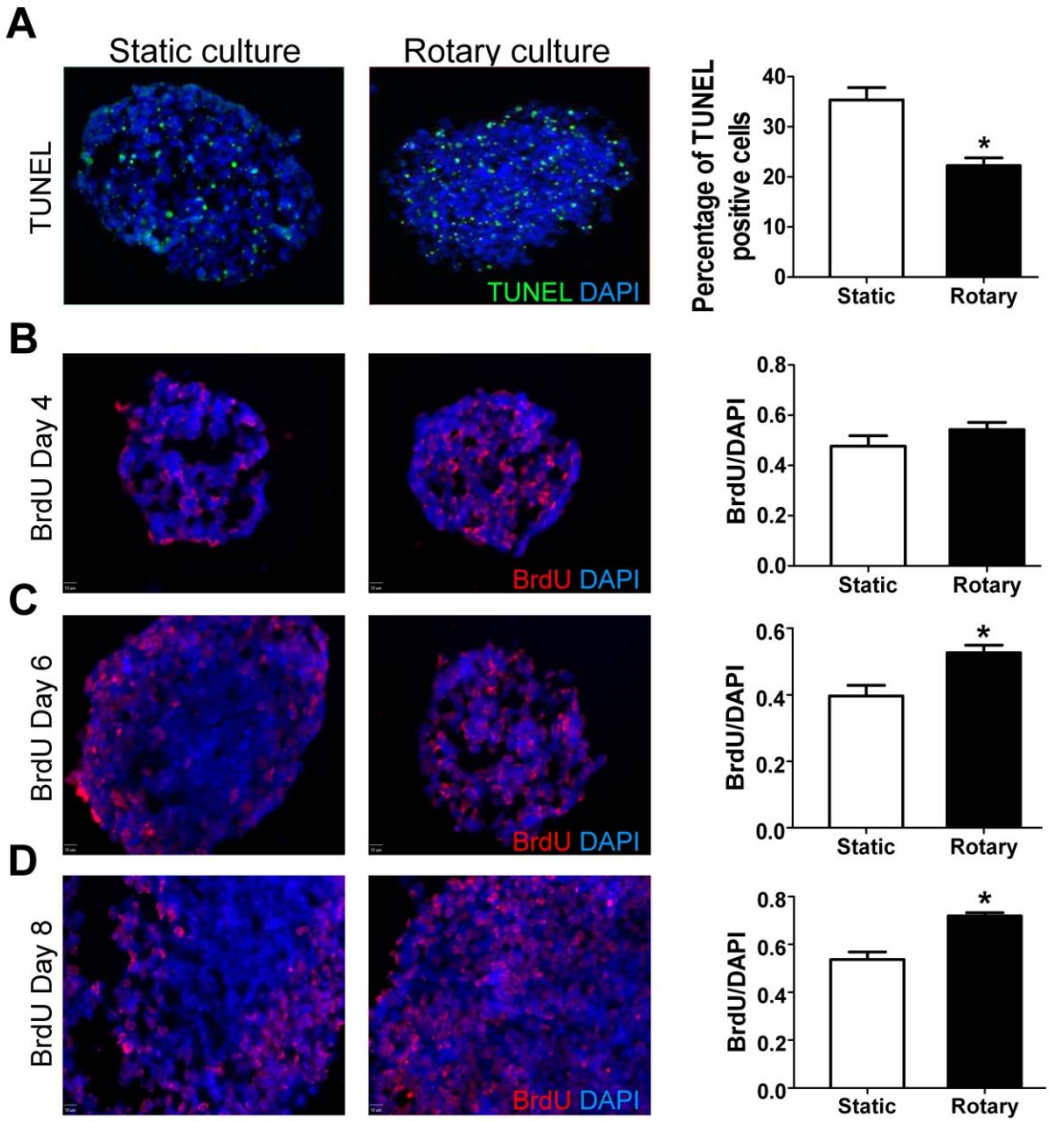

Fig. 7.2. Reduced cell death and increased proliferation in EBs and neurospheres in rotary cultures

(A) Staining of TUNEL in day 4 EBs from static and rotary cultures indicates increased TUNEL-positive cells or cell death with static cultures (n=3, *.P<0.05 using Student's *t-test*). (B, C and D) Staining of BrdU in day 4 (B), day 6 (C) and day 8 (D) EBs from static and rotary cultures indicates increased BrdU-positive cells or cell proliferation with rotary cultures especially at days 6 and 8 (n=3, *.P<0.05 using Student's *t-test*).

Increased immature neuronal marker Tuj-1expression in day 8 neurospheres of rotary cultures

To elucidate the differences between rotary and static cultures in terms of pluripotency, ectodermal or early neuronal genes, we employed immunocytochemistry and qRT-PCR analysis for selected genes. We first wanted to see how Oct-4 expression, a marker of pluripotent stem cells, changes in the two culture conditions. qRT-PCR analysis revealed that while Oct-4 gene expression significantly dropped at day 8, there were no differences in Oct-4 levels between rotary and static cultures indicating that rotary culture did not affect the loss of pluripotency in the context of neuronal differentiation (Fig. 7.3A). Since this is a neural differentiation protocol, we were mostly interested in neuroectodermal and early neuronal gene levels. We thus selected nestin, a neuroectodermal marker, and Tuj-1, an immature neuronal marker, to discern any differences between the rotary and static conditions. Nestin mRNA expression was similar between rotary and static cultures at days 4 and 8. Nestin protein expression levels were slightly elevated in the rotary cultures at both time points (days 4 and 8) but the difference was not statistically significant. These data also indicated that rotary cultures do not affect normal mES cell differentiation (Fig. 7.3B, C). Staining for Tuj-1 was negative to very low in both the rotary and static cultures at days 4 and 6 (data not shown). Tuj-1 expression was significantly increased at day 8 and was higher in neurospheres from rotary cultures (Fig. 7.3D; 0.23 ± 0.04 vs. 0.54 ± 0.06; $p<0.05$ using Student's *t-test*) indicating earlier neural commitment under rotary conditions. These data suggest that rotary cultures accelerate neural differentiation in neurospheres during differentiation of mES cells.

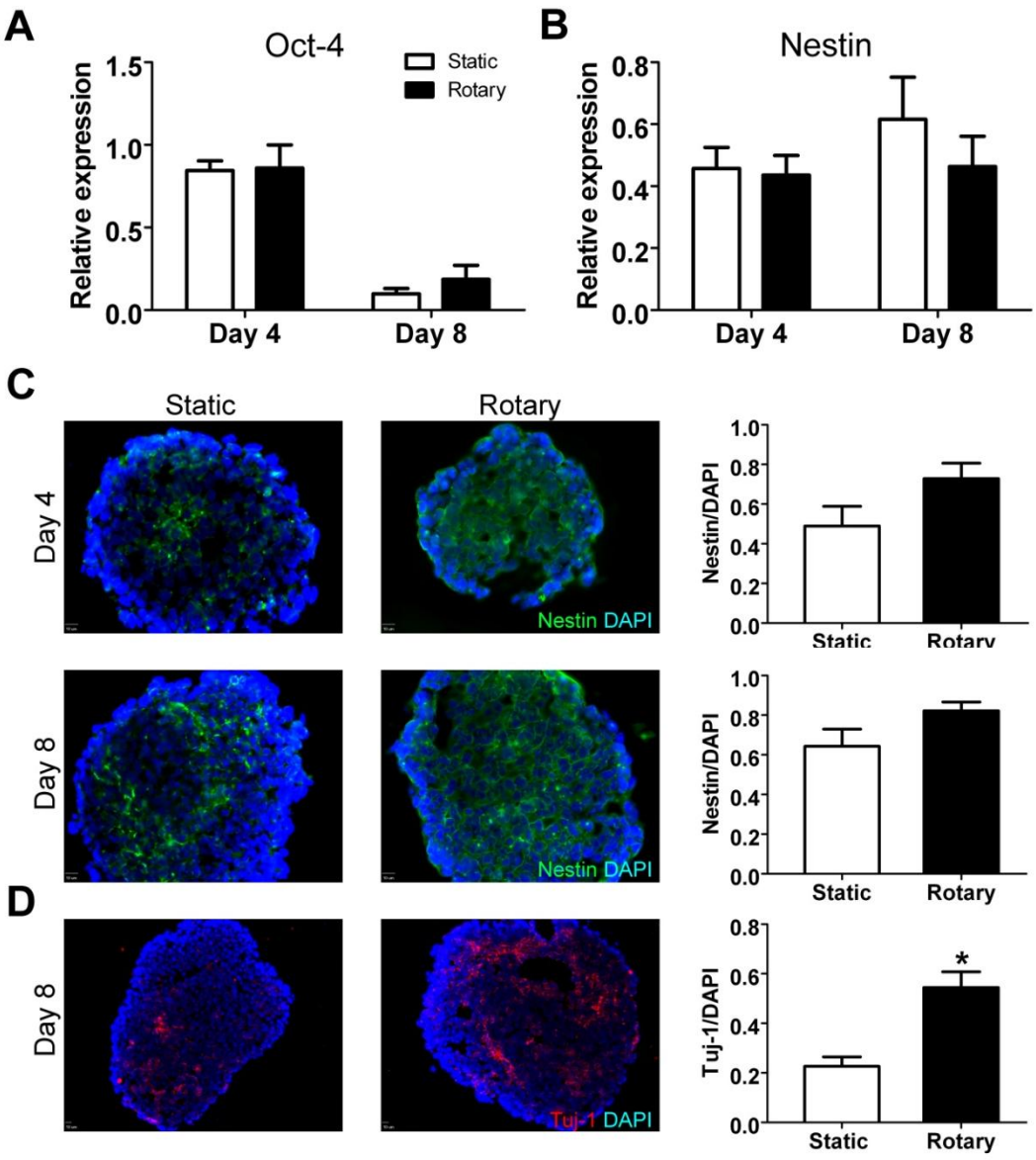

Fig. 7.3. Increased immature neuronal marker Tuj-1 in day 8 neurospheres of rotary cultures (A and B) Quantitative real-time PCR showing the relative expression of Oct-4 and nestin in day 4 EBs and day 8 neurospheres in static and rotary cultures. While Oct-4 mRNA significantly dropped at day 8, there was no difference between static and rotary cultures (A). There was no difference in nestin expression at both time points (B). (C) Staining for nestin in day 4 EBs and day 8 neurospheres showed slightly increased nestin in rotary cultures without being significant (n=3, *.P>0.05 using Student's *t-test*). (D) Staining for the immature neuronal marker, Tuj-1, in day 8 neurospheres showed increased neural phenotype in rotary cultures (n=3, *.P<0.05 using Student's *t-test*).

Rotary cultures produce neurons with similar phenotype and function as static cultures

After assessing morphology, phenotype and gene expression of EBs and neurospheres during differentiation, we wanted to examine the quantity and quality of neurons by the end of the differentiation protocol. Neurospheres were collected and dissociated and the resulting NPs were plated on laminin substrate for 5 days. We then assessed the expression of Tuj-1 and other markers of mature neurons (NeuN, NF, synapsin and MAP-2), measured neurite length and recorded action potentials under both rotary and static conditions. Although we plated the same number of NPs after neurosphere dissociation, we always ended up with more neurons from rotary cultures as shown in Fig. 7.4A and Fig. 7.4B (for example, total Tuj-1 counting; 62.80 ± 8.39 vs. 208.4 ± 28.37 in static vs. rotary cultures, respectively). Unlike Tuj-1 in neurospheres which showed higher expression in rotary cultures, the percentage of Tuj-1 expressing neurons is lower in rotary cultures (82.61 ± 3.64 vs. 63.11 ± 4.30; $p<0.05$ using Student's *t-test*) again indicating accelerated neuronal differentiation and more mature neurons under rotary conditions (Fig. 7.4A, B). While Tuj-1 expression was lower in rotary cultures, there were no significant differences in the percentage of NeuN-positive neurons, the length of neurites (MAP-2) or the amplitude of action potentials (Fig. 7.4C) from neurons under both conditions. Neurons from both rotary and static cultures stained 100% for synapsin-1. These data indicate that neurons produced from rotary cultures mature faster but are structurally and functionally similar to those from static cultures.

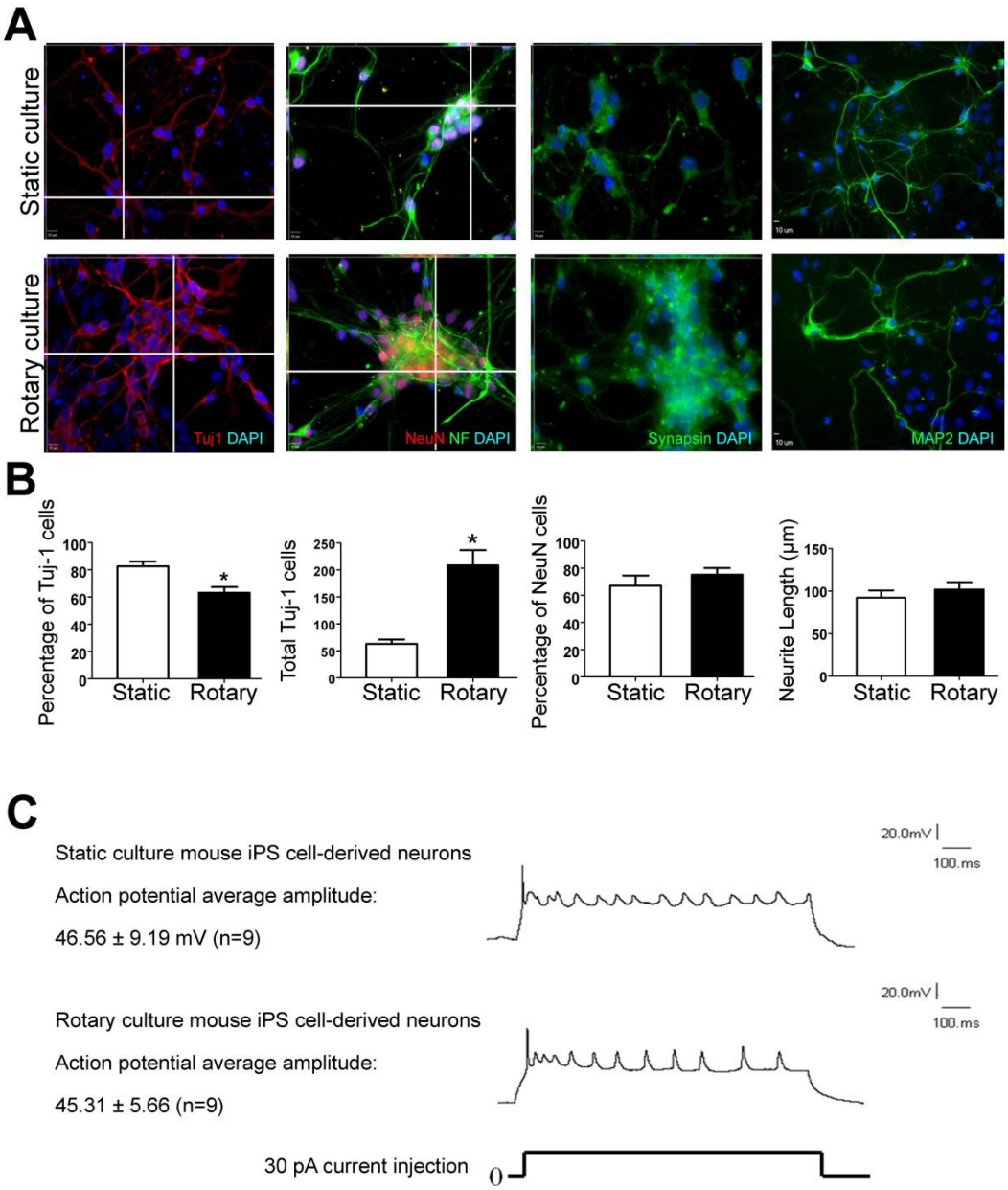

Fig. 7.4. Rotary cultures produce neurons with similar phenotype and function as static cultures

(A and B) While rotary cultures obviously produced more cells after plating, there were no differences in the percentages of NeuN- or synapsin- positive cells in both conditions. The percentage of Tuj-1 positive cells is significantly lower in neurons derived from rotary culture indicating a more mature phenotype. The total number of Tuj-1 positive cells however is significantly higher in neurons derived from rotary cultures indicating better survival after plating. Neurite length measured using MAP-2 expressing extensions was not significantly different between rotary and static conditions. (C) Recording of evoked action potentials showed there was no difference in the amplitudes of action potential between neurons derived from rotary or static conditions.

Rotary cultures increase yield of NPs from mouse iPS cell differentiation and produce mature and functional neurons

As shown above, rotary cultures increase the yield of NPs, accelerates neuronal differentiation and produce functional neurons from mES cells using the 4-/4+ differentiation protocol. However, we wanted to test whether same results could be obtained from mouse iPS cells WP5. Similar to mES cell-derived neurospheres, rotary culture neurospheres of mouse iPS cells were smaller and more uniform compared to neurospheres of static cultures which formed large aggregates of highly dense and irregular clusters (Fig. 7.5A). There were also more dead cells either floating in suspension or attached from the edges of the neurospheres and more cells and aggregates attached to the plates in static cultures. No attached aggregates were detected in rotary culture dishes. Unlike mES cells however, there was only 1.5-2 folds increase in NP yield in rotary cultures of mouse iPS cells. These data indicate that mouse iPS cell rotary cultures are more efficient in generating NPs than static cultures but the efficiency is less than that observed with mES cells. Neurons derived from mouse iPS cell rotary cultures, however, also stained positive for mature neuronal markers NeuN, NF, CaMKII and synapsin-1 (Fig. 7.5B, C) and exhibited trains of evoked action potentials when injected with 30 or 60 pA (Fig. 7.5D). These data indicate that rotary cultures also increase yield from mouse iPS cells and produce mature functional neurons.

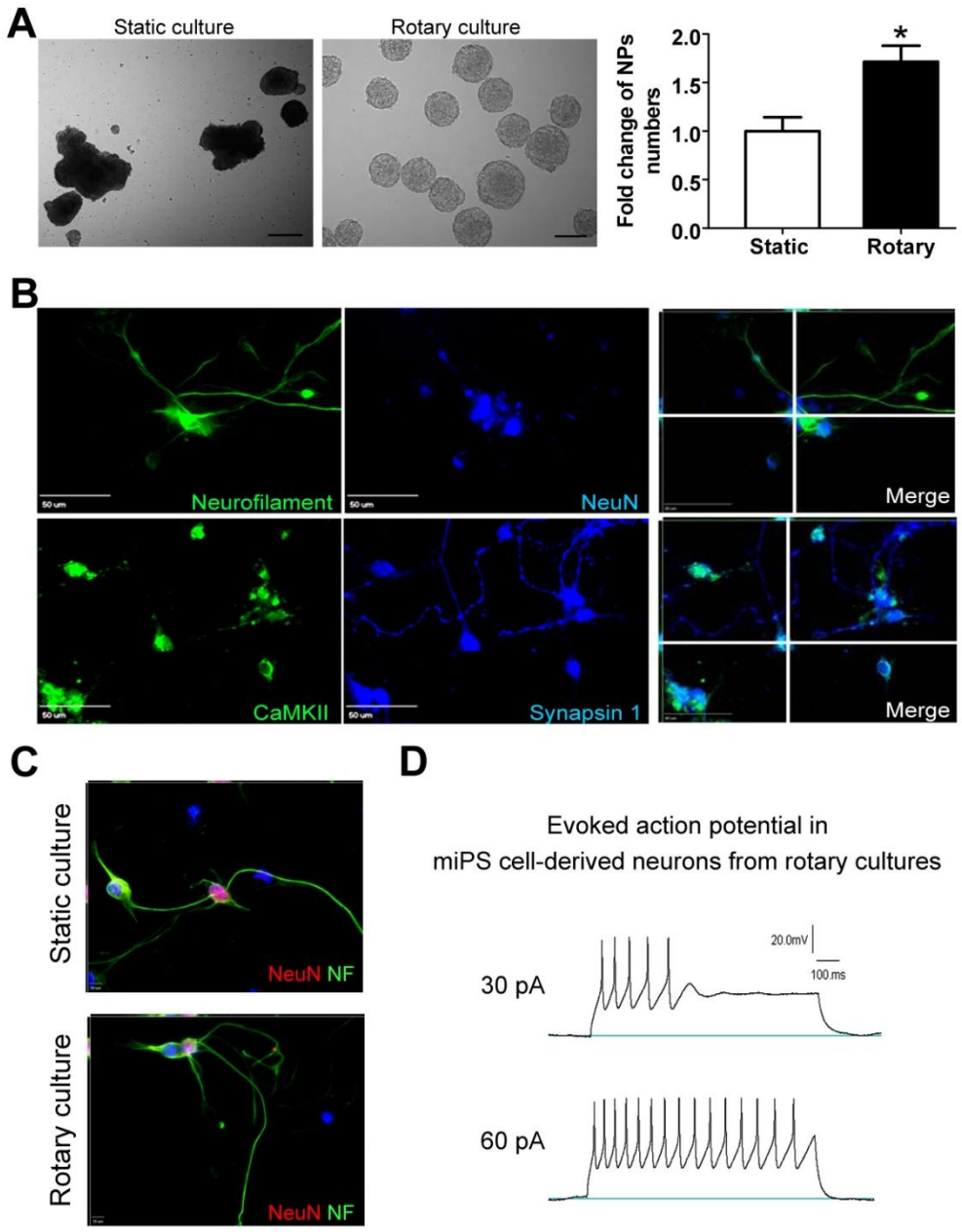

Fig. 7.5. Rotary cultures increase yield of NPs from mouse iPS cell differentiation
(A) Rotary cultures produce smaller and more uniform neurospheres from miPS cells. The yield of NPs after neurosphere dissociation is significantly higher with rotary cultures (n=3, *.P<0.05 using Student's *t-test*). (B) Neurons derived from miPS cells under rotary conditions stain positively for mature neuronal markers neurofilament, NeuN, CaMKII and synapsin-1. (C) There is no difference in neuronal morphology of NeuN-positive neurons from rotary and static cultures. (D) miPS cell-derived neurons from rotary conditions exhibit trains of action potential in response to 30 and 60 pA current injections.

C. Discussion:

In this study, we used the 4-/4+ differentiation protocol of mouse ES and iPS cells to compare the effects of rotary culture on the morphology of neurospheres, the yield of NPs and the quality of neurons relative to static cultures. Rotary cultures produced smaller and more uniformly spheroidal neurospheres compared to the large and highly irregular neurospheres of static cultures. While dependent on the initial seeding density and rotation speed, rotary cultures of mouse ES cells increased the yield of NPs after neurosphere dissociation by 4-5 folds under optimal parameters. EBs and neurospheres from rotary cultures had less cell death, increased proliferation and higher expression of Tuj-1, all of which mostly noted in the center of the spheroids. Rotary culture derived neurons survived better after plating, expressed less Tuj-1 and showed similar morphological and electrophysiological properties as those from static cultures. Rotary cultures also increased homogeneity of neurospheres and NP yield from mouse iPS cells and produced mature and functional neurons. These data suggest that rotary culture is an effective and inexpensive method to increase the yield of neuronal differentiation. Given the high quality of neurons produced, we suggest that rotary culture should be adopted for mouse ES and iPS cells neuronal differentiation *in vitro*.

Many methods have been used to control the size and homogeneity of EBs. Different techniques successfully tried to reproducibly generate EBs that are uniform in size and shape and size-controlled. For example, AggreWell™ plates have micro-wells that are hundreds of micrometers in diameter and allow users to control the size and shape of the EBs. Other techniques to form uniform EBs include the use of conical tubes [394] or agarose capsules [395]. While many of these techniques successfully generated the desired outcome, most are expensive, inefficient or quite laborious. Rotary cultures have proved to produce highly uniform EBs [344]

in a very cost- and time- effective manner. In this study, we applied rotary cultures to our differentiation protocol and we generated highly uniform neurospheres that produced 4-5 fold increase in NPs and neurons.

Rotary cultures prevent the clustering of neurospheres and increase their efficiency and homogeneity by increasing the diffusion of nutrients and small molecules into the center of neurospheres, and facilitating the removal of waste products. This is best reflected by the increased survival and proliferation especially in the center of neurospheres where diffusion is most difficult. It is clear that the highly irregular neurosphere clusters formed in static cultures receive less nutrients and are less able to remove wastes from their centers which explains the increased cell death and reduced proliferation. Indeed, we noticed increase TUNEL positive and reduced BrdU staining in neurospheres from static cultures. Moreover, the color of media in rotary cultures was less dramatically changing to yellow (not shown) indicating less acidification and slower accumulation of waste products in the rotary cultures. In addition, there were no adherent clusters in rotary compared to static cultures in which EBs/neurospheres significantly adhered to the bottom of petri dishes. This adherence not only reduces the yield of NPs but also can affect intracellular signaling and thus, influence neuronal differentiation [396-398]. All these observations indicate that the hemodynamic agitation induced by rotation has a positive influence on the nutrition and metabolic function of neurospheres which translates to an increase in homogeneity and yield.

The yield of NPs from rotary neurosphere cultures varied greatly depending on the initial seeding density and rotation speed. The more cells were seeded initially, the higher the fold change in NPs was; with the highest yield obtained starting from 4 million cells. Moreover, rotation speeds also affect NP yield with higher speeds (30 and 40 rpm) yielding more NPs

compared to 20 rpm. This is somewhat similar to what was reported earlier [344] where rotation speeds above 50 rpm exhibited relatively high shear stress and produced large numbers of free single cells in suspension. We did not try our differentiation protocols with speeds higher than 40 rpm.

Since rotary culture produce highly uniform neurospheres with high NP yield, we further investigated the quality of neurons produced from rotary suspension cultures. Neuronal differentiation is highly influenced by cell-cell and cell-environment interaction and since rotary cultures produce uniform neurospheres that do not attach to bottom of petri dishes, we anticipated higher or similar quality neurons as those from static cultures. Indeed, both rotary and static culture neurons exhibited similar neuronal morphology, immunohistochemistry (similar percentages of NeuN and neurofilament and similar neurite length) and electrophysiological profiles. It is worth mentioning that although we plated same number of NPs, there were more neurons in rotary cultures indicating better survival even after plating. Moreover, Tuj-1, a marker of immature neurons, was higher in rotary neurospheres and lower in rotary neurons indicating a faster maturation of the neuronal phenotype with rotary cultures. This increase in Tuj-1 in neurospheres of rotary cultures can be attributed to the more efficient RA gradient in the smaller and more uniform neurospheres of rotary cultures. We acknowledge the need to further characterize the neuronal subtypes and their full electrophysiological profiles. These studies are to be the subject of another investigation.

In earlier studies, it was shown that rotary culture EBs have higher expression of Alpha-Fetal Protein (AFP) compared to static culture EBs indicating that rotary cultures enhance endoderm formation. While we did not test for markers of lineages other than the ectoderm (Nestin), we assume that the addition of RA directs differentiation down a neuro-ectodermal

lineage in both static and rotary cultures. Moreover, it is important to mention that while iPS cell neurospheres were also more uniform with rotary cultures, we obtained only 1.5-2 fold increase in NP yield compared to the 4-5 increase with mouse ES cells. In fact, we tested the 4-/4+ differentiation rotary protocol with E14 mouse ES cells and with human ES and iPS cells. Indeed, each cell line responds differently to rotation. E14 cells, for example, also showed lower yields than D3 cells. These data indicate that for every cell line to be differentiated with the rotary 4-/4+ protocol, different parameters of speed and initial seeding densities should be established to obtain optimal results.

In summary, this study provides evidence for the utility of rotary suspension cultures in the differentiation of mouse ES and iPS cells. Rotary cultures increase the homogeneity of neurospheres, increase NP yield and produce functional neurons after neurosphere dissociation and terminal differentiation. We suggest the adoption of rotary culture as a routine technique for the *in vitro* differentiation of mouse ES and iPS cells. Further experiments need to be performed to characterize the subtypes of the terminally differentiated neurons and to establish optimal parameters for other mouse and human ES and iPS cells.

Chapter VIII
Summary and conclusions

Stroke is a devastating disease in the United States and world-wide. It severely affects patients' lives and their families and overwhelms the healthcare system with expensive burdens while all efforts remain short of changing the status quo. The absence of medications that drastically improve stroke patients together with the increased expenditure for post-stroke recovery is draining all aspects of healthcare. Research and development departments of major pharmaceutical companies actively avoid testing medications in clinical trials for stroke treatment because they lost hope that any neuroprotective agent can be curative. Stem cells represent a great promise in stroke research because they, theoretically, can rescue brain tissue from death, enhance endogenous repair and replace dead tissue. Basic and translational research using stem cell-based therapies for the treatment of ischemic stroke is bringing waves of optimism and increasing the belief in their power. Many clinical trials have already started using different kinds of stem cells but the only trial using embryonic stem cells was halted last year for financial reasons.

Despite the optimism, scientists and the media henceforth should be very cautious not to inflate the public hopes about the outcome. The scientific community realizes how difficult and unpredictable it is to translate therapies from the bench to bedside. The best approach to tackling this problem is through more thorough investigations at the bench and in non-human primates regarding the potential, efficacy and mechanisms of action of stem cells, and their side effects. This is the advantage of this dissertation. Being aware of the need for more basic and translational research in stem cells, we determined to test the efficacy of a great candidate for stem cell therapy, the vector-free human iPS cells, to investigate their mechanisms of action, to develop new tools to study stroke pathophysiology and augment stem cell therapy and finally to optimize protocols for stem cell differentiation and pre-transplantation rituals.

The creation of vector-free human iPS cells is a great opportunity as they seem to be perfect candidates for cell-based therapies in neurodegenerative and non-neurological diseases. Vector-free hiPS cells can efficiently differentiate to neurons *in vitro* and *in vivo,* and restore functions and behaviors lost due to stroke without any apparent risk of tumor formation. Despite these great results, we do believe that more work is needed to encompass all mechanisms of action and survey for potential side effects including long term assessments for tumor formation and the fate of the transplanted cells. Along the same lines of scientific curiosity, we developed *in vitro* and *in vivo* optogenetic tools to further dissect the mechanisms of action and enhance the functions of stem cells after transplantation. Our optogenetic experience, once applied to studying stroke and stem cells, should answer important questions related to the integration of neural cells after transplantation, and the time course of neuronal stimulation/inhibition for the treatment of stroke. Finally, realizing the importance of the *in vitro* expansion of a homogenous population of stem cells before transplantation, we developed the rotary culture technique for stem cell differentiation. We are aware that human ES and iPS cells respond differently to rotary cultures, however this study provides a proof-of-principle that simple and non-expensive techniques can efficiently be applied in stem cell related protocols.

In the battle of stem cell therapy, there are many fronts to be fought simultaneously. The ethical debate is still ongoing. Despite the relief brought by the advent of murine and consequently human iPS cells, there are still voices – legitimate sometimes – calling for a stricter control of stem cell research for fears of experimentations in human cloning. While these fears are reasonable to some, others believe that the beauty of science lies in the passion that drives some scientists to try the forbidden in their attempt to understanding nature. The most important point to remember in this field is that the presence of such a debate is the healthiest sign that

stem cell science is and will proceed in the right direction. What the future will bring is so interesting to watch as more and more achievements unfold. In addition to ethics, scientists still have a long journey at the bench to understand stem cells. Compared to molecular medicine, stem cells are live structures that affect the body in as much as they are affected by it. It will be very important to not only characterize what the cells will do to our bodies but also how our bodies will affect those cells. That said, it is essential to critically scrutinize the transformational potential of stem cells after transplantation. So far, the science in that regard is not solid; clear cut answers are not provided and conflicting reports essentially threatens the whole field. It would be our greatest responsibility, as scientists in that domain, to keep our eyes open and our emotions aside such that we realize the futility of our endeavor if stem cells showed any cancerous transformation in human studies. The well being of our fellow citizens should be always our priority even if it means that scientists give up their dreams and aspirations sometimes. The beauty in science lies in the journey; the end point is for our communities to enjoy. There will always be more journeys for us to embark on.

In all, we believe that stem cells are very promising tools for treating neurodegenerative diseases including stroke. Extreme caution however should be taken in stem cell research and their clinical translation because the public will not forgive failures and any failure due to negligence or poor design will be catastrophic in this field. More investigations are definitely needed before any stem cell is to be injected in a human patient.

Chapter IX
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