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Direct reprogramming of astrocytes to enhance recovery after stroke

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Direct reprogramming of astrocytes to enhance recovery after stroke

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Abstract

Direct reprogramming of astrocytes to enhance recovery after stroke

By: Qize Jiang

Ischemic stroke is a leading cause of death and long term disability in the United States. Ischemic stroke results in death of neurons in the affected area with limited capacity for regeneration in the adult brain. Recent advances in stem cell techniques provide the possibility of ameliorating ischemic damage by replacing lost neurons with transplanted neuronal precursor cells that can terminally differentiate into mature neurons and integrate with the host circuitry. The focus of many transplantation studies currently centers on neuronal differentiation and transplantation of ES or iPS cells into the ischemic brain. More recently, studies demonstrate the panneuronal transcription factor NeuroD1 (ND1) can reprogram astrocytes directly into neurons, a process called direct reprogramming. Lentiviral vector delivery of ND1 to astrocytes results in permanently reprogrammed neurons without the need for maintained ectopic expression of the introduced transcription factor.

Astrocytes primarily provide support to surrounding neurons but also proliferate reactively in response to pathologies including ischemic stroke. Reactive astrocytes proliferate and hypertrophy in response to ischemic stroke and form a border around the site of injury forming a glial scar. Without intervention, there are an abundance of these reactive astrocytes in the peri-infarct region around the injury. Intra-lineage direct reprogramming provides an endogenous source of new neurons from existing proliferative astrocytes and has immense potential to reduce the burden of stroke.

Compared to traditional stem cell transplantation approaches, converted neurons derived from endogenous astrocytes will have the advantage of already being "settled" in a microenvironment that is more conducive to synaptogenesis and survival. Effective reprogramming of astrocytes to neurons further acts to "melt" the glial scar which normally exerts an inhibitory barrier for synaptogenesis and axonogenesis. Finally, new neurons are autologous and post-mitotic eliminating risk associated with rejection or tumor formation. Direct reprogramming of astrocytes has not yet been explored as a therapeutic tool in a model of ischemic stroke. The following work aims to use this novel approach to directly reprogram proliferative astrocytes into neurons *in vivo* following ischemic stroke and to augment activity dependent repair using whisker stimulation resulting in enhanced functional recovery in mice. We hypothesize direct conversion of astrocytes to neurons in the peri-infarct area will improve functional recovery in a mouse model of ischemic stroke that can be enhanced with whisker stimulation. The following study utilizes a novel approach to cell replacement in a model of ischemic brain injury and evaluates the efficacy of intra-lineage direct reprogramming of astrocytes into neurons *in vivo*. Direct reprogramming with activity dependent repair may improve functional recovery and reduce the morbidity of this common but devastating disease.

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Selected 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
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
TIA	Transient ischemic attack
iPS	Induced pluripotent stem

ES	Embryonic stem
hiPS	human iPS
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IA	Intra-arterial
SSFO	Stabilized step function opsins
DIO	Double-floxed inverted open reading frame
TIA	Transient ischemic attack
EPO	Erythropoietin

Chapter One: Ischemic Stroke

1.1 Stroke

Definition and types of stroke

Stroke ranks as the fifth highest cause of death and the top cause of long term disability amongst adults in United States (Mozaffarian et al., 2015). Stroke occurs in the brain as a result of reduced or absent blood flow causing cell death. Although stroke is a complex disease, all stroke can be broadly categorized into two main classes: ischemic and hemorrhagic. Ischemic stroke comprises up to 87% of all stroke cases and is characterized by a clot which occludes a blood vessel supplying the brain. Ischemic stroke most commonly occurs as a result of an occlusion in the middle cerebral artery or one of its branches. An occlusion then leads to a complex cascade of both pathological and regenerative processes including damaging release of glutamate, Ca2+, free radicals, and inflammatory factors resulting in autophagy, excitotoxic cell death, apoptosis, necrosis, and hybrid forms of cell death involving two or more of these mechanisms (A. Durukan & T. Tatlisumak, 2007; W.-L. Li et al., 2013; Julien Puyal, Vanessa Ginet, & Peter GH Clarke, 2013; L. Wei, D.-J. Ying, L. Cui, J. Langsdorf, & S. P. Yu, 2004; Ai Ying Xiao, Ling Wei, Shuli Xia, Steven Rothman, & Shan Ping Yu, 2002). Hemorrhagic stroke is the second most common form and occurs as a result of injured brain blood vessels leading to intracranial hemorrhage (ICH) (Ferro, 2006). Transient ischemic attack (TIA) which are also known as “mini-strokes,” are characterized by short-term or temporary occlusions of brain vessels where a blockage or clot is resolved without intervention. While TIA may not

result in permanent brain damage, they are often an indication and warning for the occurrence of stroke.

Stroke is a serious threat to human life and health with very few effective treatments available to stroke patients. Up to 38% of hemorrhagic stroke victims and 10% of ischemic stroke victims do not live past 30 days following a stroke event. Survivors are often left with permanent physical and/or mental disabilities requiring comprehensive care and attention. Extensive research into this devastating disease has improved our understanding of the mechanisms involved in ischemia-induced cell death. As our scientific understanding has developed, many experimental therapeutic treatment approaches have been explored. Unfortunately, despite positive results in animal models, neuroprotective treatments have tended to fail in clinical trials. Developing clinically effective therapies for ischemic stroke remains a significant challenge and it appears that to overcome it, we will need to re-examine conventional perspectives on stroke pathology and pathophysiology towards innovative and combinatorial therapeutic strategies.

Stroke Treatment

In contrast to the research focus on the peri-infarct region, human stroke data suggest that the cortical ischemic core but not the penumbra is a determinant of clinical outcomes after acute ischemic stroke (Jovin et al., 2003; Koennecke, 2003). The percentage of penumbra tissue remains relatively constant from individual to individual. Rather than the percent penumbra it was the size of the ischemic core that was significantly associated with outcomes. This

clinical information reinforces that a more accurate understanding of cellular, molecular, and pathological events in the ischemic core may be critically important for developing stroke treatments. Unsurprisingly, clinical approaches for stroke have targeted the peri-infarcted region as a primary outcome to measure the success of stroke treatments while these same treatments have tended to ignore the ischemic core.

The success of stroke treatments in animal models but failure to translate these therapeutic strategies in human clinical trials has been a decade's long and ongoing frustration for basic scientist, medical practitioners, patients, and their families. Early approaches to mitigating stroke damage revolved around the concept of shielding vulnerable neurons from ischemic damage in a process termed "neuroprotection." Compounds such as erythropoietin (EPO) for example demonstrated significant neuroprotective effects in animals models of stroke with and without the administration of t-PA (Bernaudin et al., 1999; Keogh, Yu, & Wei, 2007; Leist et al., 2004; Y. Li, Lu, Keogh, Yu, & Wei, 2007; Y. Li, Lu, Ogle, & Wei, 2007; Prass et al., 2003; Tsai et al., 2006), (Ehrenreich et al., 2002). Despite consistent reports of successful neuroprotective effects in non-human models, EPO failed to provide similar benefits to outcomes in human thrombolysis studies (H. Ehrenreich et al., 2009). Despite enormous financial burdens incurred by stroke, only one drug has been FDA-approved for acute stroke victims. Tissue plasminogen activator (tPA) is able to break down blood clots when administered systemically and is able to restore blood flow to the brain. However, a major limitation of this compound is its short therapeutic

window of just four and a half hours. Additionally, this compound carries with it a significant risk of causing hemorrhage and cannot be used for that type of stroke. Limitations on the use of tPA results in only approximately 2% of stroke patents qualifying for this treatment leaving the vast majority of stroke victims without access.

The treatment of stroke remains an important and demanding endeavor requiring innovative and effective strategies often involving multifactorial or combinatorial treatments. Stroke is a complex disease and its resolution will also be far from simple.

1.3 Pathophysiology of ischemic stroke

Ischemic Core

Cerebral ischemia typically induces an ischemic core in the supplying territory of the occluded artery. Massive excitotoxic cell death or pannecrosis occurs several hours later and continues for a few days after the insult (D. W. Choi, 1992; J. H. Garcia, Liu, & Ho, 1995). The classical and typical definition of the ischemic core is that of “a volume of tissue with which all cells (neuronal and glial), blood vessels (arteries, veins and capillaries) and nerve fibers (myelinated and non-myelinated) have undergone necrosis” (Nedergaard, 1988). While neurons in the ischemic core typically succumb to some form of insult over time, the pathophysiology of cell death in the ischemic core is a complex and ongoing process that is slower than expected in which selective populations of cells are

affected at different time points (Jiang et al., 2017). CBF in the ischemic core is typically reduced to 10% or less(Jiang et al., 2017).

Peri-infarction

Contiguous to the core region, there is a transitional area where an intermediate severity of local cerebral blood flow (LCBF) reduction results in selective neuronal cell death (J. C. Baron, Yamauchi, Fujioka, & Endres, 2014; Kato & Kogure, 1999; Nagahiro et al., 1998; Sharp, Lu, Tang, & Millhorn, 2000). This relatively minor injury occurs more slowly (days to weeks) and cells are more likely to undergo programmed cell death (Barone, 2009; Broughton, Reutens, & Sobey, 2009; Ferrer, 2006; W. L. Li et al., 2013; L. Wei, D. J. Ying, L. Cui, J. Langsdorf, & S. P. Yu, 2004). The peri-infarcted region is the target of many therapeutic approaches as this brain region is considered to have the greatest chance to preserve otherwise lost tissue. CBF in the peri-infracted zone is typically reduced to 35% or less of normal flow.

In contrast to the ischemic core, the peri-infarcted region is a highly regulated area and the primary location where gliosis forms the glial scar to surround and contain the cytotoxic paracrine signals located in the adjacent ischemic core region.

1.4 Mechanisms and features of cell death and survival

Cell death is an important part of normal neuronal function as well as development. During normal development, neurons undergo cell death in a controlled manner usually absent of any pathological consequences. However, neurons may also die as a result of a host of pathiological causes including

traumatic brain injury, stroke, and other neurodegenerative diseases. The manner in which neurons die has been well characterized with the best understood of the mechanisms being apoptosis whereby a cell executes a caspase dependent programmed cell death. However, apoptosis is far from the only controlled mechanism governing cell death in the brain. Autophagy is another well-defined and controlled mechanism by which cells die under both normal and pathological conditions. Uncontrolled cell death resulting from insult or disease is often characterized by necrosis or necrotic cell death. Finally, the balance of unregulated and regulated cell death mechanisms can sometimes be observed together whereby cells exhibit two or more characteristics with a particular form of cell death resulting in hybrid cell death.

Cell survival

Despite extensive research on ischemic strokes, only few investigations report scattered information on cell death in the ischemic core. One such systematic pathological review in 1996 described an "incomplete infarction", referring to partial cell death in the ischemic area (J. H. Garcia, Lassen, Weiller, Sperling, & Nakagawara, 1996). Those human and animal data supported an early assumption that "tissue necrosis would affect only a portion of the cells within the ischemic area if the arterial occlusion was of short duration or if the ischemia was of moderate severity" (L. Wei et al., 1998). The incomplete infarction resulted in selective neuronal necrosis with preservation of some neurons, glia, and microvessels. Consistently, it was reported that "islands" of surviving neurons were observed in the ischemic core 2 days after stroke

(Mennel, El-Abhar, Schilling, Bausch, & Krieglstein, 2000). However, ischemic core has been ignored for the last 20 years because attention in the stroke field was drawn to the attractive concept of salvaging the penumbra. This is mostly due to the slower process of cell death in this area and increasing understanding of the programmed cell death pathways (Aronowski, Cho, Strong, & Grotta, 1999; Ferrer & Planas, 2003; Ghobrial et al., 2014; Heiss, 2011; Kidwell, Alger, & Saver, 2003). In addition to the information from early investigations, the mechanisms of death, and in particular, the survival of neuronal and vascular cells inside the core, are largely unknown.

The development of effective stroke therapies may be hindered by a lack of specific focus on the ischemic core and filling the resultant knowledge gap regarding this important area could bolster new therapeutic approaches. For instance, it is not clear whether "incomplete infarction" occurs in common clinical cases or what types of ischemic strokes may result in the incomplete infarction. The current lack of knowledge on cell fate in the ischemic core has had a negative impact on the development of targeted stroke therapies. For instance, in some cases, cells inside the core may be saved just as readily as cells in the penumbra. Moreover, many stem cell transplantation therapies after ischemic stroke are designed to avoid placing graft cells into the core region. Common justification for this practice is that there is no supporting infrastructure for grafted cells and nothing worthy of rescue inside the core region. Unfortunately, this logic translates into clinical trials that have focused on salvaging tissues in the ischemic penumbra rather than the ischemic core.

Apoptosis

Highly controlled and regulated cell death mechanisms are critical to the normal development and functioning of the brain. Cells can execute what is now thought of as “canonical” programmed cell death by initiating a caspase dependent pathway which induces distinctive, observable morphological changes in cells such as membrane blebbing, nuclear condensation, nuclear fragmentation, and a shrinking of the cytoplasmic volume. These cells eventually leave behind apoptotic bodies that can be phagocytosed by surrounding macrophages and do not characteristically induce inflammation. Just as apoptosis is inducible, so it is also suppressed by well understood mechanisms via the action of Bcl-2. Whether or not a cells engages its apoptotic machinery depends on a delicate balance between pro survival and pro death signal pathways. Neurons typically secret nerve growth factor (NGF) which binds to its cognate receptor TrkA to activate PI-3 kinase, Akt, and MAPK resulting in the suppression of apoptotic proteins including Bcl-2 associated death promoter (BAD) and activating survival proteins such as CREB and NF-kB (Yuan & Yankner, 2000).

Necrosis

When neurons die as a result of exposure to non-physiological conditions, a lack of time and preparation often results in an uncontrolled form of cell death distinct from programmed cell death known as necrosis. Cells may swell and burst open, release the entirety of their intracellular compartments including unshielded enzymes and a host of proteases or cytotoxic components. A precise

definition of necrosis is challenging due to a lack of formal structure around when, how, or why cells undergo necrosis which is not typical under normal physiological conditions.

Exposure to oxidative stress and an inability to manufacture ATP resulting in a cell's failure to keep its homeostatic mechanisms in balance result in necrosis. These cells are unable to execute apoptotic or autophagy mechanisms due to a loss of energy homeostasis. Excitotoxicity resulting from the release of excess calcium during brain injury can cause cytoplasmic spikes in Ca2+ that trigger calpains which are cytosolic calcium activated proteases capable of degrading membrane structures. The presence of these calcium activated proteases including CLP-1, TRA-3, ASP-3, and ASP-4 are critically important for necrotic cell death in *C. elegans* (Syntichaki, Xu, Driscoll, & Tavernarakis, 2002).

Autophagy

Lysosome-dependent phagocytosis is a process by which cells are able to consume and degrade membrane-bound components. This mechanism can also be induced within a cell by the formation of internal lysosomes that catabolize the degradation of cellular components as a form of regulated cell death. This process deemed autophagy is characterized by the appearance of double membrane-bound vacuoles inside of cells which includes important parts of a cell's internal organs such as the endoplasmic reticulum or mitochondria (Rubinsztein et al., 2005). These double membraned structures ultimately fuse with lysosomes to break down the cell's internal structure. Typically, autophagy is a response to

increased cellular stress, particularly resulting from starvation or environmental insults (Klionsky et al., 2003).

Under normal physiological conditions, autophagy is suppressed by insulin or insulin-like growth factor-1 (IGF-1). Not surprisingly, starvation such as in animal studies featuring caloric restriction and a lack of insulin signaling can induce autophagy. Like apoptosis, autophagy also plays an important well-conserved role during brain development. For example, the survival of visual cortex neurons requires both anterograde and retrograde signaling. During development in chicks, a lack of appropriate signaling from the tectum to the retina results in those cells undergoing autophagy (OHSAWA et al., 1998).

Hybrid cell death

Disruption in the function of critical components of programmed cell death such as the Na/K-ATPase can give rise to concurrent apoptotic and necrotic cell death characteristic in neurons. The homeostatic maintenance of K+ ions is a critical facilitator of apoptotic cell death. Blocking the Na+, K+-ATPase significantly reduces intracellular K+, while simultaneously inducing higher Ca2+ levels. Cells undergoing apoptosis which experience irregular ion homeostasis can end up with excess cytosolic Ca2+ and display ultrastructural features characteristic of both apoptosis and necrosis.

Animal models of ischemic stroke (types)

Ischemic stroke accounts for >80% of stroke cases in humans (Go et al., 2014). Rodent models of ischemic stroke are well established and feature high face validity with cerebral ischemic damage causing cortical damage to specific

brain structures including motor and sensory cortices (F. Liu & McCullough, 2011; Macrae, 2011; Tajiri et al., 2013). This is captured in the popularity of middle cerebral artery (Trueman et al.) occlusion models (Deveau, Yu, & Wei, 2012; F. Liu & McCullough, 2011). Damage can occur within minutes and can vary in severity depending on the degree of blood flow reduction and the time and permanence of occlusion (Aysan Durukan & Turgut Tatlisumak, 2007). In animal ischemic models, cerebral blood flow can be continuously monitored using laser Doppler technology (Bishop, Powell, & Rutt, 1986; Dirnagl, Kaplan, Jacewicz, & Pulsinelli, 1989). Reduction in blood flow can be induced using suture where the branches of the MCA are tied off in mice and rats (Belayev, Alonso, Busto, Zhao, & Ginsberg, 1996; Belayev, Busto, Zhao, Fernandez, & Ginsberg, 1999). Insertion of an intraluminal filament can also induce MCA occlusion in rodents (Dittmar, Spruss, Schuierer, & Horn, 2003; Trueman et al., 2011). Embolic stroke by injection of clotted blood into the MCA also induces ischemic injury in rodent models (Ahn, Zhang, Tsang, & Chopp, 1999; Henninger et al., 2006; R. L. Zhang, Chopp, Zhang, Jiang, & Ewing, 1997). Embolic rodent stroke models have been particularly effective in the evaluation of acute stroke prevention using tissue plasminogen activator (Fan, Ning, Lo, & Wang, 2013; Sumii & Lo, 2002). Chemical approaches to vascular occlusion include photothrombosis which uses intravascular photooxidation via a photosensitive dye such as rose-bengal (De Ryck, Van Reempts, Borgers, Wauquier, & Janssen, 1989; Z. Liu et al., 2014). When irradiated, singlet oxygen species cause local endothelial damage and platelet activation resulting in thrombotic ischemia (Carmichael, 2005). Hemorrhagic stroke models utilize stereotaxic injections of

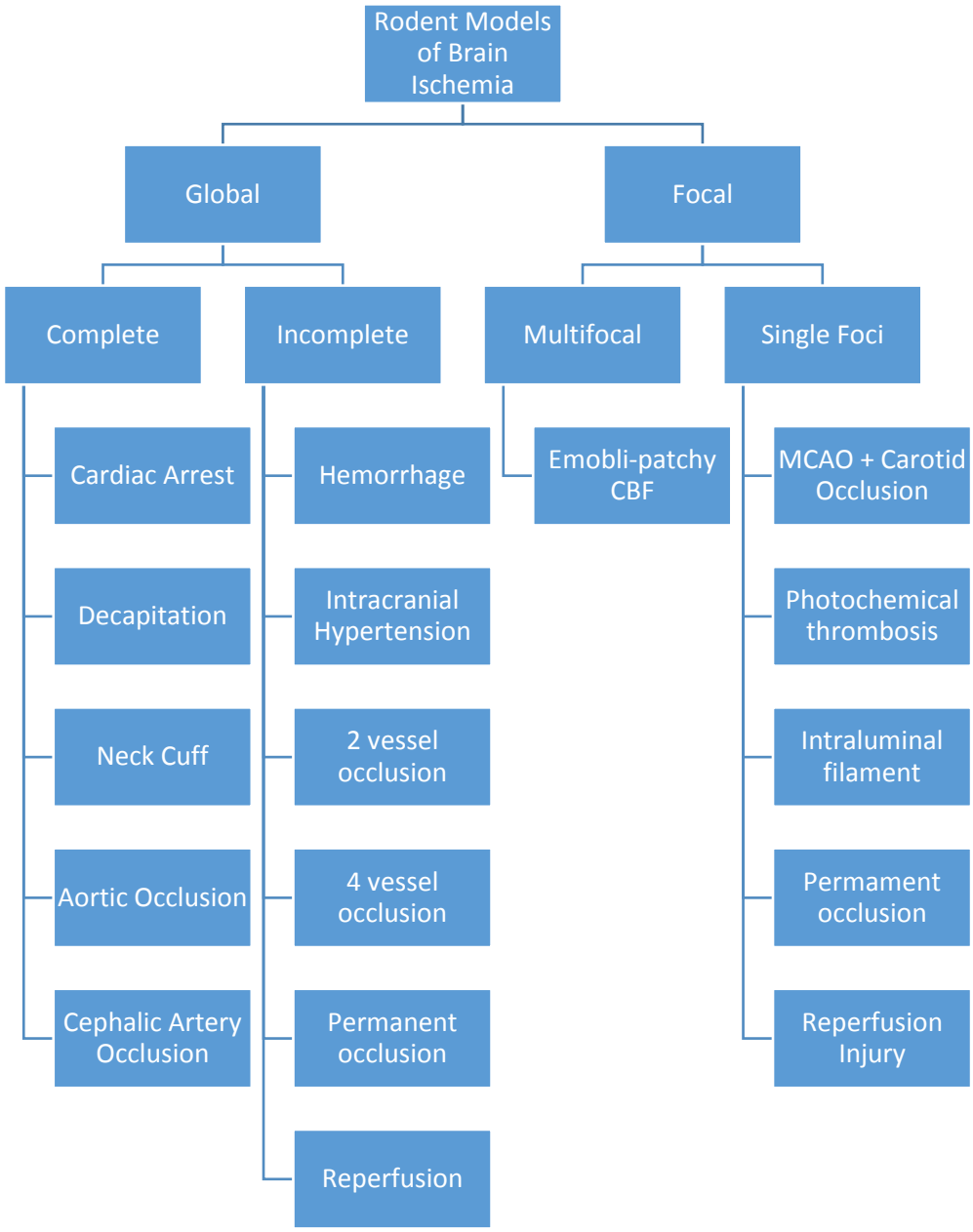
bacterial collagenase also exist (Klahr, Dickson, & Colbourne, 2014; Rosenberg, Mun-Bryce, Wesley, & Kornfeld, 1990). A variety of behavioral assays and rehabilitative strategies can be evaluated easily using rodent models.

Animal models are a critical tool to study and test the efficacy of potential therapies for use in the treatment of human disease. In addition to animal models of global ischemic damage to the cortex and subcortical tissue, focal stroke models have drawn increasing attention due to their clinical significance. The incidence rate of small strokes per 1000 person-years has been estimated at 4.7(Bos et al., 2007). A whisker barrel cortex stroke model of rats and mice was developed by us during the recent years. This model involved ligations of distal branches of the MCA and transient occlusion of both CCA, leading to selective and restricted damage to the ipsilateral sensorimotor cortex including the whisker barrel cortex (Ling Wei, Erinjeri, Rovainen, & Woolsey, 2001; Ling Wei et al., 2006; Ling Wei, Ying, Cui, Langsdorf, & Ping Yu, 2004; Vivian R Whitaker, Cui, Miller, Yu, & Wei, 2006). This relatively small and selective ischemic injury allows morphological and functional assessments before, during and at different times after the ischemic insult. We will later review how whisker stimulation induces activity dependent repair in the rodent somatosensory cortex.

In particular, animal models of stroke have great face validity when compared to in vitro models of ischemia which are useful for studying the effects of hypoxia and anoxia on cells and their responses but do not replicate the systems wide effect of ischemia on the neurovascular unit. In humans, ischemic

strokes can result from local blood clots which are mimicked by focal ischemic stroke models in animals. However, humans can also suffer brain ischemia as a result of cardiac arrest resulting in global ischemia which has also been modeled in animals. Experimental therapies for stroke are often first modeled in mice which offer several unique advantages to investigative studies including a diverse range of transgenic animals which we make use of in our studies. The mouse brain is favorable to use over those of larger mammals such as rabbits, cats, dogs, pigs, or monkeys due to fewer concerns. The smaller size of their brains but sophisticated sensorimotor and motor circuitry makes the evaluation of damage and recovery convenient and compelling. The size of the tissue is also favorable for handling throughout a myriad of scientific procedures including the ability to quickly fix, section, and extract tissues for biochemical analysis(Ponten, Ratcheson, Salford, & Siesjö, 1973).

While small rodent models of ischemic stroke offer several advantages, they are by no means the best disease model for translational research in humans. However, their efficiency will guarantee continued utilization in research for its consistency and reproducibility. Additional animal models developed in larger mammals are both available and necessary throughout the investigative process for new therapeutic approaches(de Leciñana, Díez-Tejedor, Carceller, & Roda, 2001).



Types of rodent ischemia models adapted from (Traystman, 2003).

Global ischemia

Models of global brain ischemia limit or restrict cerebral blood flow (CBF) to all areas of the brain resulting in pan-neuronal injury affecting first selectively susceptible areas followed by all neurovascular units in the brain. Complete global ischemia limits all sources of blood flow to the brain whereas in

incomplete global ischemia models, collateral circulation may supply limited blood flow that is still capable of resulting in ischemic injury. Reversible global ischemia or global reperfusion injury models typically require surgical sutures which can be applied and removed. Four vessel occlusion is capable of reducing blood flow to less than 3% of normal controls in the frontal cortex, striatum, and hippocampal areas (Pulsinelli, Levy, & Duffy, 1982). Global ischemia models are often accompanied by strong central hyperemia throughout reperfusion with hypoperfusion lasting up to 24 hours in some brain regions. Global ischemia is less common than focal ischemia in humans and typically occurs during either suffocation or cardiac arrest. Use of global ischemia models in rodents has been challenging primarily due to high levels of mortality and a currently accepted model involves bilateral common carotid occlusion combined with anoxia (Murakami, Kondo, Kawase, & Chan, 1998). A final note on global ischemia models is that consistency in damage is vital to producing reproducible results and global ischemia models often have larger variation in pathogenesis than focal ischmia models.

Focal ischemia

Human often suffer from focal ischemia located to one or more occlusions of the middle cerebral artery (MCA). Consequently, MCAO models in animals, and particularly in mice and rats are widely used due to their relatively high validity in mimicking human disease(JULIO H Garcia, 1984; K.-A. Hossmann, 1991; Khodanovich & Kisel, 2015). Proximal MCAO models in rats damaged both the

striatum and the cortex using sutures. Alternatively, a nylon intraluminal filament may be inserted into the MCA via a neck incision through the carotid artery(Badr, Yin, Mychaskiw, & Zhang, 2001; Dittmar et al., 2003).

In mice, a robust model of focal ischemia is the whisker barrel mini-stroke variation of the MCAO model. This model, developed by Wei at al. 1995, specifically targets the whisker barrel cortex in rodents to produce a specific and easily assessable deficit in sensory motor function. Because it is located near the brain's surface, the barrel cortex can be imaged using optical signal imaging, and corresponding flux of blood can be measured by stimulation of the contralateral whiskers (Ling Wei, Rovainen, & Woolsey, 1995). The occlusion of one major blood vessel supplying a subsection of the brain is not only more clinically relevant, but it is also accompanied by lower mortality rates and higher reproducibility in animal models. Additional forms of MCAO, which alter the duration and severity of CBF reduction. Permanent or temporary (with reperfusion) models exist which may also ligate proximal or distal sections of the MCA also produce variable severity injury models appropriate for a wide range of studies.

Aside from occlusion by suture, focal ischemia in rodents is commonly produced by three additional methods. The first is electrocoagulation of the MCA to limit CBF which results in irreversible pathological injury to the blood vessels. A second method which is rising in popularity is the use of a photochemical model where by the photosensitive dye rose Bengal is used to induce a reduction in CBS by the administration of focused light through a small craniotomy

window. Lack of specificity by the light may induce damage to microvessels near the site of injury resulting in less specific damage. Finally, an embolus may be injected through the common carotid artery to reduce CBF. Blood can be coagulated ex vivo and administered through the use of a syringe and placed to block the MCA. All of the above methods of reducing CBF via occlusion of the MCA may be supplemented by temporary common carotid artery occlusion to reduce blood flow to the required ischemic range below 10% normal flow (Mao, Yang, Zhou, Stern, & Betz, 1999).

Chapter Two: Direct Reprogramming

2.1 Direct Reprogramming

The brain is a wonderful organ, curiously complex, and only lightly explored. When it works, it produces all the wonders of human achievement but with its demise comes human pain and suffering. In this regard only damage to the brain afflicts both our bodies and characters. Although well protected, it remains the subject of blunt trauma in the form of TBI and numerous other internal insults encompassing a host of neurodegenerative diseases including Alzheimer's, stroke, Parkinson's (Hamer & Chida, 2009). Despite its clear importance to our survival, the brain is an organ with severely limited regenerative capacity with small and selective neurogenesis niches occurring only in the sub-ventricular zone and the subgranular zone of the dentate gyrus(Altman & Das, 1965; C. Zhao, Deng, & Gage, 2008). Ischemic stroke stands out as a particularly concerning disease as not only is it often lethal otherwise permanently disabling, it is also severely limited in its available treatment options(Ling Wei, Wei, Jiang, Mohamad, & Yu, 2017). There seems to be a paradox between the brain's importance and our bodies own programming to limit its repair in response to injury.

Michangelo once said that he "saw the angel in the marble and carved until [he] set him free." Much in the same vein, the brain during development can be compared to an uncut block of marble, densely packed, containing within it, every person to be revealed by chipping away at that grey block. Just as with time, the sculptor chips away at the marble, our development prunes away our

neuronal connections. As the sculptor sharpens certain features of the sculpture, learning and memory sharpen certain circuits in the brain. By this analogy it becomes obvious that in order for life to create the complex circuitry that comprises mammailian brains – it necessarily cannot regenerate or else that work would be undone.

We begin our lives with more or less the majority of our neurons. These cells are post mitotic, they do not divide and no longer renew. Instead they settle and begin to shape themselves, to form memories, and to develop personalities. However a neuron just as every other cell, still contains all the DNA encoding the cellular machinery required for division and repair. Unfortunately when it is required in the case of neurodegenerative disease, it is permanently switched off.

However this is not the case in all animals, certain animals such as salamanders or the rainbow trout are able to regenerate nerves and even entire hemispheres of brain. They behave like simple machines which are easy to rewire and reset to nature's "factory settings." In contrast, most organisms with larger and complex nervous systems capable of extensive learning and memory all share a certain loss of this regenerative capacity.

Aside from the neurons, brains are also comprised of a number of other cell types whose function is to aid and support their work. The basic functional unit of the brain is the neurovascular unit comprised of vasculature which acts as access to the other cells to supply blood and nutrients, glial cells that act to transfer nutrients and support and aid the function of neurons, and the neurons themselves whom conduct information. Of these, it is only the neurons which

display severely limited regenerative capacity. In contrast, glia are able to rapidly divide and migrate, particularly when they sense neuronal injury in a process known as gliosis. Vasculature is similarly able to both remodel as well as divide and form new vessels in a process known as angiogenesis.

Complex multicellular organisms function based on the coordination and cooperation of an immense variety and number of individual cells. These cells differentiate and occupy their specialty niche throughout life. All human cells derive from three lines: endoderm, mesoderm, and ectoderm. The entire nervous system comprised of the central nervous system (brain and spinal chord) and peripheral nervous system (ganglia and nerves) are derived from the ectoderm germ layer. These cells, which can differentiate into specialized cell types, are known as stem cells and they are categorized by several classifications based on their ability to differentiate. Totipotent stem cells are able to differentiate into all other cell types including the extra-embryonic, otherwise known as placental, cells. Only the first few cell divisions beyond the zygote are considered totipotent. Pluripotent cells are capable of differentiating into all somatic cell types from the three germ layers. An example of pluripotent stem cells are those derived from embryos or embryonic stem cells. Finally, multipotent stem cells are more limited than pluripotent stem cells, but are still able to give rise to two or more different cell types, including adult stem cells, bone marrow mesenchymal stem cells, and umbilical cord blood stem cells.

As cells mature throughout development into their respective biological niches, they become more and more highly specialized. Once a cell has reached a

state where it no longer divides or is only able to divide into a single type of cell, it is considered as fully differentiated. From the embryo stage and onwards, cells tend to differentiate or maintain their potency to differentiate, but do not normally reverse in their specialization. This observation led scientists such as Waddington to propose the ball rolling down a landscape analogy for cell development. That is that a cell's differentiation journey can be thought of as a ball rolling down a hill with valleys and crevices. Once a ball rolls finds its biological niche it tends to stay and does not tend to roll back up the hill to a less differentiated state.

Direct reprogramming involves a vast alteration in the transcriptome and cellular components of a target cellular substrate. This is particularly pronounced when cells undergo interlineage reprogramming involving the crossing of germ lines(Arlotta & Berninger, 2014; Graf, 2011; Xu, Du, & Deng, 2015). The process cells must undergo when administered transcriptional master switches via exogenous application of gene transfer techniques is only beginning to be elucidated. Current approaches use single-cell RNA sequences at various time points across the reprogramming period to examine a transcriptomic analysis of changing gene heterogeneity(Smith, Nachman, Regev, & Meissner, 2010). Thus far, the process appears to be a continuous rather than all or nothing procedure whereby the administration of gene products gradually alters the transcriptome of a cell over time to redirect its fate (Treutlein et al., 2016). Upon infection, host cells appear to respond uniformly, however this homogenous response does not later translate into homogenous reprogramming. In fact, during later time points,

complete myogenic programs and variable induction of transgenes appear to produce heterogeneity amongst reprogrammed targets that results in only a fraction of infected cells undergoing a full transformation into the target cell type (Treutlein et al., 2016). Rather than a transcriptome, which is distinctly the original cell type or the target cell type, cells undergoing direct reprogramming adopt a hybrid of the two throughout the reprogramming process (Buganim et al., 2012). This allows cells to share characteristic proteins of two otherwise segregated cell populations such as expression of both GFAP (characteristic of astrocytes) and NeuN (characteristic of neurons).

Both inter-lineage and intra-lineage conversion of endoderm-derived fibroblasts and ectoderm-derived glia are able to be reprogrammed directly into neurons. As few as a single transcription factor is capable of both inter-lineage and intra-lineage reprogramming from fibroblasts or astrocytes to neurons (Ambasudhan et al., 2011, Caiazzo et al., 2011, Kim et al., 2011, Ladewig et al., 2012, Liu et al., 2012, Liu et al., 2013, Meng et al., 2012, Pang et al., 2011,Pfisterer et al., 2011, Qiang et al., 2011, Son et al., 2011, Torper et al., 2013, Vierbuchen et al., 2010 and Yoo et al., 2011). Intra-lineage cell fate is controlled by a limited number of master switch transcription factors that control the expression of a set of cell type specific proteins.

2.2 Comparison with current cell therapy approaches: Hematopoietic stem cells

The mammalian hematopoietic system

Mammalian bone marrow is host to an active stem cell population with the responsibility of maintaining and replenishing a variety of high turnover cells that are vital for survival, regeneration and support of the rest of the body. The major outputs of this hematopoietic system are the erythrocytes and platelets of the blood and the granulocytes and macrophages of the immune system. These stem cells also give rise to antibody producing B-lymphocytes and T-lymphocytes that consume foreign or inflammatory substrates in the body. As all of these substituent cell types have a very limited lifespan, they must be constantly and consistently replenished. This rapid turnover of up to one hundred billion new cells from progenitors forms the hematopoietic system (Mohammadi, Mohammadnejad, & Yavari, 2014).

History of hematopoietic research

Hematopoietic stem cells have been studied for more than a half century as a therapeutic approach for patients suffering from compromised immune systems (Lidman & Cohn, 1945). The hematopoietic system was particularly sensitive to acute radiation with death resulting from either pathogenic invasion or loss of blood due to low levels of platelets or other reasons. In 1952, Lorenz et al. demonstrated that radiation damaged hematopoietic systems could be rescued by injections of bone marrow cell suspensions (Lorenz, Congdon, & Uphoff, 1952; L. Wang et al., 2002). This discovery formed the foundation of modern day

treatment for hematopoietic system failure by transplantation of bone marrow hematopoietic stem cells (Deeg et al., 2003).

The selective vulnerability of the hematopoietic system to low doses of radiation also gained relevance as it was compromised in patients with the advent of chemical or radioactive chemotherapeutic agents that target rapidly dividing cells (Siena et al., 1991; Stadtmauer et al., 2000). The hematopoietic system is comprised of cells that divide rapidly as is the case of many cancer cells. Therefore, drugs and therapies used to treat cancers can cause patients suffering a weakened immune system that requires secondary hematopoietic therapies to restore the immune activity (Stadtmauer et al., 2000). Much work in the hematopoietic system originated and continues in murine models. Hematopoietic stem cells (HSCs) can be dissected from the yolk sac or the intra-body aorta-gonad-mesonephros (AMG) region of mouse embryos (Matsuoka et al., 2001; Ohneda et al., 1998). Surprisingly, adult hematopoietic stem cells are not present during the earliest stages of development, but arise after the formation of the hematopoietic system (Orkin, 1996).

Hematopoietic stem cells and stroke

Stroke remains a leading cause of death and disability in the United States and around the world (Mozaffarian et al., 2015). The current limited availability of effective therapeutics for human stroke patients has inspired multifaceted and increasingly complex approaches to limit ischemic damage and/or enhance post stroke regeneration. Treatments that can do both have become attractive candidates in stroke research (Lo, 2008). Among these are various cell-based

transplantation therapies which have had demonstrable success in animal models of stroke (Bang, Lee, Lee, & Lee, 2005; Bliss, Guzman, Daadi, & Steinberg, 2007; L.-R. Zhao et al., 2002). The investigation has looked at the effects of transplanting different stem cells and neural progenitor cells, timing of the transplantation, and the delivery method of the desired cells. Cellular therapies have multifaceted effects during both acute and chronic phases of neurological disease. Major cell types currently under investigation including, but not limited to, induced pluripotent stem (iPS) cells (Oki et al., 2012), embryonic stem cells (Erdö et al., 2003), bone marrow derived mesenchymal stem cells (Yi Li et al., 2000) and bone marrow hematopoietic stem cells (HSC) (Paczkowska et al., 2005), each offers unique advantages with its own set of drawbacks. Among the various potential cell types used for cell-based stroke therapeutics, HSCs maintain the advantage being available from autologous donation without risk of graft rejection, low immunoreaction, and self-renewal (Aggarwal, Pompili, & Das, 2009; Domen & Weissman, 1999).

Ischemic stroke is characterized by rapid dysregulation of normal cellular processes resulting from lack of oxygen. The resulting cell loss due to excitotoxicity and various apoptotic and/or necrotic mechanisms generates a highly toxic environment with compromises access to the hematopoietic system (Castillo, Dávalos, & Noya, 1997). As such, a temporary ischemic insult can result in a chronic condition within a brain loci that mimics many of the pathological hallmarks characteristic of systemic hematopoietic failure. Interestingly, ischemic stroke and myocardial infarction can mobilize CD34+ hematopoietic

stem cells into blood circulation (Paczkowska et al., 2005). Augmenting the body's hematopoietic system by stimulating bone marrow stem cells into the blood and homing to the ischemic region is an attractive means of assuaging stroke pathogenesis during acute and chronic phases of injury.

Brain ischemia is known to trigger immune responses including activation of resident microglia within 24 hours (Ekdahl, Kokaia, & Lindvall, 2009). Bone marrow-derived monocytes can enter the brain through the blood brain barrier near the ischemic region where they also exhibit a microglia phenotype (Hill et al., 2004). Both local immune cells and ischemic tissues are known to release cytokines, complement factors, and free radicals that escalate the immune response (Kamel & Iadecola, 2012). While some of these factors are anti-inflammatory such as IL-10 (Frenkel et al., 2003), the general environmental tone is pro-inflammatory. Consequently, both the ischemic and the peri-infarct regions following stroke harbor a pro-apoptotic and/or pro-necrotic tone. In the days to weeks following ischemia, this toxic environment leads to the expansion of neuronal and glial loss (Furlan, Marchal, Derlon, Baron, & Viader, 1996)

Hematopoietic stem cell transplantation

Ischemia results in a massive loss of not only neuronal cells, but also a host of supportive cell types necessary for the maintenance and survival of brain tissue (Del Zoppo, 2010). Together, the entire neurovascular units which includes cell types such as endothelial cells, pericytes, astrocytes, and other glial cells are affected by this loss (Del Zoppo, 2010; Hawkins & Davis, 2005). One of the major goals of transplantation of hematopoietic stem cells for stroke is to augment the

body's natural ability to supply supportive substituent cell types to the ischemic region. Rather than delivery of a single differentiated cell type to the ischemic region, HSCs have been demonstrated to differentiate into a variety of supportive cell types including astrocytes, oligodendrocytes, neural precursors, macrophages, and microphages (Kopen, Prockop, & Phinney, 1999; Spangrude, 1991). The multipotent characteristic of HCSs makes them a preferable cell therapy without need for combination with other cell types.

Systemic injection of HSCs can be traced using pre-labeling of injected cells to differentiate them from host tissues. Injected GFP tagged HSCs can be observed in the spleen within the first 24 hours of delivery (Y Li et al., 2002). HSCs typically home to the bone marrow and spleen regardless of delivery methods (Henschler, Fehervizyova, Bistrian, & Seifried, 2004; Plett, Frankovitz, & Orschell, 2003). A limited number of injected HSCs can be observed in the brain where their most common phenotype is that of microglia-like cells reminiscent of host microglia found in a healthy state (Henschler et al., 2004; Recio et al., 2011; Simard & Rivest, 2004). Modification of HSCs by exogenous overexpression of Sca-1, Thy-1, and c-kit in a spinal cord injury model demonstrated differentiation of HCSs into astrocytes, oligodendrocytes, and neuronal precursors(Weissman, Anderson, & Gage, 2001).

As a cardiovascular disease, ischemic stroke damages both the brain and the circulatory arteries and veins in and around the infarct. Regenerative therapy for ischemic stroke favors approaches that enhance both neurogenesis and angiogenesis. Toward the latter goal, HSCs include a subset of CD34+ endothelial

stem, and progenitor cells. HSC therapy via system injection or local intracerebral injection after stroke are both known to increase angiogenesis in the peri-infarct region. Along with angiogenesis, these studies also indicate that HSC therapy promotes enhanced functional recovery and reduced infarct size. Specifically, CD34+ cells from HSCs which provide the strongest angiogenic effects can be enriched using immunoselection for acute ischemic stroke (Taguchi et al., 2004).

Post-ischemia neurogenesis in the brain stems from two well-studied regions: the subventricular zone and the subgranular zone of the dentate gyrus (Gage, 2000). While neurogenesis has been well documented to play a role following ischemic stroke in animal models (John J Ohab, Sheila Fleming, Armin Blesch, & S Thomas Carmichael, 2006; Parent, Vexler, Gong, Derugin, & Ferriero, 2002), its effect has been minimal with reports of only 0.2 % of neurons being replaced by endogenous neurogenesis (Arvidsson, Collin, Kirik, Kokaia, & Lindvall, 2002). These results can be verified by delivery of CD34- cells from HSC preparations to the post ischemic brain. Furthermore, consistent with these findings are reports that pro-angiogenic agents such as erythropoietin (EPO) produces similar results as CD34+ HSC delivery (Hannelore Ehrenreich et al., 2009). Suppression of endothelial proliferation by endostatin can also directly mask the beneficial effects observed from CD34+ cell delivery(Taguchi et al., 2004). These findings indicate a role for HSC therapy in augmenting endogenous post stroke regeneration by enhancing neovascularization to support new

neurons which have otherwise shown to migrate but quickly die in the toxic environment surrounding stroke(Arvidsson et al., 2002).

The relationship between vascular support and neuro-regeneration can be likened to effective angiogenic therapies studied in other organs including liver and thyroid. By supporting regenerating tissues with neovascularization can significantly improve endogenous cell replacement both in vivo and in vitro(Ross, Sander, Kleeb, Watkins, & Stolz, 2001; Toda et al., 1999). It is hypothesized that improved vascularization may help brain regeneration by providing additional routes for the removal of dead tissue which also doubles as additional migration routes into the ischemic zone. Examination of this hypothesis and various factors that may play a role in promoting the survival of both endogenous and transplanted neuro-progenitors has been extensively investigated including but not limited to fibroblast growth factor 2(Jin et al., 2003), platelet derived growth factor(Su et al., 2008), brain derived neurotrophic factor(J. Chen et al., 2005), interleukin-8(L. Wang et al., 2002), vascular endothelial growth factor(van Bruggen et al., 1999), insulin growth factor-1(Zhu et al., 2008), and focal adhesion kinase(Hu et al., 2011). Generally, multifactorial therapies aim to improve both the migration and trophic support provided to the ischemic tissue following stroke but in animal models augmenting just one of these by aforementioned and other factors have consistently demonstrated enhanced functional behavior and reduction in stroke severity(Z. G. Zhang & Chopp, 2009).

The advantage and disadvantage of using BMSCs

Stem cells are characterized not only by their multipotent differentiation ability but also by their faculty for self-replication (Clarke & Frampton, 2013). Hematopoietic stem cells are able to reproduce themselves while also differentiating into hematopoietic progenitor cells which are still multipotent but without the ability for self-replication (Reya, Morrison, Clarke, & Weissman, 2001). HSCs removed from bone marrow of rodents are capable of surviving and perpetuating in in vitro cultures beyond the lifespan of the original host. However, self-replication in HSCs is not indefinite and this phenomenon has been the focus of studies examining the effect of telomere length and telomerase activity (Morrison, Wandycz, Akashi, Globerson, & Weissman, 1996). Once removed from murine hosts, in vitro HSC preparations can survive up to three or four lifetimes of the original host but cannot be maintained much past this length. The inability for indefinite self-renewal may be related to telomerase activity (Morrison, Prowse, Ho, & Weissman, 1996).

Unlike induced pluripotent stem cells or bone marrow mesenchymeal stem cells, HSC's exhibit limited capacity for expansion in vitro. Consequently, reproducing large quantities of HSCs is challenging as they must be harvested from bone marrow itself (Parmar, Mauch, Vergilio, Sackstein, & Down, 2007), umbilical cord blood (Broxmeyer et al., 1989), or mobilized blood (Broxmeyer et al., 2005). HSCs are penchant to differentiation in cell culture rather than self-perpetuity (Terada et al., 2002). Investigation into HSC self-renewal mechanisms in vitro that are absent in vivo are currently ongoing.

While systemic delivery of HSC via intravenous injection has proven effective in the treatment of both myocardial infarction and ischemic stroke, the mechanisms governing separate protective and restorative or regenerative effects are difficult to isolate. A key role of HSC infiltration to the ischemic brain is the anti-inflammatory effects conveyed by HSCs differentiated glia and immune cells. Tracking of transplanted BM-derived cells was traditionally conducted by Till and McCulloch by irradiating HSC from donors to induce chromosomal repairs and breaks making them distinguishable from host HSCs. In this way, fully differentiated cells with various fates could be mapped back to a single transplanted donor colony bearing the same radiation induced chromosomal marker (Becker, McCulloch, & Till, 1963). In contrast, recent studies examining HSC infiltration after stroke used HSCs isolated from green fluorescent protein (GFP) expressing transgenic mice. Once injected into C57/BL6 recipient mice, GFP+ cells can be observed to migrate throughout the body include infiltration into the brain {Schwarting, 2008 #35}.

Unfortunately, CD34+ cells make up approximately just 1% of BMHSCs preparations making it difficult to discern the role played by these cells. In support of the effect of CD34+ cell fraction from HSC preparations, Taguchi et. al. demonstrated that cell delivery in the CD34+ group but not CD34 negative group after stroke had enhanced neurogenesis via angiogenesis. Using human CD34+ cells in an immunocompromised cell line, Taguchi et. al. reported enhanced neovascularization in the peri-infarcted tissue along with enhanced neurogenesis(Taguchi et al., 2004).

In addition to HSCs, two other bone marrow derived cell types have been studied for the treatment of ischemic stroke: hematopoietic progenitor stem cells, and bone marrow derived mesenchymal stem cells. Interestingly, injection of bone marrow stem cell conditioned media alone has been demonstrated to convey functional recovery benefits in a rodent model of stroke (X. Chen et al., 2002). Thus, it has been proposed that HSC transplantation can act as an augmented source for paracrine trophic support to the ischemic brain(Caplan & Dennis, 2006; Hess & Borlongan, 2008). This hypothesis supports the efficacy observed using HSC transplantation studies despite relatively poor homing of the transplanted cells to the brain and ischemic region itself. Despite this, the study of HSC therapy continues to seek methods for improving survival, homing, and efficacy of transplanted HSCs.

An effective approach to improving HSC transplantation therapy is the preconditioning of transplanted cells by hypoxia immediately prior to administration in an ischemic stroke model(Aggarwal et al., 2009). Hypoxic preconditioning (HP) has been leveraged in stem cell therapies for its multifactorial effects on improving cell survival following transplantation(Hu et al., 2008). Both embryonic stem cells and bone marrow stem cells which undergo this hypoxia preconditioning protocol are significantly more resistant to necrotic and apoptotic insults making them more capable to survive in vitro and in vivo ischemic conditions (Francis & Wei, 2010; Pacary et al., 2006; Theus et al., 2008). Preconditioning bone marrow-derived stem cells is also an effective means of improving the homing of intravenously injected stem cell preparations

for the treatment of stroke and myocardial infarction(Hu et al., 2008). Hu et. al. describe a potassium channel-kinase interaction (Kv2.1-FAK) mediated mechanism whereby hypoxia enhances the expression of Kv2.1 thereby phosphorylating FAK and boosting BMSC migration ability. Selective knockdown of the Kv2.1 channel or applying a K+ channel inhibitor significantly dampened the ability of BMSCs to migrate to the infarcted tissue. Altering gene expression in HSCs related to survival, migration, and differentiation of transplanted cells remains a popular avenue for investigation.

2.3 Viral delivery of transcription factors

AAV

Adenovirus vectors are among the most popular methods of gene transfer due to the combination of several advantageous characteristics. The key highlights of this approach are the ability of AAV's to infect both dividing and non-dividing cells. Manipulating and editing AAVs can be executed with relative ease using standard cloning techniques. Their packaging capacity is highly variable from 5 Kbp and under for ssDNA vectors to up to 7.5 Kbp in dsDNA vectors. Advantages of the ssDNA vectors is their relatively lower immunogenicity compared to dsDNA AAVs but their lower packing capacity and lack of genome integration makes them favorable to use in in vivo models. In contrast, dsDNA AAVs with a higher Kpb delivery potential are risky to use in vivo due to their ability to elicit a high immunogenic response and cause cytotoxic effects mediated by host T-cells response (Asad et al., 2017; S. Li & Huang, 2006).

Lentivirus

Lentiviruses have been tested in human clinical trials with efficacy and positive safety profiles. Lentiviruses offer several unique advantages and also some challenges to the field of direct reprogramming by gene transfer. The first is the relatively large packing capacity of 7.5 Kbp and the ability to infect a wide variety of cell types. Secondly, lentiviruses are capable of long term stable expression, which is necessary for direct reprogramming due to the time it takes for cells to undergo all the genetic changes required to successfully mature into their target cell type. The latest lentiviruses are well tolerated in humans and other animals including the rodent models of ischemic stroke. These advantages are combined with a relatively high titer of 10^7-10^8 IFU/mL and the ability to infect dividing and non-dividing cells. Together, the safety, stable expression, titer, and capabilities of lentiviruses makes them a superior choice as a method of gene transfer for direct reprogramming (Asad et al., 2017).

Chapter Three: Engaging reprogrammed cells via activity dependent repair

Functional deficits including behavioral and motor impairments are a significant cause of morbidity amongst stroke survivors but therapies addressing paresis remains unsatisfactory and is an active research field. Rodent models of stroke have provided some insights into the mechanisms responsible for spontaneous recovery and provided potential therapeutic targets for improved functional recovery. The complexity and heterogeneity of stroke has yielded a suite of varying approaches to rehabilitation including intrinsic and extrinsic stimuli. Many rehabilitation strategies are based on endogenous neuronal plasticity. Specifically, activity-dependent rehabilitation therapies involve the establishment or strengthening of unmasked or redundant cortical connections following stroke. Intensive, repetitive, and controlled exercise may result in morphological and functional improvements via neurogenesis, angiogenesis, and synaptogenesis. Forced use of paretic upper extremity by constraint known as Constraint Induced Motor Therapy (Rijntjes, Hamzei, Glauche, Saur, & Weiller) paired with intensive exercise has been practiced as an effective treatment which improves functional recovery by leveraging endogenous neuroplasticity while future investigations will help to understand the cellular and molecular mechanisms of neural network remodeling and enhance the efficacy and efficiency of the stroke rehabilitation therapy.

3.1 Introduction

Stroke is a heterogeneous disease resulting from a reduction in blood supply to the brain. Stroke is a major public health concern as the third leading cause of death and leading cause of disability in the United States (Go et al., 2014). Brain tissue is sensitive to loss in energy and nutrient supply which often results in permanent damage to neurons and the disruption of existing brain circuitry. Neurons sustain numerous insults during the acute phase of a stroke leading to excitotoxicity and selective neuronal loss (J.-C. Baron, Yamauchi, Fujioka, & Endres, 2013). While on time reperfusion of ischemic tissue can reduce cell death and improve recovery, inflammation often causes significant secondary injury along with reperfusion. In the hours to days that follow, inflammatory factors and sustained depressed perfusion to the affected regions result in further apoptotic cell death (Ahmad & Graham, 2010; Sims & Muyderman, 2010). While stroke is often associated with significant morbidity, many patients experience mild to moderate recovery with and without intervention(Kong, Chua, & Lee, 2011).

The brain has come to be appreciated for a remarkable ability to adapt and exhibits plasticity and regeneration under both healthy and diseased states (W. L. Li, Yu, Ogle, Ding, & Wei, 2008; Ying Li, Yu, Mohamad, Genetta, & Wei, 2010; Winner, Kohl, & Gage, 2011). Treating disruptions to motor control and motor learning are central to recovery from stroke with basic and clinical researchers adopting multiple strategies to enhance recovery(B. Johansson, 2011). Effective therapeutic approaches leverage and augment endogenous plasticity and other

mechanisms involved in spontaneous recovery. Here we will evaluate various strategies used in animal and clinical trials to increase stroke recovery. We will also review mechanisms underlying spontaneous recovery that are targeted by therapeutic strategies.

Functional recovery after stroke

While stroke patients experience some level of functional recovery following mild to severe stroke, such recovery is a mix of "true" recovery via restoration of neural circuitry and task-specific compensation (Kitago et al., 2012). In animal models, functional recovery is measured by a wide array of behavioral tests which assess motor and sensory ability by presenting a challenge. Assessment paradigms in animals for post ischemic sensory and motor function include the neurological deficit score which assesses a suite of behaviors including hind limb retraction, beam walking, forelimb flexion, and forelimb grasping. Other tests include forelimb reaching tasks including the Montoya staircase test where animals must reach for food pellets on staircases (Klein & Dunnett, 2012). Tests have also been designed to measure asymmetry such as the cylinder test where the use of forelimbs for body support on the wall of a vertical cylinder can indicate deficits in motor ability and recovery (Clarkson et al., 2013; Fleming & Schallert, 2012). Typically, rodent models of stroke exhibit recovery within weeks of injury while in humans, post stroke recovery may take extended periods of time up to years (Schaar, Brenneman, & Savitz, 2010). Neuroimaging techniques including functional magnetic resonance imaging (fMRI) and diffusion tensor imaging (DTI) can be effectively employed for investigating

neurophysiological post stroke recovery in human brains. Specifically, fMRI provides information regarding task/stimulus neuronal activation, functional connectivity, and neurotransmitter release while DTI can allow us to determine the integrity and connectivity of white matter (Dijkhuizen et al., 2012). Studying post-stroke recovery using animal models has both advantages and disadvantages due to the disparity in the recovery time window. Evaluating post-stroke repair in humans and animal models presents challenges when determining the amount of recovery resulting from restoration of lost neuronal circuits and compensatory behaviors which also improve performance in behavioral assessment suites.

Neuroplasticity, circuits repair and functional recovery

Cerebral ischemic stroke disrupts the somatotopic maps in the cortex where clusters of sensory and motor neurons form groups which are specifically coupled to respective afferent nerves via dedicated pathways (Bernardo, McCasland, & Woolsey, 1990). Loss of cortical neurons disrupts the circuits required for sensory and motor control despite the preservation of neurons in unaffected brain regions and spinal cord (central pattern generators) and peripheral neurons. Recovery from such damage occurs with the restoration of these disrupted circuits or neuroplasticity of functional takeover by the nearby tissue. Unmasking and redundancy in the brain are two endogenous phenomena which form the basis for spontaneous neuroplasticity following stroke (R. G. Lee & van Donkelaar, 1995). The maintenance of synapses requires persistent neuronal activity, but there are also latent sub-threshold synapses which do not normally induce action potentials (Isaac, Nicoll, & Malenka, 1995; Zeiler et al.,

2013). Sudden loss of cortical neurons can unmask these "silent" synapses (Font, Arboix, & Krupinski, 2010; Otsuka, Miyashita, Krieger, & Naritomi, 2013). Without other synaptic competition, these once sub-threshold synaptic inputs are free to strengthen and establish new pathways resulting from motor learning (Chipchase, Schabrun, & Hodges, 2011; Font et al., 2010). A second characteristic of synapses in the CNS which contributes to stroke recovery is redundancy (Chklovskii, Mel, & Svoboda, 2004; Petersen, Panzeri, & Diamond, 2001). Cortical projections are both specific and diffuse under healthy conditions (Chmielowska, Carvell, & Simons, 1989). Neurons in the sensory and motor cortices feature cortico-thalamic projections but also corticocortical projections (Hoffer, Hoover, & Alloway, 2003). Competition and development define adult neural circuits but not all diffuse projections are pruned during development (Arbib, 1981; Petreanu et al., 2012). As such, motor control lost from stroke can experience some level of spontaneous recovery by utilizing backup and previously redundant pathways (Wen, Ma, & Weng, 2014). For example, although motor control of any ipsilateral muscle groups is commonly believed to be controlled by neurons in the contralateral cortex, ipsilateral afferent and efferent motor pathways exist. Recent research suggests that these ipsilateral connections are strengthened following stroke recovery is paired with intensive use thus playing a key role in spontaneous recovery following stroke (Wen et al., 2014; Xerri, Zennou-Azogui, Sadlaoud, & Sauvajon, 2014).

Vascular plasticity in stroke recovery: angiogenesis and arteriogenesis

Ischemic stroke results in permanent, insufficient blood supply to cortical neurons. Vascular plasticity like neuroplasticity plays an integral role in stroke recovery (Edvinsson & Povlsen, 2011; Hermann & Chopp, 2012). Restoration of lost blood vessels by angiogenesis supports metabolic demand required in the poorly perfused peri-infarct region around the infarcted core of a stroke. General exercise and physical activity are known to induce physiological and metabolic processes after injury (C.-C. Chen, Chan, & Yang, 2013; Shaughnessy, Michael, & Resnick, 2012). In rodent models of sensorimotor/whisker barrel cortex strokes, whisker stimulation using a bar or brush under a repetitive regimen has demonstrated the therapeutic effect of sensory inputs in promoting angiogenesis within the ischemic cortex (W. L. Li et al., 2008; Vivian R Whitaker et al., 2006). Additionally, overuse of contralateral whiskers by trimming ipsilateral whiskers can increase use-dependent recovery in rodents (Vivian R Whitaker et al., 2006). Whisker stimulation can significantly increase functional recovery from unilateral barrel cortex stroke in rats in whisker texture discrimination tasks (Hoffman et al., 2003). Adult mice suffering loss of cortical whisker barrels have been shown to restore lost somatotopic maps over just weeks using voltage sensitive dyes and peripheral stimulation (Brown, Aminoltejari, Erb, Winship, & Murphy, 2009). Specifically promoting angiogenesis using vascular endothelial growth factor can reduce neurodegeneration and immune cell infiltration after stroke, while increasing vessel volume. In a neonatal rat stoke model, a single 1.5 μg/kg icv injection of VEFG significantly ameliorated ischemic injury and

promoted endothelial cell proliferation(Dzietko, Derugin, Wendland, Vexler, & Ferriero, 2013).

Enriched environment for activity-dependent repair and stroke recovery

Rodent models can be used to study activity dependent repair using social housing and enriched environments. Complex environmental settings have been established as an effective method for increasing capillary branching, synaptic density, and cortical thickness (Diamond, 2001; Saito et al., 1994; Sirevaag, Black, Shafron, & Greenough, 1988; Will, Galani, Kelche, & Rosenzweig, 2004). Enriched environments for rodents feature running wheels, complex housing, and toys. Rats given access to early rehabilitation during the acute phase after ischemia (5 to 14 days) experienced significant recovery compared to delayed (30 days after stroke) access. Importantly, at the physiological level, early access animals had greater branching of layer V cortical neurons (Murphy & Corbett, 2009). Rather than reducing ischemic volume, enriched environments promote post stoke structural changes to neurons including increasing spine density, dendritic branching, and neurogenesis (Biernaskie & Corbett, 2001; B. B. Johansson & Belichenko, 2002; Komitova, Perfilieva, Mattsson, Eriksson, & Johansson, 2002; Nygren & Wieloch, 2005). These effects are mediated by increased and sustained levels of local brain activity (such as by exercise or physical therapy) that up regulates expression of immediate early genes such as nerve growth factor-induced gene A (NGF-I) compared to control rats (Nygren & Wieloch, 2005). Targeting VEFG receptors, angiopoietin-1 pathway and

endothelial nitric oxide synthase can also promote angiogenesis and arteriogenesis in the peri-infarct region to generate a more conducive microenvironment for therapy induced neuroplasticity(J. Chen & Chopp, 2012).

Rodent models of stroke rehabilitation

In both animal models and human patients, motor learning after stroke is induced by intensive, repetitive, and meaningful exercises. Forced exercise is featured in a wide array of rehabilitation programs, but the parameters regarding training vary. Moderate to high intensity aerobic exercise releases, in a dose dependent manner, brain-derived neurotrophic factor (BDNF), insulin-like growth factor-I (IGF-I), nerve growth factor (NGF), and enhances diffuse synaptogenesis (Ploughman, Austin, Glynn, & Corbett, 2014). These exercises can generate additional dendritic spines and synaptogenesis, but also strengthen redundant and unmasked cortical connections. When exercises lack any of these three properties, physiological changes indicative of motor learning are significantly subdued (Daly & Ruff, 2006). Asynchronous motor therapy in rats administered concurrently with anti-Nogo-A antibody can experience marked recovery in paretic forelimbs. Along with intensive forelimb reach tasks (100 reaches per day), rats improved in both the Montoya staircase grasping test and the horizontal ladder crossing test within just two weeks(Wahl et al., 2014).

3.2 Current activity-dependent repair therapies

Constraint-Induced motor therapy for stroke patients

For human patients, meaningful activities include daily necessities such as reaching and grasping when intensively and repetitively practiced everyday can

generate permanent and significantly improved motor function (Hubbard, Parsons, Neilson, & Carey, 2009; Kleim & Jones, 2008). Early stroke rehabilitation developed by Bobath sought to maximize recovery using a neurophysiological approach which included therapy involving both the affected and unaffected limb (Chan, Chan, & Au, 2006; Dobkin, 2004; Paci, 2003). This was due to the prevalent notion that motor skill was reflexive and hierarchical (Loeb, Brown, & Cheng, 1999). However, it is now evident that motor ability is distributed throughout the brain (Doyon & Benali, 2005; Shumway-Cook & Woollacott, 2007). Recovery of upper extremities has been particularly challenging as functional improvement is more difficult to achieve with and without therapy compared with lower extremity recovery (Rand & Eng, 2012). Constraint-Induced Motor Therapy (CIMT) was initially developed by Edward Taub in response to the phenomenon known as "learned non-use" (Wen et al., 2014). Stroke patients suffering from hemi-paresis often preferentially use the unaffected limb, while ignoring the paretic limb, even when the paretic limb may have some limited ability to move. To counteract this effect, clinicians recommended artificial restriction of the unaffected limb to force the use of the paretic limb. Early applications of CIMT involved putting the healthy limb in a sling or patients were asked to wear a mitten that restricted use of the digits in order to promote the use of the paretic limb (Caimmi et al., 2008). Rigorous CIMT involves use of a splint for the patients waking hours (<90% of awake time) to force use to the paretic limb except when necessary. CIMT has since developed into one of the most effective rehabilitative strategies for treating hemi-paretic limbs. The reason for its success lies in how this approach leverages

neuroplasticity following injury. Large multicenter randomized clinical trials investigating the efficacy of CIMT in human stroke patients with ipsilateral upper extremity paresis have concluded that this therapy effectively improves functional recovery up to two years after stroke (Wolf et al., 2006).

CIMT induces neurophysiological changes

CIMT induces detectable cortical reorganization after stroke in patients suffering from upper extremity hemiplegia (Levy, Nichols, Schmalbrock, Keller, & Chakeres, 2001). The extremity constraint-induced therapy evaluation (Wolf et al.) trial reported that compared with usual control care or no therapy, CIMT conferred greater improvements when evaluated using the Wolf Motor Function Test and the Motor Activity Log (MAL) Amount of Use evaluation(Van der Lee, Beckerman, Knol, De Vet, & Bouter, 2004; Wolf et al., 2001). fMRI studies provide further evidence regarding the mechanism underlying functional recovery. Specifically, CIMT enhances ipsilateral afferent and efferent pathways such as those from the motor cortex to the hand which have been demonstrated using fMRI studies in patients. CIMT also benefits from intensive, meaningful, and repetitive exercises which Physiological changes in patients whom underwent CIMT compared those who did not include increased metabolic rate and cortical blood flow (Schaechter et all 2002). A recent study of recovery from CIMT provided evidence using TI-weighted MRI pathology that stroke volume was a poor predictor of recovery from this therapy (Gauthier et al., 2014).

CIMT for chronic stroke patients

CIMT's effectiveness has primarily been demonstrated in patients suffering from the chronic phase of stroke, but its success has merited investigation for acute stroke patients. Traditionally, treating acute or sub-acute stroke with rehabilitation presents more challenges than recruiting chronic stroke patients in a clinical trial. The 2009 Very Early Constraint-Induced Movement during Stroke Rehabilitation (VECTORS) trial with a total of 52 participants found that CIMT was an effective therapy compared to control, but not significantly more effective than traditional therapy after inpatient stroke rehabilitation. Dromeric et al. reported that HI CIMT conferred lower motor improvement 90 days after therapy (A. Dromerick et al., 2009). Strenuous activity soon after ischemia may cause damage or impede stroke recovery. As such, CIMT can be adapted to a lower intensity (LO CIMT) for acute administration compared with high intensity (HI CIMT) or regular CIMT. In a systemic review, Nijland et al. report that of five randomized clinical trials featuring 106 total patients, there is an overall positive trend to the effects of both LO and HI CIMT during the acute/sub-acute phase after stroke. Recovery was detected using Action Research Arm Test, Motor Activity Log, and Grooved Pegboard Test with patients significantly improving in these tasks compared with no therapy controls. A limitation of acute studies with CIMT is the existence of wide variation in inclusion criteria between studies. Some studies require both proximal and distal motor ability which suggests the preservation of central to distal connections (Boake et al., 2007; Bonaiuti, Rebasti, & Sioli, 2007). Other studies only included proximal motor ability in their inclusion criteria where

recovery of distal digit function is a more reliable indicator of recovery (A. Dromerick et al., 2009).

Evaluation of CIMT across clinical trials has yielded predominantly positive results for this therapeutic strategy. However, stroke recovery resulting from CIMT is often highly variable within studies. This may be a consequence of two factors. The first owing to the heterogeneity in severity and presentation of stroke induced deficits. The second being patient specific factors including variable brain activation patterns. Chronic stroke patients recruited in clinical trials for CIMT experience variable degrees of recovery which persists for months to years after therapy. Using fMRI data, Rijintjes et al. found significantly increased activation in the motor cortex of CIMT-treated individuals which could be detected after 6 months (Rijntjes et al., 2011). Augmenting CIMT with an intensive, meaningful, and repetitive exercise regimen can bolster the effectiveness of the therapy. Patients participate in training programs which feature repetitive every-day tasks such as screwing, turning faucets, and flipping light switches. In a meta-analysis on the effect of exercise intensity and frequency, there is some support for the notion that a higher dose of the same exercise confers greater motor recovery following stroke (Cooke, Mares, Clark, Tallis, & Pomeroy, 2010). A limitation of CIMT is that it is dependent on a minimum level of movement ability of the paretic limb. This recruitment of patients is highly dependent of stroke severity and thus eliminating those with complete paresis in the upper extremity. Human trials using CIMT cannot control for several important factors contributing to motor recovery such as

patient motivation, initial level of motor ability, and family support (Winstein et al., 2003).

Exercise and transcranial magnetic stimulation for stroke recovery

Low-frequency repetitive transcranial magnetic stimulation has also been used in conjunction with intensive exercise training for upper extremity paresis (Conforto et al., 2012). In a multicenter trial with 204 patients, daily low frequency rTMS applied over the motor cortex contralateral to the paretic limb followed by 120 min of training and 60 min of self-exercise resulted in a mean improvement in both the Fugl-Myer Assessment (Hoffman et al.) and the Wolf Motor Function Test (WMFT). This effect could be observed in 79 patients one month after discharge. Importantly, there were no differences in improvement as a function of age nor did any patients experience any adverse side effects (Kakuda et al., 2012).

Clinical assessments of stroke recovery

In humans, an important metric for evaluating recovery from stroke-induced upper extremity hemiparesis is a patient's performance on the Wolf Motor Function Test developed by Wolf et al. to specifically assess the therapeutic effectiveness of CIMT (Wolf et al., 2001). The test assesses functional recovery of arm and hand joints using tasks of varying difficulty. Two assessments of strength and 15 assessments of speed result in three scores named the Functional Ability (FA) score based on the quality of motor ability, a Time score based on speed, and a Grip Strength (Wen et al.) score based on strength. This test has been used widely to assess recovery using CIMT (A. Dromerick et

al., 2009; A. W. Dromerick, Edwards, & Hahn, 2000; Wolf et al., 2005). Another functional test of performance of the upper extremity after stroke used in CIMT trials is the Action Research Arm Test (ARAT). Developed in 1981, the ARAT uses a 4 point scale on 19 functional tasks including grasping, gripping, pinching, and gross movement (Lyle, 1981). The aforementioned two tests are often used in conjunction with the widely used Neurological Severity Score (NSS)(Brott et al., 1989).

Virtual reality

Virtual reality is an emerging computer based technique used for stroke rehabilitation by simulating multimodal sensory inputs to users with real time feedback. While traditional neurophysiological techniques help improve motor recovery following stroke, there remains a gap for patients during the acute phase of stroke when participation in exercise programs is not possible. Furthermore, virtual reality addresses shortages of rehabilitation services for a group of stroke survivors without access to more expensive and intensive programs (Jutai & Teasell, 2003; Teasell, Foley, Salter, & Jutai, 2008). Virtual reality simulated exercise have the potential to apply the same concepts to stroke recovery and neuroplasticity as physical exercise programs and apply similar approaches which include simulations which are intensive, repetitive, and meaningful (Langhorne, Coupar, & Pollock, 2009). To address accessibility issues, computer based rehabilitation programs take advantage of common household entertainment systems such as the Nintendo Wii. Non-immersive video games are being adapted for clinical rehabilitation despite not having been designed

with such a purpose in mind (Mouawad, Doust, Max, & McNulty, 2011; Saposnik et al., 2010). A key advantage of virtual reality systems is their ability to facilitate recovery in the acute/subacute phase following stroke. Clinical trials investigating the efficacy during the acute phase (4-6 weeks) have demonstrated significantly benefits for using virtual reality rehabilitation whether it is immersive (true virtual reality) or non-immersive (gaming) (Casserly & Baer, 2014; Joo et al., 2010; Piron et al., 2003; Saposnik et al., 2010). Virtual reality has also proven to significantly improve upper extremity recovery following stroke in the chronic phase. Specifically when compared to equal intensity conventional training, virtual reality conferred an equal benefit to recovery (Fritz, Peters, Merlo, & Donley, 2013; Prange, Kottink, Krabben, Rietman, & Buurke, 2013).

Chapter Four: Rationale, Aims, and Experimental Methods

4.1 Rationale and significance

Stroke is the third most common cause of death worldwide and a leading cause of long term disability. This year, over 795,000 people in the United States will experience a stroke with 610,000 cases being new cases. On average, someone has a stroke every 40 seconds in the United States(Go et al., 2014). Stroke is a major public health concern with direct and indirect costs totaling 36.5 billion in 2010(Go et al., 2014). Promising clinical trials using pharmacological drugs effective in animals fail to be efficacious in humans(De Keyser, Sulter, & Luiten, 1999; Sacchetti, 2008). Cell replacement therapies made available using stem cell techniques hold promise for restoring lost brain circuits and improving recovery following ischemia(Bang et al., 2005; Haas, Weidner, & Winkler, 2005; J. S. Lee et al., 2010; Ling Wei et al., 2005; Ling Wei et al., 2006). Recent advances in cell lineage reprogramming has uncovered several neuronal master transcriptional switches controlling lineage specification and differentiation including BAM (Brn2, Ascl1, and Myt1l), ND1, Sox2, and Ngn2 (Guo et al., 2014; Qiang et al., 2011; Torper et al., 2013; Thomas Vierbuchen et al., 2010; S.-C. Zhang, Wernig, Duncan, Brüstle, & Thomson, 2001)(Fig. 1).

An important challenge is to investigate whether cell replacement using direct cell reprogramming will functionally engage host circuitry and result in enhanced functional recovery. Few recent studies have utilized this approach as a potential therapy for Alzheimer's, trauma, and spinal cord injury(Guo et al.,

2014). These emerging data suggest *in vivo* direct reprogramming can be an effective source of cell replacement for neurological pathologies. This approach remains unreported in a stroke model which features its own unique pathophysiology. Ischemic stroke results in a focused astroglial reaction that can be leveraged as a substrate for reprogramming. In addition, reprogramming astrocytes into neurons effectively acts to melt the glial scar enhancing synaptogenesis and axonogenesis (Lu, Bradley, & Zhang, 2014).

Direct reprogramming

Under the Waddington model, cell specification was once thought of as an intransigent finality comparable to a ball obeying the laws of gravity as it rolls down a hill(Ladewig, Koch, & Brüstle, 2013). The transformative discovery of redefining cell fate using transcription factors which generated pluripotent stem cells from fibroblasts resulted in a paradigm shift regarding cellular differentiation and specification(Takahashi & Yamanaka, 2006). Advances in transcription factor-mediated cell fate specification have demonstrated that a stem cell intermediate can be bypassed using lineage specific transcription factors which directly convert terminally differentiated cells into other cell types (Ieda et al., 2010; J. E. Lee et al., 1995; Thomas Vierbuchen et al., 2010). Specifically, the neuronal basic helix-loop-helix transcription (bHLH) factor NeuroD1 is capable of intra-lineage reprogramming of astrocytes into neurons(Thomas Vierbuchen et al., 2010)(Fig. 1). Such reprogramming is possible *in vivo* by packaging transcription factors into viral vectors expressed under tightly controlled promoters to target specific cellular subpopulations in

the brain. Neurons converted by ectopic transcription factor expression appear as soon as 14 days following infection and persist for at least 8 weeks following administration of the viral vector (Pang et al., 2011). Furthermore, direct reprogramming has generated both glutamatergic and GABAergic neurons from astrocytes and oligodendroctye precursor NG2 cells, respectively(Lu et al., 2014). The ability to redirect cell fate *in vivo* has profound implications for neurodegenerative disease including but not limited to ischemic stroke.

Barrel cortex ministroke model in mice

In my study, I will use the barrel cortex mini-stroke model in mice developed by Wei and Woolsey(Ling Wei et al., 1995)(Fig. 2). This model targets the sensory cortex where individual cortical barrels functionally map to contralateral vibrissa on a mouse's face(Woolsey & Van der Loos, 1970). The tight correlation between cortical substructure and readily observable behavior makes this a suitable model to investigate stroke pathophysiology and the effectiveness of cell replacement by direct reprogramming. Whisker stimulation therapy in mice has been an effective approach to enhancing activity-dependent repair, angiogenesis, and neurogenesis following stroke (Bao et al., 2011; Ling Wei, Fraser, Lu, Hu, & Yu, 2012). Whisker stimulation further enables the possibility of enhancing the ability of directly converted neurons to engage with host circuitry (Thomas Carmichael, Wei, Rovainen, & Woolsey, 2001; Vivian R Whitaker et al., 2006).

Innovation: Current cell based therapies for ischemic stroke focus on cell replacement with exogenously introduced neuronal precursor cells. Direct reprogramming *in vivo* offers cell replacement from endogenous glial substrates.

This enables the generation of autologous, post-mitotic neurons in a supportive, non-hostile environment, while melting the glial scar. Although direct reprogramming is a promising therapy for several CNS pathologies, it has yet to be investigated for stroke. This investigation will explore whether direct reprogramming of astrocytes into neurons will integrate into host circuitry resulting in functional recovery.

4.2 Specific aims

Ischemic stroke results in death of neurons in the affected area with limited capacity for regeneration in the adult brain. Recently, exciting studies demonstrate the pan-neuronal transcription factor **NeuroD1 (ND1)** can reprogram astrocytes **directly** into neurons via a process called direct reprogramming/conversion. Lentiviral vector delivery of ND1 to astrocytes results in **permanently** reprogrammed neurons without the need for maintained ectopic expression of ND1. Without intervention, astrocytes provide support to neurons and proliferate reactively in the peri-infarct region after ischemic stroke. **Intra-lineage** direct reprogramming provides an endogenous source of **new** neurons by leveraging **existing** proliferative astrocytes.

Reprogrammed neurons arise within the host tissue extracellular matrix in a microenvironment that is conducive to synaptogenesis and survival. Reprogramming astrocytes into neurons also **reduces the glial scar load** which is inhibitory for axonogenesis and synaptogenesis. New neurons are **autologous** and **post-mitotic** eliminating risk associated with rejection and tumor formation. Direct reprogramming of astrocytes has not been reported as a

therapeutic tool in a model of ischemic stroke. Directly reprogrammed neurons bolster activity-dependent repair induced by whisker stimulation and these techniques used in conjunction may significantly alleviate stroke-associated morbidity. In this thesis, I hypothesize that direct Neuro-D1-mediated reprogramming of astrocytes into neuronal cells in the peri-infarct area, augmented with whisker stimulation, will improve functional recovery in a mouse model of ischemic stroke. Results from these experiments demonstrate the efficacy of **intra-lineage direct reprogramming** of astrocytes into neurons *in vivo*. In order to test this central hypothesis, I proposed the following specific aims:

Specific Aim 1. To study the *in vitro* reprogramming of astrocytes into neurons using NeuroD1 lentivirus

Hypothesis: Ectopic expression of the transcription factor NeuroD1 in astrocyte cell cultures will result in intra-lineage direct reprogramming into neurons.

1.1 Generate NeuroD1 plasmid by cloning NeuroD1 under GFAP promoter with mCherry tag into the FUGW plasmid

1.2 Evaluate NeuroD1 lentivirus titer, purification, and infection efficiency

1.3 Characterize direct reprogramming *in vitro* using NeuroD1 lentivirus in astrocyte cell cultures using electrophysiological and histological techniques

Specific Aim 2. To study the *in vivo* reprogramming of astrocytes into neurons and activity dependent repair in stroke mice

Hypothesis: Ectopic expression of ND1 following stroke reprograms reactive astrocytes into neurons and whisker stimulation will mediate activity dependent repair resulting in enhanced functional recovery.

2.1 Assess timeline for reprogramming and survival of neurons converted from reactive astrocytes after ischemia *in vivo*

2.2 Examine functional integration of converted neurons *in vivo*

2.3 Enhance functional recovery by activity dependent repair using whisker stimulation

4.3 Materials and methods

Plasmid Construction and Viral Production

To express genes together with mCherry using a single plasmid, pEGIP was modified by constructed by replacing the GFP-IRES-Puro sequence with *BamHI-EcoRI*-IRES-mCherry-WPRE and named EIMW. pEGIP was a gift from Linzhao Cheng (Addgene plasmid # 26777) (Zou et al., 2009). For expression of Ngn2-myc, the mouse DNA sequence of *Ngn2* was amplified from Ngn2 vector and cloned into EIMW through *BamHI* and *EcoRI* cutting sites. Ngn2 vector was a gift from Malin Parmar (Addgnen plasmid #34999) (Pfisterer et al., 2011). Ascl1 (amplified from Tet-O-FUW-Ascl1), Brn2 (amplified from Tet-O-FUW-Brn2) and Myt1l-myc (amplified from Tet-O-FUW-Myt1l) were cloned into EIMW though *EcoRI or BamHI* and *EcoRI*. Tet-O-FUW-Ascl1, Tet-O-FUW-Brn2 and Tet-O-FUW-Myt1l were gifts from Marius Wernig (Addgene plasmid # 27150, #27151 and #27152) (T. Vierbuchen et al., 2010). Plasmids were isolated using

Primers:

Ngn2-BamHI-F	5'-GGG GGATCC ATGTTCGTCA AATCTGAGA-3'

Ngn2-KpnI-myc-EcoRI-R	5’-CCCGAATTCT CACAGATCCT CTTCAGAGAT GAGTTTCTGC TCGGTACCGA TACAGTCCCT GGCGAGGG-3’
Ascl1-EcoRI-F	5’-GGG GAATTC ATGGAGAGCT CTGGCAAGA-3’
Ascl1- EcoRI-R	5’-GGG GAATTC TCAGAACCAG TTGGTAAAG-3’
Brn2- EcoRI-F	5’-GGGGAATTCATGGCGACCGCAGCGTCTAACCACTACA GCCTGCTCACCT -3’
Brn2- EcoRI-R	5’-GGG GAATTC TCACTGGACG GGCGTCTGC-3’
Myt1l-BglII-F	5’- GGG AGATCT ATGGACGTGG ACTCTGAGG-3’
Myt1l-3554-EcoRI-R	5’-GACCTGAATT CCTCTCACAG-3’
Myt1l-3554-EcoRI-myc-F	5’-CTGTGAGAGGAATTCAGGTC GAGCAGAAACTCATCTCT-3’
Myt1l-myc-EcoRI-R	5’-GGG GAATTCT CACAGATCCT CTTCAGAGAT-3’

Note: F, Forward primer; R, Reverse primer;

EIMW-Ngn2-myc, EIMW-Ascl1, EIMW-Brn2 and EIMW-Myt1l-myc lentiviruses were produced and purified as described (G. Tiscornia, O. Singer, & I. M. Verma, 2006).

Cell Culture and Viral Infection

Astrocytes were dissected and cultured as described (Berninger et al., 2007). Briefly, cerebral cortex from postnatal day 5 (P5) to P7 was dissected, and cells were cultured in a medium consisting of Dulbecco's Modification of Eagle's Medium (DMEM, Corning, Manassas, VA), 15% FBS (Sgima), MEM non-essential amino acids (Life Technologies), 3.5 mM glucose (Sigma). One week post plating, cells were dissociated using trypsin-EDTA (Life Technologies) and passaged into poly-D-lysine (Sigma) and laminin (Sigma) coated coverslips (80,000 cells per well for 24-well plates) in the same medium. Lentivirus was added into astrocytes culture immediately after passage. One day post transfection, the medium was completely changed into a medium consisting of DMEM/F-12 (Life Technologies), 3.5 mM glucose, penicillin/streptomycin (Life Technologies)), B27 ((Life Technologies)), and 20 ng/ml brain-derived neurotrophic factor (BDNF, Sigma). Media were changed every 3-4 days.

Generate NeuroD1 plasmid by cloning NeuroD1 under GFAP promoter with mCherry tag into the FUGW plasmid

Ectopic expression of NeuroD1 can directly convert astrocytes into neurons and significantly increases the rate of reprogramming of fibroblasts into neurons when co-infected with the transcriptional cocktail Brn2, Ascl1, Myt1l (BAM)(Thomas Vierbuchen et al., 2010). We generated a mCherry tagged NeuroD1 lentivirus under a mouse GFAP promoter which can be used for both *in vitro* and *in vivo* infection. Lentiviral vectors have stable expression when infecting HEK 293FT cells and cultured astrocytes.

The mCherry tag was ligated to FUGW using two step overlap PCR. A purified NeuroD1 fragment was ligated into the FUGW plasmid. Verification of correct ligation and plasmid generation was confirmed using PCR and DNA sequence analysis. Plasmid production utilized Stbl3 bacteria and DNA was purified using Qiagen Miniprep and Maxiprep kits. Transfection efficacy was assayed compared to control GFP-FUGW in HEK 293FT cell cultures that were fixed and stained for both mCherry and NeuroD1.

Cloning experiments were performed in triplicates. A minimum of 8 samples were required during cloning steps and 3 bacterial strains were sequenced to isolate a mutation free strain. Of these, two contained point mutations and one was mutation free. All subsequent experiments used the mutation free clone. In transfection experiments, 3 wells of HEK cells were used to examine efficacy. We used the t-test to compare NeuroD1 plasmid transfection

efficiency to a control. DNA concentrations were assessed using a Gen5 ultraviolet spectrophotometry by BioTek. (Ansorge et al., 2009)

NeuroD1 lentiviral production, purification, and titer calculation

Lentivirus has been established as an effective method of infection both *in vitro* and *in vivo (Blömer et al., 1997; Naldini, Blömer, Gage, Trono, & Verma, 1996; Taoufik et al., 2007)*. Under an ubiquitin promoter, a NeuroD1 LV was an effective vector to observe direct reprogramming *in vitro*. We packaged control GFP-FUGW and NeuroD1-FUGW into a lentiviral vector. HEK 293FT cells were used as the substrate for lentiviral production (Ansorge et al., 2009; Gustavo Tiscornia, Oded Singer, & Inder M Verma, 2006). We achieved a viral titer of at least $2.48x10^7$ which was suitable for use in vitro and in vivo. Virus was harvested from culture media two times resulting in 144mL total. To reach a high titer for *in vivo* use, virus was concentrated using two round ultracentrifugation to concentrate first from 144mL to 1.2mL and then from 1.2mL to 30µL. Phage titer was calculated by infecting triplicate HEK 293FT cell cultures with 1:10 and 1:100 dilutions of concentrated virus and then fixed and stained for mCherry

Electrophysiological and histological examination of direct reprogramming *in vitro* using NeuroD1 lentivirus

Rationale: Direct reprogramming of astrocytes into neurons results in cells which fire action potentials from cells that do not. Whole cell patch clamp electrophysiology can be used to evoke action potentials from converted cells. Converted neurons should also express both intermediate histological markers of neurogenesis (Tuj1, DCX), but also markers for mature neurons (NeuN). This is

in contrast with unconverted astrocytes or contaminant microglia which will express GFAP and Iba1, respectively. Neurons contaminating initial astrocytes cell cultures will die within 10-14 days allowing for converted neurons to be identified at later time points.

Methodology: Mouse astrocytes were dissected from p1 C57BL mice and purified by centrifugation. NeuroD1 virus was diluted according to titer and fixed and stained at time points of 14 and 42 days to quantify viral expression. For converted neuron function, we applied a whole cell patch clamp to 14-42 d.i.v. cells with neuronal morphology in astrocyte cell cultures. Using the current clamp mode with a -60mV holding potential, we elicited action potentials with voltage injections. To verify the type of synaptic current elicited, we applied Bicuculline (20uM). Further, immunoctyochemistry for VGluT1, marker of glutamatergic neurons, or GAD67, marker of GABAergic neurons, provided information about the neurochemical phenotype of neurons astrocytes were reprogrammed into. Staining for neuronal lineage markers Tuj1, DCX, Map2, and NeuN also provided further evidence of the various neuronal stages reprogramming passes through from GFAP-positive astrocytes. To examine whether ND1-infected astrocytes pass through a neural progenitor or stem cell state, we monitored cells in vitro from 24 hrs until 42 days for neural stem cell markers including Sox2 and Oct4. BrdU cannot be used to label if reprogrammed cells pass through a stem cell stage as astrocytes would take up BrdU during their proliferative stage.

Enhancing functional recovery by activity dependent repair using whisker stimulation.

Aim 2.3 used a separate group of animals that received surgery in conjunction with those in groups in aims 2.1 and 2.2. However rather than sacrificing this group of animals at the aforementioned time points, this group was subject to daily whisker stimulation as detailed in Whitaker et al. 2007. These animals underwent the corner test and the adhesive removal tests which challenge sensorimotor function impaired by barrel cortex ischemia. After behavior testing, animals were sacrificed and their brain were cryogenically frozen to observe histological evidence of activity dependent repair.

Animal Protocol

The animal protocol (DAR 2003027) was approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University School of Medicine. Animal procedures followed institutional guidelines that meet NIH standards. Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) were followed, as well as specific national laws where applicable.

Focal ischemic stroke in mice

C57/BL adult mice (male, 26-28 g) were used in this investigation. In the experiment of collecting cortical cells for neuronal cultures, Nestin-GFP transgenic adult mice were used to track neural progenitors. A focal cerebral ischemic stroke involving the right sensorimotor cortex was induced as previously described (K. E. Choi et al., 2012; W. L. Li et al., 2013) Mice were anesthetized with 3% isofluorane and maintained using 1.5% isoflurane

supplemented with regular air during surgery. The cortex ischemia was achieved by permanent occlusion of the distal branches of the right middle cerebral artery (MCA) supplying the sensorimotor cortex. The MCA occlusion was paired with 10-min ligation of both common carotid arteries (CCAs). Local cerebral blood flow (LCBF) was applied to verify the reduction of flow to the targeted brain region (see below).

Body temperature was monitored during surgery and recovery period using a rectal probe and maintained at 37°C on a homoeothermic blanket in a ventilated incubator. The body temperature for mice that received pharmacological hypothermia treatment was not altered by any other intervention. Different time points after stroke, mice were anesthetized with 4% chloral hydrate and sacrificed by decapitation. Before and after surgery, the animals were housed at 4-5 animals per cage, with *ad libtum* access to food and water.

Permanent embolic ischemic stroke in mice

A severe stroke model of permanent embolic MCA occlusion was also tested (n=10). Clot preparation followed earlier reports with a few modifications (Bereczki et al.). Briefly, the blood collected by cardiac puncture was supplemented with human fibrinogen (10 mg/ml), and immediately clotted in PE-50 tubing for 6 hrs at room temperature followed by storage at 4^{o}C. Before use, the clot (2.5 cm) was transferred into a PE-10 tube filled with sterile saline and retracted. A single clot was transferred to PE-10 catheter for embolization. Mice were anesthetized with 3% isofluorane and maintained using 1.5%

isoflurane during surgery. The right CCA, the right external carotid artery (ECA) and the internal carotid artery (ICA) were exposed via a ventral midline neck incision. The PE-10 catheter containing a clot was introduced into the CCA lumen through a small hole, advanced into the ICA, and the clot was gently injected with saline. The catheter was removed immediately after thrombo-embolization.

Animal temperature control and animal care during/after surgery were the same as in the focal cortical stroke.

Local cerebral blood flow (LCBF) measurement

We used two different methods of LCBF measurement: laser Doppler perfusion imaging using the PeriScan PIM II scanner system (Perimed AB, Stockholm, Sweden) and autoradiography of ^{14}C-iodoantipyrine.

Laser Doppler scan imaging

This measurement was performed before and during surgery, 5, 10 minutes, and 24 hrs after reperfusion of CCAs, as previously described (L. L. Wang et al., 2014). Briefly, under anesthesia, a crossing skin incision was made on the head to expose the whole skull. Laser scanning imaging measurements and analysis were performed using the PeriScans system and LDPIwin 2s (Perimed AB, Stockholm, Sweden) on the intact skull. The scanning region had a center point of ML+ 4.1mm, and the four edges of the infarct area were ML+ 2.9mm, ML+ 5.3mm, AP–1.5mm, and AP+ 2.0mm, respectively. In laser scanning imaging, the 'single mode' with medium resolution was used to scan the photo image of LCBF. The laser beam was pointed to the center of the ischemic core (ML + 4.1 mm, AP 0 mm), the scan range parameter was set up as 5×5 and

the intensity was adjusted to 7.5 to 8.0. The conventional 'duplex mode' was used to record the Doppler image with the laser beam pointed to exact the same point on the border of the stroke core (ML- 0, 5 mm, AP 0 mm). Corresponding areas in the contralateral hemisphere were similarly surveyed as internal controls. This scanning measurement largely avoids inaccurate or bias results caused by inconsistent locations of the traditional single point measurement.

[^{14}C]Iodoantipyrine Autoradiography:

Regional LCBF was measured according to the established method of iodoantipyrine autoradiography (Bereczki et al., 1993; Majid et al., 2000). Mice were anesthetized with a mixture of 1.5% halothane, 69% nitrous oxide, and 29.5% oxygen. Under the operating microscope, the femoral artery and femoral vein were catheterized on both sides of the animal with polyethylene tubing (PE-10; 3.0 cm long). The wound was infiltrated with lidocaine-HCl and closed with sutures. Body temperature was monitored and maintained at 37.0°C to 37.5°C with a heat lamp. Arterial blood pressure was continuously recorded, and arterial blood samples were taken for blood gas assays before the start of the actual measurement of flow. The measurement of cortical cerebral blood flow followed the procedures described by Jay et al (Jay, Lucignani, Crane, Jehle, & Sokoloff, 1988) and Wei et al, (L. Wei et al., 1998) with some modifications. In brief, 5 to 10 μCi of [^{14}C]iodoantipyrine (American Radiochemical) was infused into 1 femoral vein for 20 seconds; 6 well-timed blood samples were collected on pre-weighed pieces of filter paper over this period. At 20 seconds, the mice were decapitated. The brains were removed from the severed heads and frozen in 2-

methylbutane cooled to −45°C within 30 seconds of decapitation. Frozen brains were stored at −80°C until the time of sectioning. ^{14}C radioactivity was determined in the reweighed samples of blood by liquid scintillation counting. Tissue radioactivity was assayed by quantitative autoradiography in the same part of the cortical field of the MCA in all 3 strains. Coronal sections (20 μm thick) were serially cut in a cryostat set at −17°C, starting at the level of the area postrema and ending at the rostral end of the caudate putamen. These sections were placed in x-ray cassettes along with an appropriate set of standards and a sheet of x-ray film (BRS Kodak, New York, NY). Commercial standards were used for ^{14}C quantification (American Radiolabeled Chemicals, St. Louis, MO). The exposure period of the sections and standards was 7 to 9 days. The optical densities of the brain images and of the standards were measured on the autoradiograms with an MCID image analysis system (Imaging Research Inc., Ontario, Canada)). Cortical cerebral blood flow was determined from the blood and tissue radioactivities and the equation of the method (Jay et al., 1988; L. Wei et al., 1998).

Infarct volume measurement

Twenty-four hours or 4-5 days after the onset of MCAO, animals were sacrificed for assessment of brain infarct formation. 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) staining was used to reveal damaged/dead brain tissue as previously described (L. L. Wang et al., 2014). Brains were removed and placed in a brain matrix then sliced into 1-mm coronal sections. Slices were incubated in 2% TTC solution at 37°C for 5 min, then stored in 10% buffered

formalin for 24 hrs. Digital images of the caudal aspect of each slice were obtained by a flatbed scanner. Infarct, ipsilateral hemisphere, and contralateral hemisphere areas were measured using ImageJ software (NIH, Bethasda, MD, USA). The indirect method (subtraction of residual right hemisphere cortical volume from cortical volume of the intact left hemisphere) was used for infarct volume calculation. Infarct measurements were performed under double-blind conditions.

TUNEL staining and cell death assessments

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit was used to examine cell death by detecting fragmented DNA in 10-μm-thick coronal fresh frozen sections as described previously (n=8 per group) (J. H. Lee et al., 2014). After fixation (10% buffered formalin for 10 min and then ethanol:acetic acid (2:1) solution for 5 min) and permeabilization (0.2% Triton X-100 solution), brain sections were incubated in equilibration buffer for 10 min. Recombinant terminal deoxynucleotidyl transferase (rTdT) and nucleotide mixture were then added on the slide at 37°C for 60 min in the dark. Reactions were terminated by 2x SSC solution for 15 min. Nuclei were counterstained with Hoechst 33342 (1:20,000; Molecular Probes) for 5 min. Cell counting was performed as described previously (J. H. Lee et al., 2014). Cell counting was performed following the principles of design based stereology. Systematic random sampling was used to ensure accurate and non-redundant cell counting. Eight brain sections per animal were collected at 90 μm distance between

sections for non-overlapping multistage random sampling. For each animal, 8 "area of interest" regions per slide were selected. Each field was scanned at 200x magnifications for cell counting. ImageJ (NIH) was used to analyze each picture. All experiments was performed in a double-blinded fashion so the data collector and data analysis were performed without knowledge of experimental groups.

Western blot analysis

Western blot analysis was used to detect the expression of trophic factors brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF). Brain cortical tissue was lysed in a lysis buffer containing 0.02 M $Na_4P_2O_7$, 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton, 1 mM EGTA, 2 mM Na_3VO_4, and a protease inhibitor cocktail (Sigma-Aldrich). The supernatant was collected after centrifugation at 15,000 g for 10 min at 4 °C. Protein concentration was determined with a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA). Equivalent amounts of total protein were separated by molecular weight on an SDS-polyacrylamide gradient gel, and then transferred to a polyvinyl difluoride (PVDF) membrane. The blot was incubated in 5% bovine serum albumin (BSA) for at least 1 hr and then reacted with primary antibodies at 4°C for overnight. The primary antibodies used in this investigation included: rabbit anti-cleaved caspase-3 (1:500; Cell Signaling, Danvers, MA, USA), anti-BDNF antibody (1:2000; Cell Signaling) and anti-VEGF antibody (1:5000; Sigma). After washing with Tris-buffered saline with Tween (TBST), membranes were incubated with AP-conjugated or HRP-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ, USA) for 1-2

hrs at room temperature. After final washing with TBST, the signals were detected with bromochloroidolylphosphate/nitroblue tetrazolium (BCIP/NBP) solution (Sigma-Aldrich) or film. Signal intensity was measured by ImageJ and normalized to the actin signal intensity.

Immunohistochemical staining and cell counting

Frozen brain tissues were sliced into 10 μm-thick coronal sections using a cryostat vibratome (Leica CM 1950; Leica Microsystems, Buffalo Grove, IL, USA). Sections were dehydrated on a slide warmer for 30 min, fixed with 10% formalin buffer, washed with -20°C precooled ethanol: acetic acid (2:1) solution for 10 min, and finally permeabilized with 0.2% Triton-X 100 solution for 5 min. All slides were washed 3 times with PBS (5 min each) after each step. Then, tissue sections were blocked with 1% fish gelatin (Sigma-Aldrich) in PBS for 1 hr at room temperature, and subsequently incubated with the primary antibody: mouse anti-NeuN (1:400; Millipore, Billerica, MA, USA), rabbit anti-NeuroD1 (1:400; Millipore, Billerica, MA, USA){Guo, 2014 #34}, rabbit anti-Glut-1 (1:400, AB1341, Chemicon, Temecula, USA), and rabbit anti-Beclin (1:5000; Abcam, Cambridge, MA, USA){Huang, 2017 #36}, goat anti-collagen type IV (1:400; Millipore, Billerica, MA, USA) overnight at 4°C. Next day, the slides were washed 3 times with PBS for 5 min, then reacted with the secondary antibodies Alexa Fluor®488 goat anti-mouse or rabbit (1:300; Life Technologies, Grand Island, NY, USA) and Cy3-conjugated donkey anti-rabbit (1:300; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or Cy5-conjugated donkey anti-mouse or rabbit (1:400; Jackson ImmunoResearch Laboratories) for 80 min

at room temperature. After 3 washes with PBS, nuclei were stained with Hoechst 33342 (1:20,000; Molecular Probes, Eugene, OR, USA) for 5 min as a counterstain. And then the brain sections were mounted, coverslipped, imaged, and photographed under a fluorescent microscope (BX51, Olympus, Japan) and laser scanning confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA).

Primary cell cultures and immunocytochemistry

Wild type and Nestin-GFP adult mice were subjected to the sensorimotor focal ischemia. The Nestin-GFP mouse permanently express GFP under the Nestin promoter, consequently labeling all newly generated neurons green. This works as a reporter for neurogenesis *in vitro* assays. Primary cortical cells were isolated from the ischemic core region following the dissection procedure described previously (Zeng, Yu, Zhang, & Wei, 2010). For labeling of all newly divided cells, 5-bromo-2'-deoxyuridine (BrdU, 50 mg/kg, i.p.; Sigma) was administered daily starting from 1 day before stroke to 6 days after stroke. Seven days after stroke, the mice were anesthetized with isoflurane. The cortical tissue of core region and the corresponding area of the contralateral cortex tissue were isolated by an ophthalmic scissor and a forceps under a dissection microscope. Dissociated cells from the ipsilateral and contralateral cortex were plated separately under the same culture condition. Trypsin-treated dissociated cells were plated on poly-D-lysine and laminin-coated glass (VWR, West Chester, PA, USA) at the density of 8×10^4 cells/ml in MEM containing 10% horse serum (Invitrogen, Carlsbad, CA, USA). One hour later, medium was changed to

Neurobasal-medium (Invitrogen) supplemented with B-27 (Invitrogen), L-glutamine (0.5 mM, Invitrogen) and 20 ng/mL NGF (Sigma-Aldrich). Cells were cultured at 37^{o}C and 5 % CO_2 for 3-7 days. No inhibitor of glial cell proliferation was added in order to allow growth of all cell types. Half amount of the medium was changed with fresh medium every two days.

Transmission Electron Microscopy

The cellular and sub-cellular structures of the brain cells was examined by electron microscopy. Seven days after stroke, brains were perfusion fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and then stored in the same fixative overnight at 4 C. Brains were then sliced into 100 μm sections using a vibrating microtome. After washes with 0.1 M cacodylate buffer (pH 7.4), sections were post-fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide in the same buffer for 1 hour, dehydrated in an ethanol series up to 100%, infiltrated with Epoxy resin, and then flat-embedded in the pure resin. Ultrathin sections of stroke affected cortical area were cut at 70 nanometers on a Leica Ultracut S ultramicrotome (Buffalo Grove, IL) and counterstained with 5% aqueous uranyl acetate followed by 2% lead citrate. Ultrathin sections were examined on a JEOL JEM-1400 transmission electron microscope (Tokyo, Japan) equipped with a Gatan US1000 CCD camera (Pleasanton, CA).

Statistics

All data were analyzed for normality using the D'Agostino and Pearson omnibus normality test (where N>7) and Kolmogorov-Smirnov normality test (where

N<7). Unpaired Student's t-test or Fisher's test were used for pairwise comparisons. Comparisons of multiple groups were analyzed using one- or two-way ANOVA followed by post hoc Tukey's test. All results are expressed as mean ± S.E.M. Statistical comparisons were generated using Graph Pad Prism 6 (Graph Pad Software, Inc., San Diego, CA). $P<0.05$ was considered significant for all comparisons.

Stereotaxic Administration

Stereotaxic injection of control and experimental lentivirus into the sub-ventricular zone (SVZ) in mice was performed using a 10 uL Hamilton GASTIGHT™ syringe (Hamilton Company, NV). Viral particles were delivered to targets in the mouse cortex including the SVZ (Fig. 4) and barrel cortex. For barrel cortex post-stroke injections, the borders of the ischemic core were observed and virus was delivered to the bordering peri-ischemic area. Infected cells can be observed up to a distance of 3-4 mm from the injection site.

Chapter Five: Long-term Survival and Regeneration of Neuronal and Vasculature Cells inside the Core Region after Ischemic Stroke in Adult Mice

5.1 Abstract

Focal cerebral ischemia results in an ischemic core surrounded by the peri-infarct region (penumbra). Most research attention has been focused on neuronal and vascular cell fate in penumbra. Meanwhile, the cellular fate of these cells in the ischemic core is poorly defined. In the present investigation, we tested the hypothesis that, inside the ischemic core, some neuronal and vascular cells could survive the initial ischemic insult and that this population forms a regenerative niche which exists many days after stroke. Adult male mice were subjected to focal cerebral ischemia induced by permanent occlusion of distal branches of the middle cerebral artery (MCA) plus transient ligations of bilateral common carotid artery (CCA). The ischemic insult uniformly reduced the local cerebral blood flow (LCBF) to the barrel cortex by 90%. Massive cell death occurred due to multiple mechanisms and a significant infarction was cultivated in the ischemic cortex 24 hrs later. Nevertheless, normal or even higher levels of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) remained in the core tissue, some NeuN-positive and Glut-1/College IV-positive cells with intact ultrastructural features still resided inside the core 7-14 days post stroke. BrdU-positive but TUNEL-negative neuronal and endothelial cells also existed in the core where extensive extracellular matrix infrastructure were observed. Concurrently, GFAP-positive astrocytes accumulated in the

penumbra and Iba-1-positive microglial/macrophages invaded the core several days after stroke. Up to 5% of neuronal and vascular cells inside the ischemic core were observed after severe ischemic stroke induced by permanent embolic occlusion of the MCA. Furthermore, we demonstrate that therapeutic intervention via pharmacological hypothermia could further increase survival in an embolic stroke to 6% inside the core. These data suggest that the ischemic core remains an actively regulated brain region with residual and newly formed viable neuronal and vascular cells acutely and chronically after at least some types of ischemic strokes.

5.2 Results

Focal ischemic stroke in the mouse and ischemia-induced cell death

A focal ischemic stroke model of adult mice was tested first in this investigation. The ischemic stroke was induced by permanent MCA occlusion and transient bilateral CCA ligations. The MCA/CCA blockade markedly decreased the local cerebral blood flow (LCBF) to the lethal level in the sensorimotor cortex involving the barrel cortex as well as the surrounding motor cortex. In Laser Doppler scanning imaging, the LCBF in the targeted cortical region was 11.5 ± 1.8 % of the basal level. Release of CCA ligations returned the flow to 44.0 ± 1.0 % of the original LCBF (Fig. 1A). In experimental assays, the center area within the white color area of TTC staining was identified as the ischemic core (Fig. 1C). The peri-infarct region or penumbra was defined as previously described by a 500 μm boundary extending from the edge of the infarct core, medial, and lateral to the infarct (J. J. Ohab, S. Fleming, A. Blesch, & S. T. Carmichael, 2006).

Figure 1
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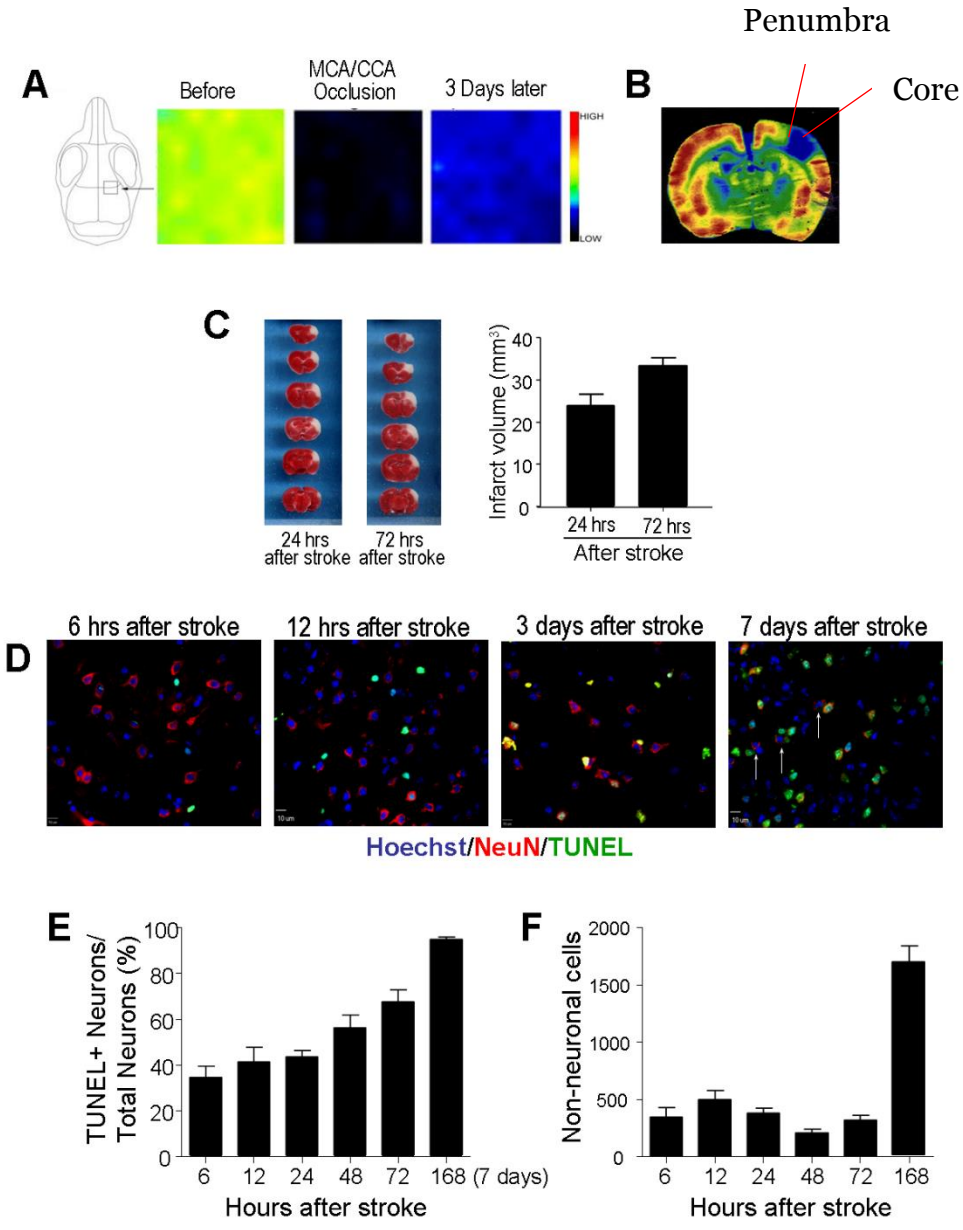

Figure 1. Focal ischemic stroke in the mouse and ischemia-induced cell death

Focal cerebral ischemia targeting the right sensorimotor cortex was induced by permanent occlusion of the distal braches of the right MCA and transient (10 min) ligation of both CCAs. **A**. The Laser Doppler scanner was used to measure the local cerebral blood flow (LCBF) in the ischemic region shown on the skull sketch (squire box) on the left. LCBF was reduced to about 10% of basal LCBF during the MCA/CCA blockage. **B**. ^{14}C-iodoantipyrine autoradiography was applied to assess regional flow in the brain after MCA/CCA occlusions. The blue area in the right cortex shows a marked and uniformed reduction in LCBF, surrounded by less reduced penumbra. **C**. One and 3 days after the ischemic insult, TTC staining revealed a well-defined ischemic core region in the senserimotor cortex. N=12 animals for 24 hrs and 15 for 72 hrs after stroke. **D**. DNA damage representing neuronal cell death was identified using TUNEL staining (green) different times after stroke. In the ischemic core, Hoechst 33342 (blue) was applied to label nuclei of all cells, NeuN (red) was used as a mature neuronal marker. As late as 7 days after stroke, there were still some NeuN+ cells that were not overlaid with TUNEL labeling (arrows). **E**. NeuN+/TUNEL+ cells were counted in the ischemic core region. Quantified cell numbers were from 6 random survey fields and 6 brain sections. Neuronal cell death increased from 6 hrs after stroke and reached to more than 90% at 7 days after stroke. **F**. Non-neuronal cells

were identified as Hoechst 33342-labeled NeuN-negative cells. The number of these cells was relatively stable during the first day after stroke, but showed a reduction at 2 days after stroke. A significant increase of non-neuronal cells was seen at 7 days after stroke when massive invasion of inflammatory immune cells into the ischemic core($p<0.05$, $N = 10$). Six brain sections were obtained from each animal and 6 random fields on the core of each section were counted according to Hoechst fluorescence and analyzed by One-way ANOVA.

Using ^{14}C-IAP autoradiography, we verified that there was a marked and homogenous reduction in LCBF within the ischemic core area (Fig. 1B). Fourteen days later, the LCBF in this ischemic region was 60.0±5.5% of the basal flow. The ischemic insult induced significant and consistent infarct formation revealed by the white area in TTC staining (Fig. 1C). The cortical infarct volume progressed from 24 mm^3 to 33 mm^3 at 1 to 3 days after stroke and the infarct ratio was around 10% of the hemispheric volume (Fig. 1C). These data indicated that the permanent MCA occlusion paired with transient CCA ligations was severe enough to decrease LCBF in the targeted region, resulting in a sizable infarction.

Acute and chronic neuronal cell death in the ischemic core

The ischemic insult to the cortex triggered massive neuronal cell death revealed by TUNEL staining of DNA damage. In the ischemic area, about 40-60% of NeuN-positive (NeuN+) cells were marked with TUNEL staining from 6 to 48 hrs after the cerebral ischemia (Fig. 1D-1E). This data suggests that a large population of neuronal cells die within hours after the ischemic stroke. On the

other hand, it also implies that about a half population of neurons in the ischemic area survived the initial ischemic insult. Seven days after stroke, the majority (>90%) of neurons inside the core became TUNEL positive (Fig. 1E). During the 7 day period after stroke, we also examined non-neuronal cells by counting NeuN-negative cells that were labeled with nuclei staining of Hoechst 33342. These cells showed a trend of gradual reduction and reached the lowest level at 48 hrs after stroke (Fig. 1F). There was, however, a marked increase of non-neuronal cells 7 days after stroke, resulting from a massive invasion of inflammatory cells into the ischemic core (Fig. 1F and see below).

Figure 2
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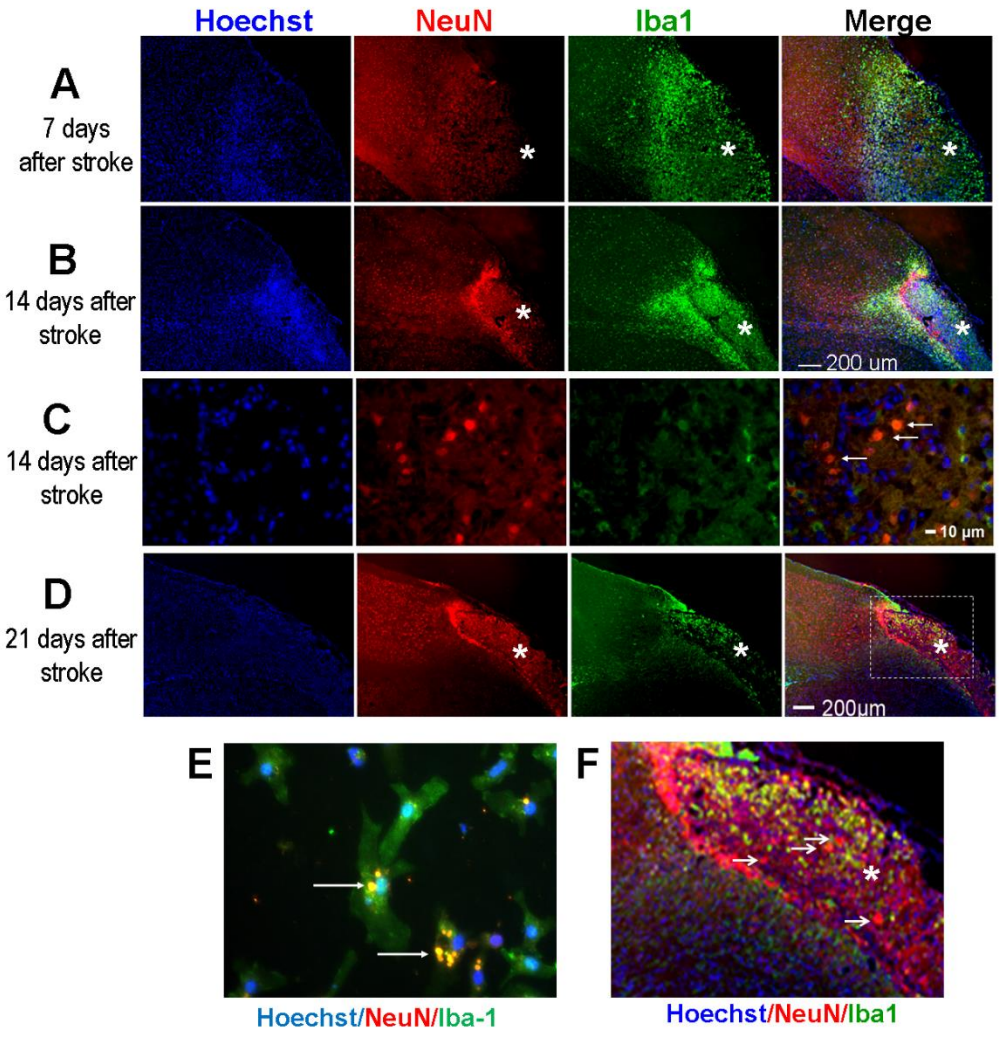

Figure 2. Neuronal cell fate in the ischemic core.

Immunohistochemical staining was performed in brain sections using NeuN antibody to label neurons and Iba-1 antibody to label microglial cells in the ischemic cortex. A to D. Neuronal and microglial cells at 7, 14 and 21 days after stroke. NeuN+ cells can be seen inside the ischemic core (*). The enlarged images in C illustrate intact NeuN+ staining (red, arrows) in the core region 14 days after stroke. Meanwhile, there were significant numbers of Iba-1+ microglia and macrophages (green) located into the core. Hoechst 33342 staining (blue) marks the nuclei of all cells. E. A magnified image showing Hoechst 33342, NeuN and Iba-1 labeling in a core area. The NeuN immunoreactivity appears inside the Iba-1+ cells (arrows), suggesting that neuronal cells and/or debris were cleaned up by microglial/macrophages in the process of phagocytosis. This appears the case for most NeuN-positive staining in the core region. F. Enlarged image from the framed area in D, showing there were some intact NeuN+ cells (arrows) that were not overlaid with Iba-1 staining in the core 21 days after stroke.

Several days after stroke, there were around 5% of NeuN+ cells that were TUNEL-negative in the ischemic core. A close look revealed, however, that some of them exhibited their NeuN immunoreactivity inside of Iba-1-positive (Iba-1+) macrophages/microglial cells (Fig. 2E). These cells appeared to be actively

undergoing the clearing process via phagocytosis. Surprisingly, we detected a population of NeuN+ cells that were neither overlaid with Iba-1 nor with TUNEL staining as late as 7 to 14 days after stroke. These cells are suggestive of surviving neurons in the ischemic core lasting many days after an ischemic insult (Fig. 1D and 2A-2D and 2F).

Figure 3
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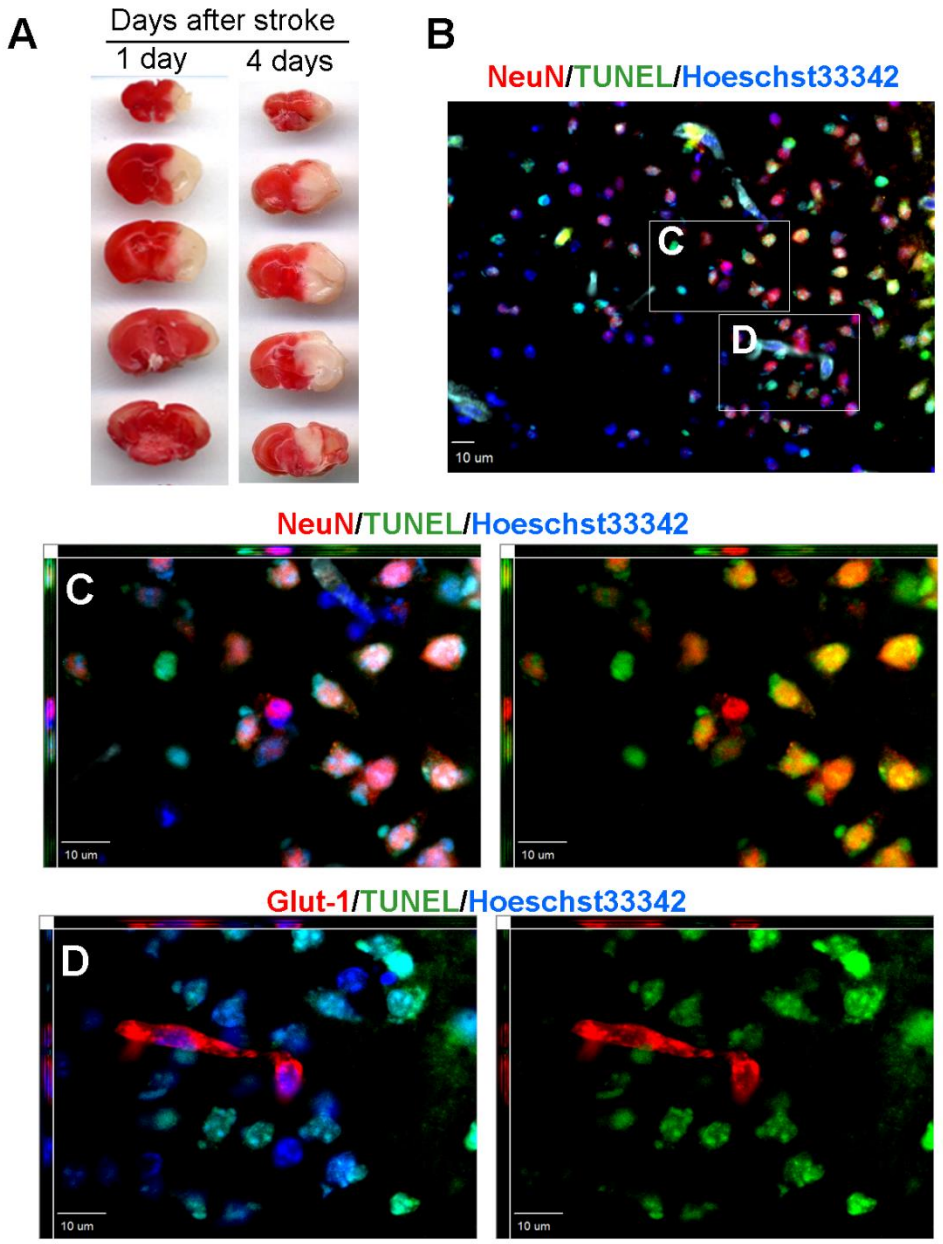

Figure 3. Cell death and survival in the ischemic core 5 days after embolic stroke of sever ischemia.

A severe ischemic stroke was induced in adult mice by permanent occlusion of the MCA using an autologous blood clot. **A**. TTC staining of brain sections at 1 and 4 days after the ischemic insult. **B**. Immunohistochemical staining of NeuN (red), TUNEL (green) and Hoechst 33342 (blue) in the core region for the inspection of neuronal cell death at 5 days after stroke. NeuN-positive, but TUNEL-negative, cells can be seen in the region. The frames show the enlarged areas in C and D. **C**. Three dimensional images show a NeuN- positive, but TUNEL-negative neuron (arrow). **D**. Glut-1 staining was used for vascular endothelial cells. The 3-D images show endothelial cells that were TUNEL-negative. Representative of 3 animals.

To verify this conclusion obtained from the partial reperfusion model, some examinations were repeated in a permanent ischemic stroke model with damage to the most parts of the right hemisphere (Fig. 3). The MCA was permanently occluded using an autologous blood clot; the embolic ischemia lead to an infarction that occupied most of the right hemisphere in TTC staining (Fig. 3A). As a permanent ischemia model, the infarct formation reached to a maximal volume around 2-3 days after the onset of ischemia (J. H. Garcia et al., 1993). At 5 days after stroke, we observed surviving neurons and vascular cells in the center of the core region (Fig. 3B to 3D). Data presented below were obtained from the partial reperfusion stroke model.

Mechanism of neuronal cell death in the ischemic core

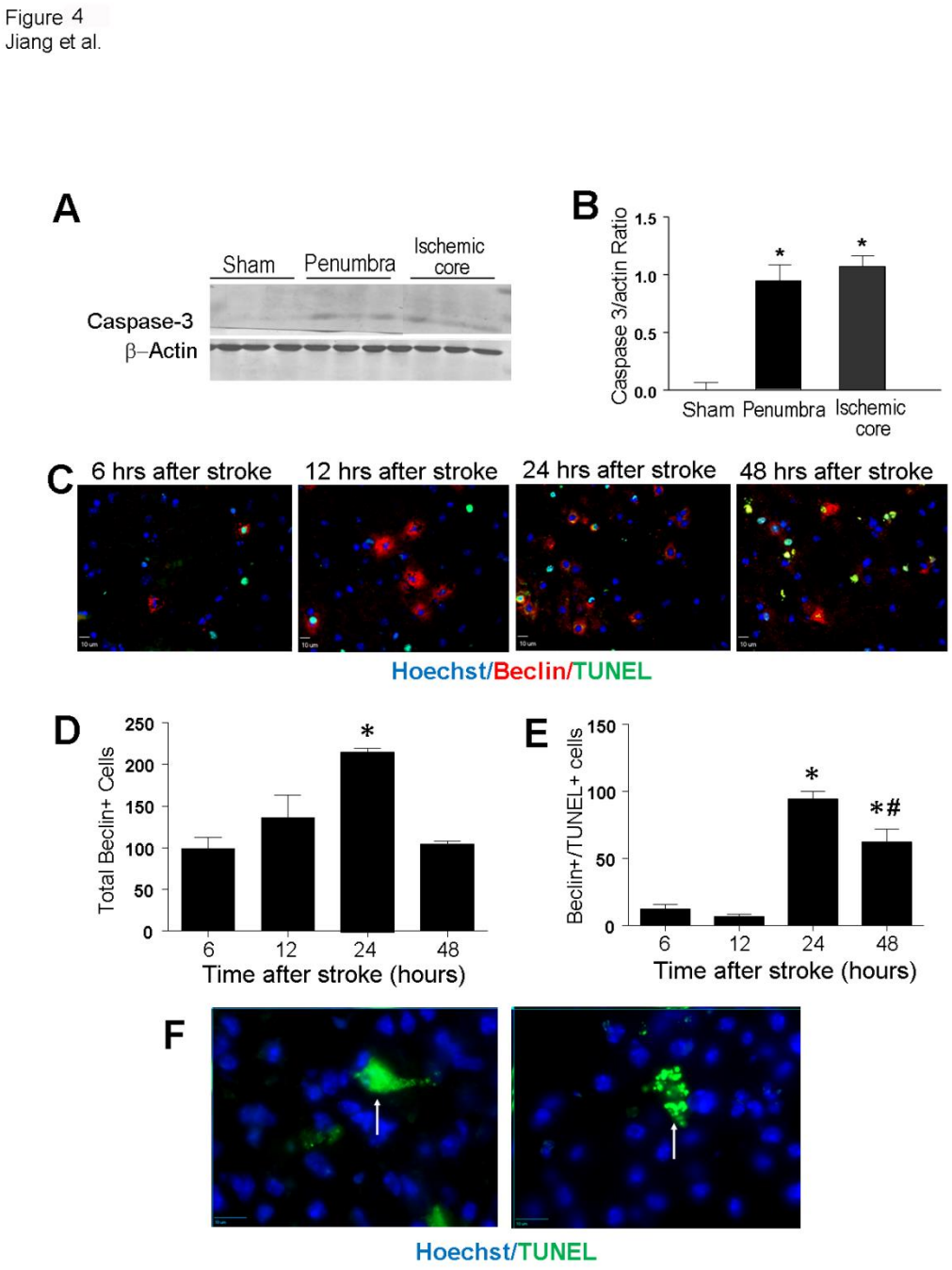

Figure 4. Apoptosis and autophagy contributed to cell death in the ischemic core

Signals in the apoptotic and autophagic cascades were examined in the ischemic cortex. **A** and **B**. Western blotting shows activation of caspase-3 in the penumbra and core regions 1 days after stroke. N=4-5. * $p<0.05$ vs. sham controls. **C**. The autophagy marker beclin 1 was detected in immunostaining of the ischemic core area (arrows). **D**. Quantification of total beclin 1+ cells per animal (6 brain sections and 6 random fields on each section) in the ischemic core different time points after stroke. **E**. Double labeling of beclin 1 and TUNEL revealed a relationship between the beclin 1 immunoreactivity and cell death, especially 24 to 48 hrs after stroke. Comparing to the bar graph in D, approximately 50% of beclin cells were also TUNEL+ at these time points. N=3 animals per time point. * $p<0.05$ vs. 6 hrs, # $p<0.05$ vs. 24 hrs. **F**. Differential morphology of TUNEL+ neurons. The image on the left show smear TUNEL staining without noticeable nucleus fragmentation (Type 1 TUNEL+ cells; arrow). The image on the right shows fragmented DNA of TUNEL staining (Type 2 TUNEL+ cells; arrow).

It was shown that acute cell death after ischemic stroke is due to excitotoxicity involving increased glutamate release, intracellular Ca^{2+} accumulation, production of reactive oxygen species, and a number of other injurious factors (D. W. Choi, 1992; Mattson, 2003; Niizuma, Endo, & Chan,

2009). This acute cell death in the core was widely believed to be necrotic in nature. However, we detected significant activation of caspase-3 in the ischemic core at 1 day after stroke, similar to the caspase-3 activity in penumbra (Fig. 4A and 4B). Caspase-3 activities were also detected in the core tissue 7 days after stroke (data not shown). These results suggested that apoptotic events played a noticeable part in the ischemic core as a mechanism of cell death.

Meanwhile, immunohistochemical examination in the ischemic core region revealed beclin-1 activity as early as 6 hrs after stroke, suggesting that autophagy took place at this time (Fig. 4C and 4D). Most beclin-1 reactive cells at 6 and 12 hrs after stroke were not TUNEL positive (Fig. 4D and 4E). Thus, this early autophagic activity was likely a physiological event of clearing away dead cells. The beclin-1 activation reached to its peak at 24 hrs after stroke, while many beclin-1+ cells now became TUNEL positive, indicating that, one day after stroke, excessive autophagy contributed to cell death in the ischemic core (Fig. 4D and 4E). We also noticed different morphological features of TUNEL+ cells. Some TUNEL+ cells showed smear fluorescence of TUNEL staining in cell nuclei, which was named Type I TUNEL+ cells against Type II TUNEL+ cells that had fragmented TUNEL staining (Fig. 4F) (L. Wei et al., 2004). These cellular and subcellular changes indicate that a mixed form of cell death takes place in the ischemic core, which may include necrosis, apoptosis as well as autophagy.

Endothelial cell and vasculature fate in the ischemic core

Figure 5
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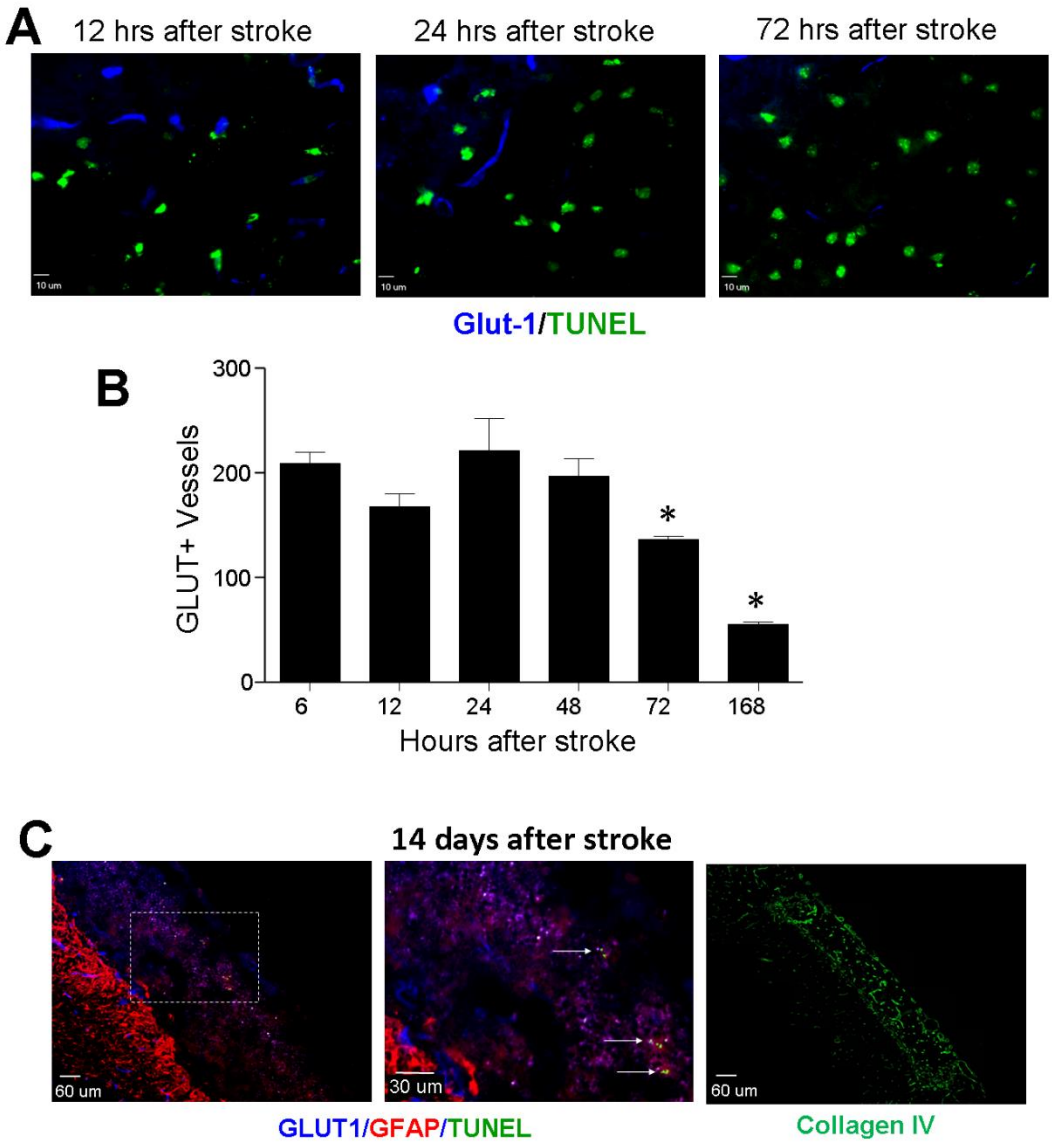

Figure 5. Vascular endothelial cells and microvessel structures in the ischemic core

Vascular endothelial cells were examined using the glucose transporter 1 antibody in immunohistochemical examination. **A.** Immunofluorescent images of Glut-1 (blue) and TUNEL (green) staining in the ischemic core at different days after stroke. **B**. Quantified data of the experiment in A. The number of Glut-1+ endothelial cells/vessels remained relatively constant for up to 2 days after ischemia. Glut-1+ cells decreased gradually from 3 to 7 days after stroke. N=7 per time point, * $p<0.05$ vs. 6 hr data). **C**. At 14 days after stroke, Glut-1 (blue) markers was still widely detectable inside the ischemic core, while GFAP labeled glial cells (astrocytes) were mostly located in the peri-infarct region. In the enlarged image from the frame shown in the left, TUNEL staining revealed some cell death process at this much delayed time point. The Collagen IV staining verified the extramatrix networks developed in the ischemic core 14 days after stroke.

Glut-1 is a uniporter protein expressed in the endothelial cell membrane. The Glut-1 55-kDa isoform is specifically expressed in brain microvascular endothelium and has been used as a specific marker of microvessel/capillary in the brain (V. R. Whitaker, Cui, Miller, Yu, & Wei, 2007). We noted that Glut-1+ vessels remained at about the same level for up to 2 days after stroke and then

started to decrease and reached a lower level by day 7 after stroke (Fig. 5A and 5B). Interestingly, Glut-1+ microvessel structures increased significantly 14 days after stroke and the majority of Glut-1+ cells were not labeled by TUNEL (Fig. 5C). Furthermore, Collagen IV staining revealed that extensive extra-matrix networks formed at this time throughout the ischemic core (Fig. 5C). This extra-matrix infrastructure forms the foundation of a favorable microenvironment for angiogenesis.

Invasion of microglial/microphage into the ischemic core after stroke

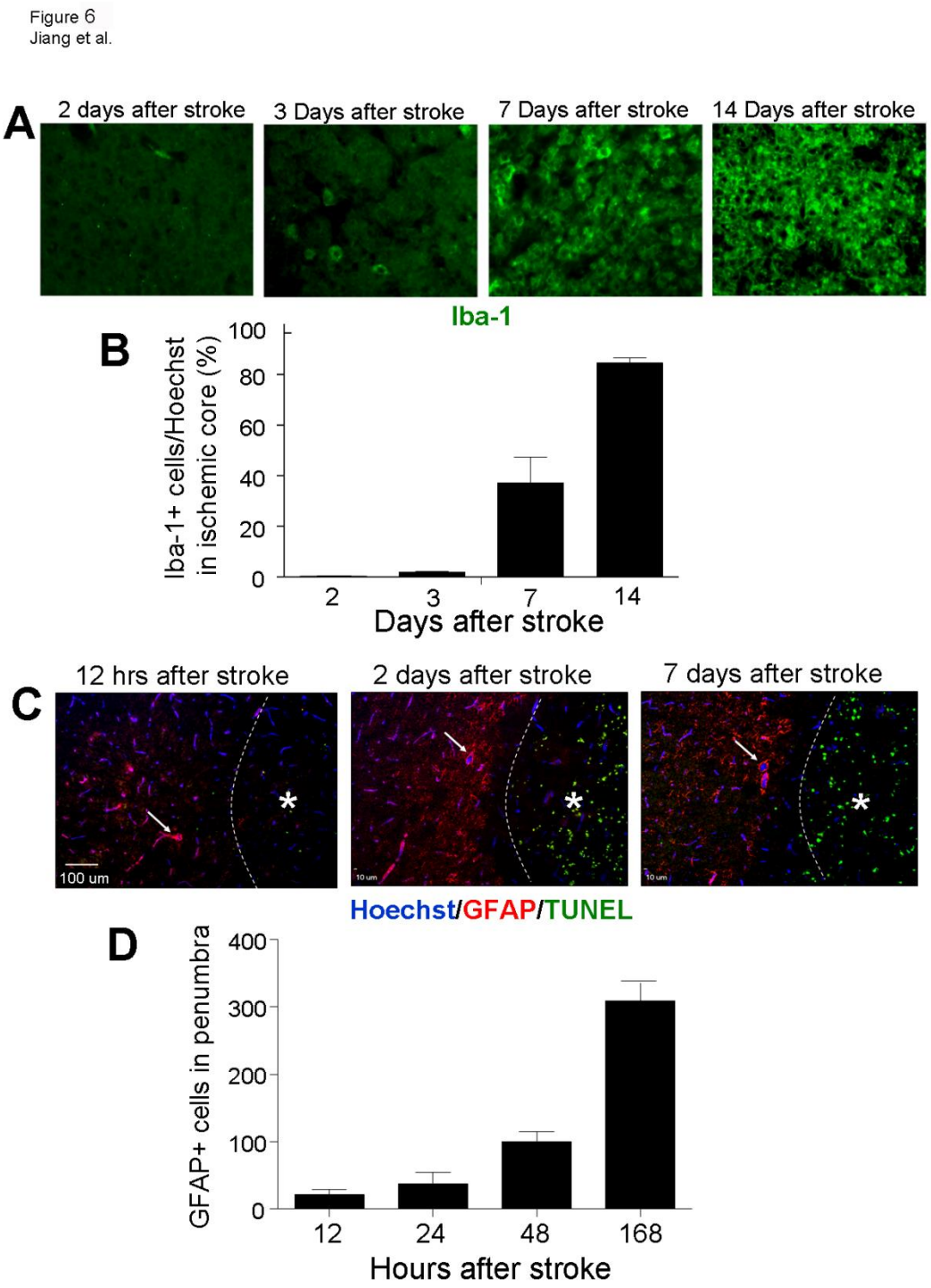

Figure 6. Distribution of microglia/macrophages and astrocytes in the ischemic and penumbra regions after stroke

Immunohistochemical staining was performed to track the invasion and localization of Iba-1+ and GRAP+ cells in the ischemic cortex. **A** and **B**. Iba-1 staining (green) in the ischemic core at different days after stroke. Iba-1+ microglial/macrophages were rare in the core during the first few days after stroke. These cells started to occupy the ischemic core tissue several days after stroke, by day 14 about 90% of cells in the core were Iba-1+ cells. N=10 animals. Six brain sections were obtained from each animal and 6 random fields on the core of each section were counted. **C** and **D**. GFAP staining (red) was applied to identify activated astrocytes and TUNEL staining (green) was used to detect dead cells. Hoechst 33342 (blue) labeled nuclei of all cells. GFAP+ astrocytes started to accumulate in the peri-infarct area 12 hrs after stroke, and the number increased gradually as long as 7 days after stroke. Arrows point to some GFAP/Hoechst positive cells in the peri-infarct area. TUNEL staining was concentrated in the ischemic core (*), demonstrating massive cell death in the core. The bar graph shows the average total counted numbers of cells per animal. N=8-12 animals. Six brain sections were obtained from each animal and 6 random fields on the core of each section were counted.

One of the main cellular events after stroke is the invasion of activated microglia and macrophages into the ischemic core. Using the anti-Iba1 antibody, we detected a gradual migration and accumulation of microglial cells and macrophages in the core region from 2 to 21 days after stroke (Fig. 6A and 6B). By day 14, it was estimated that more than 80% of cells resident in the core region were microglia or macrophages and the rest of cells were some NeuN+ cells and non-neuronal cells such as vascular endothelial cells and a few astrocytes (Fig. 5C, 6B and 6C).

Astrocytes fate in the ischemic core and penumbra

As shown in previous investigations, activated astrocytes accumulated in the peri-infarct region surrounding the ischemic core but not inside the core. GFAP staining illustrated that at 12 to 48 hrs after stroke, an increasing number of astrocytes started to locate in the area around the ischemic core (Fig. 6C). The number of astrocytes steadily increased and formed an astrocytic scar around the core 7-14 days after stroke (Fig. 6D).

Ultrastructural examination of surviving cells in the ischemic core

Figure 7
Jiang et al.

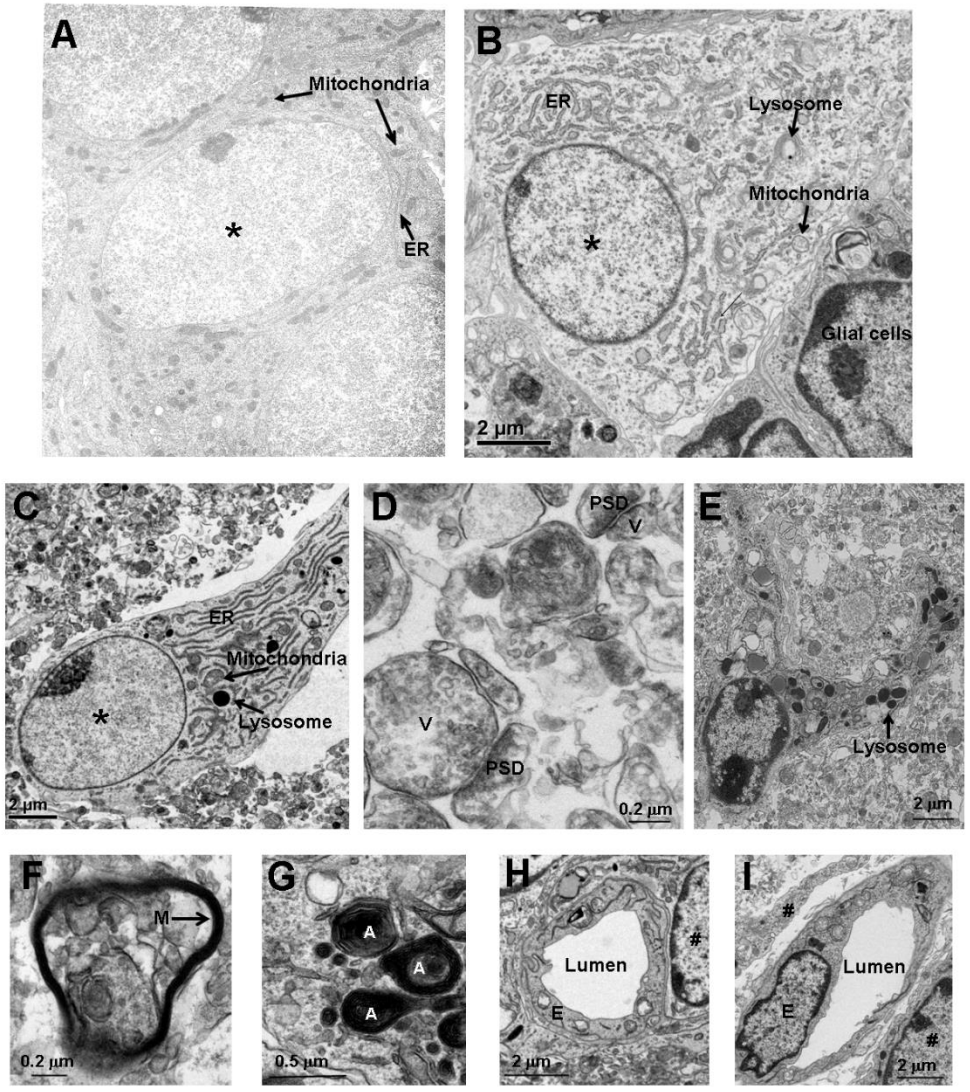

Figure 7. Ultrastructural evidence of surviving cells in the ischemic core 7 days after stroke

A transmission electron microscope was used to examine the control brain section and the ischemic core of brain sections 7 days after ischemia. **A**. A control EM image of normal cortical neurons in the contra lateral cortex. The neurons had clear boundaries and a large nuclear (*), chromatin was uniformly distributed, the plasma membrane was continuous and clear, and many organelles including mitochondria and endoplasmic reticulum (ER) were visible. **B.** A representative surviving neuronal cell in the ischemic core. As in the contralateral hemisphere, the cell contains a relatively large nucleus delineated by an intact nuclear membrane surrounded by abundant and well preserved cellular organelles in the cytoplasm. **C**. Another example of a surviving neuronal cell in the ischemic core. In addition to the near normal nucleus (*) and chromatin, there were numerous intracellular organelles including mitochondria and ER (arrows) that extended from the cell body in a proximal dendrite. Some lysosomes were also found in the cytoplasm, suggesting some cellular degeneration. **D.** Remaining synaptic structures in the ischemic core region. A presynaptic axon terminal filled with synaptic vesicles (V) in contact with a post-synaptic element can be seen in this image. The dark post-synaptic density (PSD) was located opposite to the pre-synaptic membrane. **E**. A microglial or microphagic cells showing two processes

containing many lysosomes in the cytosol. Note the small size of this type of cells. **F**. Damaged myelinated axon as shown in this image can be seen in the ischemic core. The image shows a crossing section of the myelin (M) surrounding nerve fibers. **G**. Some autophagolysosomes (A) were identified, suggesting the process of autophagy. **H** and **I**. Surviving microvessels and endothelial cells (E) as well as the infrastructure of the neurovascular unit were easily detectable in the ischemic core. Astrocytes and/or pericytes (#) and basement membrane were surrounding endothelial cells. The lumen space was clearly formed inside the neurovascular unit.

To further understand the viability of the residual cells in the ischemic core, surviving cells in this region were examined using electron microscopy at 7 days after stroke. Under an electron microscope, viable cells were spread throughout survey fields. Some of them showed neuronal features including a large nucleus with an intact plasma membrane and numerous cellular organelles in the cytoplasm (Fig. 7B and 7C). Moreover, there were still synaptic contacts between cells, composed of intact synapses with pre- and post-synaptic features such as presynaptic vesicles and a postsynaptic density (PSD) (Fig. 7D). Some glial-like cells were observed and most of them appeared to be microglia or microphages (Fig. 7E). Interestingly, we also identified myelinated axons (Fig. 7F) and many

vessel like structures composed endothelial cells and astrocytes/pericytes that resembled the neurovascular unit (Fig. 7H and 7I). Consistent to the earlier observation of the autophagy marker, typical autophagosomes were observed in microglial cells (Fig. 7G).

Regenerative factor expression in the ischemic core

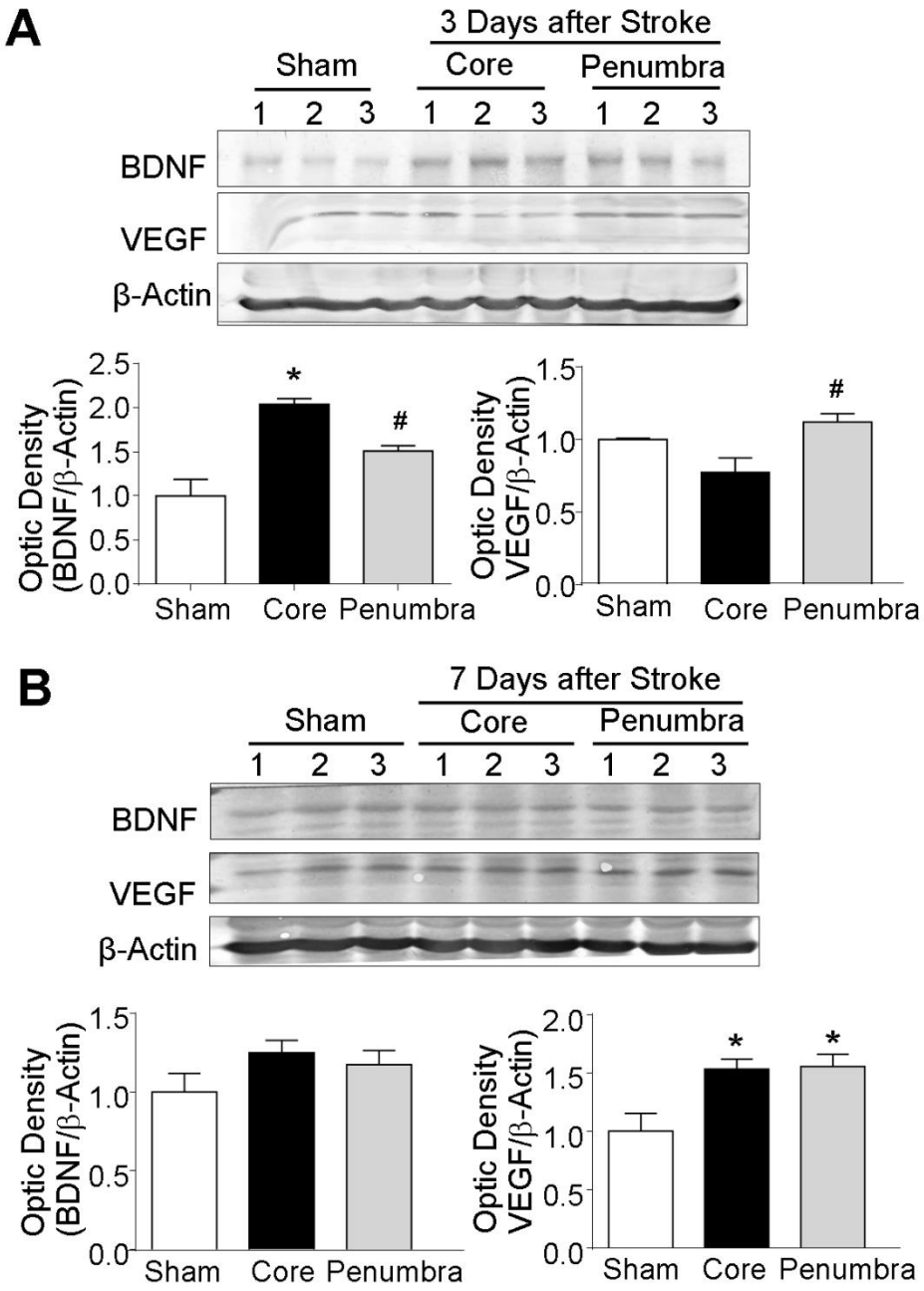

Figure 8. Expression of trophic factors in the ischemic core and penumbra

Western blotting was applied to assay the expression levels of BDNF and VEGF in the ischemic core and penumbra regions 3 and 7 days after stroke. **A**. BDNF and VEGF expression levels 3 days after stroke in sham control, core and penumbra brain tissues. **B**. BDNF and VEGF levels at 7 days after stroke in the three brain regions. The bar graph show the expression ratios normalized to sham control after correction with loading controls. *. $p<0.05$ vs. sham, #. $p<0.05$ vs. core; n = 6 animals per group.

Consistent with the existence of different cell populations in the ischemic core, Western blot detected significant expression of trophic/growth factors including brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF). At 3 days after stroke, BDNF expression in the core tissue showed a two fold increase compared to the expression in the corresponding cortex of the sham control mice (Fig. 8A). BDNF in the penumbra also increased, but to a lower extent (Fig. 8A). The VEGF expression was not significantly altered in the core, but increased in the penumbra (Fig. 8A). Seven days after stroke, the BDNF level returned to about the sham control levels, while the VEGF level significantly increased in both the core and penumbra tissues, which was consistent with the extensive vasculature formation in the ischemic region around this time (Fig. 8B and 5C). These different regulations of BDNF and

VEGF also verified that the tissues examined were distinct from the core and peri-infarct regions.

Regenerative activities in the ischemic core

Figure.9
Jiang et al.

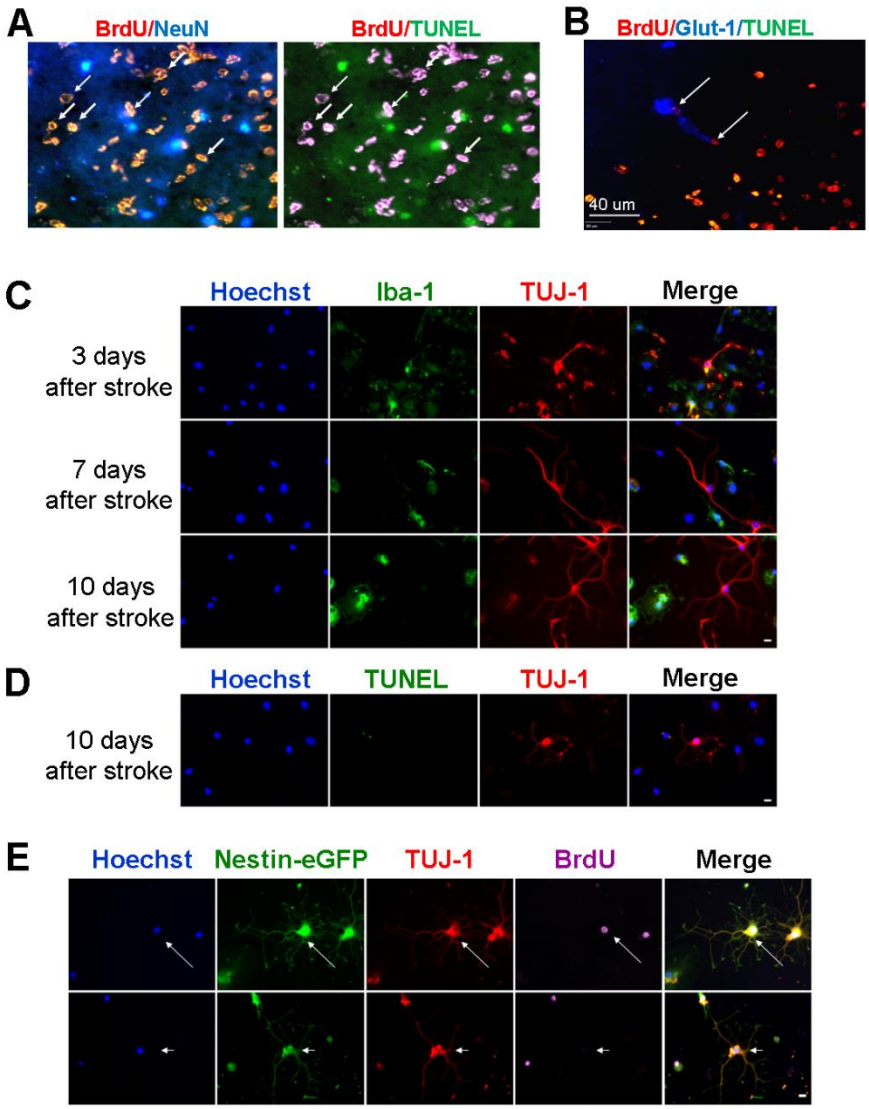

Figure 9. Regenerative niche exited in the ischemic tissue

Regenerative activities in the ischemic core were inspected in the brain and under cultured conditions. **A** and **B**. Stroke animals received BrdU injections daily to label proliferating cells. Seven days after stroke, immunostaining of the core region revealed the existence of BrdU/NeuN double-positive cells that were TUNEL-negative (A). BrdU/Glut1-positive, but TUNEL-negative, endothelial cells were also observed in the core (B). Arrows point to BrdU/NeuN or BrdU/Glut1 double positive cells. To detect regenerative niche that might reside in the ischemic core tissue, we dissected the core tissue at 7 days after stroke and plated the dissociated cells in PDL/Laminin coated dishes. **C**. Different days after in vitro, cells were fixed and stained with immature neuronal marker TUJ-1 (red) and microglia cell marker Iba-1 (green). There were increasing numbers of TUJ-1+ cells from 3 to 10 days after stroke. Many of them were not overlaid with iba1. **D**. In these images of 10 days after stroke, TUJ-1+ cells (red) were negative to TUNEL (green) staining suggesting they were viable cells. Scale bar = 10 μm. **E**. Nestin-eGFP transgenic mice were subjected to the focal ischemic stroke. BrdU (50 mg/kg) was injected daily from 1 day after stroke. Cells from the ischemic core of 7 days after stroke were cultured for 7 days. Nestin-eGFP (green) was easily detectable in this culture and it overlaid with the immature neuronal marker TUJ-1 (red) as well as with the proliferation marker BrdU (purple) (arrow). There were a few cells that were eGFP-

and TUJ-1-positive but BrdU-negative (arrowhead), implying that they might surviving original cells. Scale bar = 10 μm.

Since we observed normal or even increased levels of BNDF and VEGF as well as dense vasculature structures inside the ischemic core many days after stroke, we speculated that regenerative niches existed in this region. To label newly formed cells, BrdU (50 mg/kg, i.p.) was injected daily from day 1 after stroke until the day before sacrifice. In immunohistochemical assays of the ischemic cortex 7-14 days after stroke, BrdU/NeuN positive but TUNEL negative cells were detected in the core region, indicating newly generated neuronal cells (Fig. 9A). Some Glut-1+ vasculatures in the ischemic core were also BrdU positive, suggesting newly formed microvessels (Fig. 9B). This was consistent with the abundant collagen IV expression on extra-matrix basal lamina in the core (see Fig. 5C).

To verify the regenerative niche in the core region, we dissected the ischemic core tissue at 7 days after stroke and cultured the cells in a neuronal culture media for 3-10 days. As a control, the corresponding cortex region in the contralateral hemisphere was dissected and similarly cultured. As commonly known, neurons acutely dissected from the adult brain could not survive long-term cell cultures. All neuronal cells from the contralateral side die by 7-10 days in culture (data not shown). In the cultures from the ischemic core region, the

majority of cells were Iba1-positive microglia and macrophage cells, which excluded the possibility of contamination by peri-infarct tissue in the assay. Initially, we noticed some NeuN immunoreactivity in day 1 cultures. The NeuN staining, however, mostly appeared inside the cytoplasm of Iba1-positive cells, similarly as seen in the core region where neuronal debris were phagocytized by immunoinflammatory cells (Fig. 2E). In day 3 cultures, we started to see the immature neuronal cell marker TUJ-1 (Fig. 9C). These TUJ-1+ cells increased with time and grew extensive neuronal processes (Fig. 9C). Importantly, these cells were TUNEL negative when examined after 10 days in culture (Fig. 9D), supporting the idea that viable neural progenitor cells and neurons existed in the ischemic core tissue.

To detect newly generated neuroblasts, Nestin-GFP transgenic mice were similarly tested after ischemic stroke. In day-10 cultures of the ischemic core tissue from this mouse, there were Tuj-1+/Nestin-GFP+ cells, and some of these cells also showed BrdU immunoreactivity (Fig. 9E). These observations illustrated that significant regenerative niche existed in the post-stroke environment of ischemic core region. Under favorable conditions, regenerative activity including cell proliferation and neural differentiation can take place in the core region.

The possibility of protecting neuronal and vasculature cells in the ischemic core

Figure 10
Jiang et al.

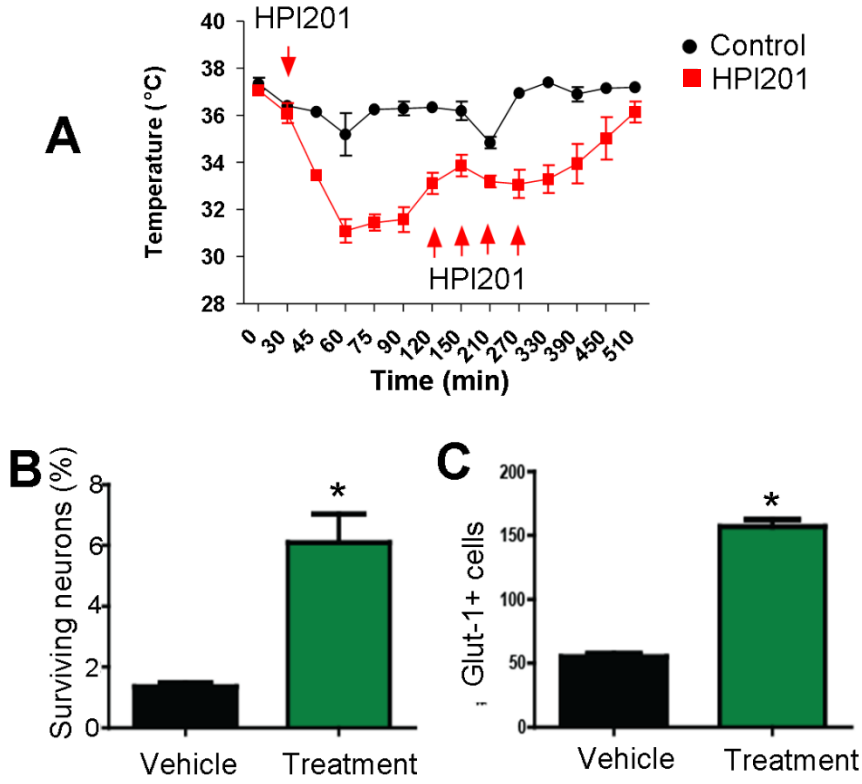

Figure 10. Protection of the neuronal and vascular cells in the ischemic core

A. The neurotensin receptor 1 agonist HPI-201 was injected 60 min after the onset of ischemia. It effectively reduced the body temperature of the stroke animals from 37^{o}C to 32^{o}C for 6 hrs. **B** and **C**. HPI-201 treatment significantly increased surviving NeuN+ neurons (B) and endothelial cells (C) in the core.

To delineate whether it was possible to promote the cell survival in the ischemic core after stroke, we tested pharmacologically induced hypothermia (PIH) as a brain protective therapy (K. E. Choi et al., 2012; J. H. Lee et al., 2014; N. Wei et al., 2013). The hypothermic compound neurotensin receptor 1 (NTR1) agonist HPI-201 (2 mg/kg, i.p.) was injected 60 min after the onset of ischemia and it reduced the animal's body temperature from the normal 37 ^{o}C to around 32 ^{o}C in 30-60 min (n=6 per group). The hypothermia was maintained for 6 hours before it gradually returned to normal temperature (Fig. 10A). Seven days after stroke, NeuN, Glut1 and TUNEL staining in brain sections showed that the hypothermia treatment markedly enhanced the survival of neuronal cells and endothelial cells in the ischemic core (Fig. 10B and 10C).

5.3 Discussion

The present investigation systematically evaluated the cellular fate in the ischemic core acutely and chronically after ischemic stroke. We demonstrate that a small percentage of neurons can survive in this region for at least 7 days after

stroke. Moreover, many vascular endothelial cells and neurovascular structures in the core survive the ischemic insult. Regenerative paracrine factors BDNF and VEGF remain at normal or even higher levels in this region. Furthermore, regenerative activities including proliferation of endothelial cells and formation/invasion of neural progenitor cells takes place in the core many days after stroke. As a result, extensive neurovascular networks formed in the ischemic core 14 days after stroke. Although the surviving cells in the core after stroke are few and the neurovascular structures may be imperfect or immature, they could provide a minimum but vital infrastructure for possible regeneration either from endogenous mechanisms or exogenously transplanted cells. For regenerative therapies using exogenous stem cells and neural progenitors, our data suggest that the microenvironment of ischemic core several days after stroke provides certain cellular and trophic supports for cells to survive while the remaining and regenerating neurovascular infrastructure may be utilized for repair of damaged neural networks.

A short duration of LCBF decrease that reaches 30-70% of normal flow usually does not lead to neuronal cell death and infarct formation (Back, 1998; K. A. Hossmann, 2006). An infarction develops when LCBF decreases by more than 80-90% for sustained periods(Back, 1998; K. A. Hossmann, 2006). Typically inside a ischemic core, LCBF is reduced to 15% or less of the control flow (K. A. Hossmann, 2006). In our focal ischemia model, the LCBF was reduced to around 11% of basal level during MCA/CCA occlusion, leading to lethal damage to the territory of the MCA branches. The autoradiography data illustrated a uniform

reduction of the LCBF in the core region. The infarct is formed 1-3 days after stroke as in other stroke models (Carmichael, 2005). We thus conclude that the cerebral ischemia in our models was homogenous and severe enough to form infarction in the targeted brain tissue.

In the focal ischemia model tested, MCA was permanently occluded, paired with 10 min CCA ligation. The duration of the lethal ischemia induced by MCA/CCA occlusion seems to be relatively short. Longer MCA/CCA occlusion was tested but it resulted in enhanced mortality likely because mice are more sensitive than rats to cerebral ischemia (Carmichael, 2005). In our preliminary experiments, MCA/CCA occlusion could be extended to 20 min when 100% O_2 gas was used instead of room air for isoflurane anesthesia. The high oxygen condition, however, resulted in large variations of the infarction volume in C57/BL mice and we decided to use regular air in the inhalant anesthesia. Long-term cell survival in the core was also observed in the permanent embolic ischemia model of MCA occlusion, suggesting that the cellular fates we observed are not limited to a particular model. The pathological feature of surviving cells in the core of different ischemic insults needs to be further compared and characterized. For example, there is a small possibility that in the embolic stroke model, spontaneous thrombolysis might occur sooner or later, leading to transient ischemia. In a future investigation, survival of cells in the ischemic core may be tested in a filament insertion model which features permanent MCAo.

Partial reperfusion due to incomplete (spontaneous and postthrombolytic) recanalization after an ischemic attack occurs in 30% and up to 70% of clinical

cases at different times after the onset of ischemia (Barber et al., 1998; Hakim et al., 1987; Jorgensen, Sperling, Nakayama, Raaschou, & Olsen, 1994; Neumann-Haefelin et al., 2004). An MRI study of 82 stroke patients with MCA occlusion revealed that incomplete reconalization occurred in 39 patients, complete reconalization in 10, and persistent occlusion in 33 patients (Neumann-Haefelin et al., 2004). In another CT/SPECT scan study on 354 stroke patients, the incidence of spontaneous reperfusion was 77% in patients with cortical infarcts (Majid et al., 2000). In this regard, the release of CCAs ligation while MCA was permanently occluded in our stroke model may resemble a partial reperfusion condition relevant to many clinical stroke cases.

Previous and current investigations have focused on the peri-infarct or penumbra region of the ischemic brain. It is believed that only this region is savable due to the slow process of programmed cell death. This approach makes sense considering that the majority of cells in this region survive the initial ischemic attack, providing a reasonable time window to rescue cells in following hours and days (Aronowski et al., 1999; Ferrer & Planas, 2003; Ghobrial et al., 2014; Heiss, 2011; Kidwell et al., 2003). Conversely, the ischemic core has been widely regarded as a pan-necrotic tissue deteriorating soon after stroke (Claus et al., 2013; Nedergaard, 1988). Due to this historical focus on the peri-infarct region, the temporal and spatial patterns of cell fate process in the ischemic core has been poorly defined. Our data indicate that up to 5-7 days after ischemic stroke, a small but quantifiable number of neurons and vascular cells are still surviving in the ischemic core. Considering that maximal infarction normally

forms 3 days after permanent MCA occlusion (J. H. Garcia et al., 1993), the survival of these cells persist long after maximal infarct formation.

In a few previous investigations, neurons have been reported to survive in ischemic tissue longer than 3-7 days (Goldberg & Choi, 1993). In an ischemic stroke model of rats, about 80% neurons became necrotic in the core region 7 days after stroke (J. H. Garcia, Wagner, Liu, & Hu, 1995). Newly formed vessels can be seen in the ischemic core after ischemic stroke and Nestin expression was detected with the vasculature-associated cells 14 to 28 days after ischemia (DeGirolami, Crowell, & Marcoux, 1984; Mennel et al., 2000). In a more recent investigation, McMarthy et al. showed that about 5% NeuN+ cells could still be counted in the ischemic core 3 days after transient ischemic stroke in rats (McCarthy et al., 2012). Moreover, a recent investigation on biochemical parameters in the ischemic core and penumbra showed that protein synthesis was only partly inhibited by 36% at 3 days after transient ischemic stroke (Bonova, Burda, Danielisova, Nemethova, & Gottlieb, 2013), suggesting that the cellular/molecular machinery of protein synthesis was largely preserved at this time.

These reports differ from the popular belief that neurons in the ischemic core die from pannecrosis, several early investigations on the cell death in the ischemic region indicate that many cells die with shrunken cell body (J. H. Garcia & Kamijyo, 1974), which is strikingly different from the swollen cell body seen in glutamate/NMDA-induced excitotoxicity *in vitro* (D. W. Choi, 1987; Goldberg & Choi, 1993). Garcia et al. reported that neurons in the ischemic tissue showed

early shrinkage and scalloping followed by appearance of Ghost neuron morphology observed up to 7 days after stroke (J. H. Garcia et al., 1993). “Ghost cells” have a shadowy appearance in hematoxylin-eosin (H&E) stained sections. Later, the Ghost cell was linked to apoptosis in sympathetic neurons (Tomkins, Edwards, & Tolkovsky, 1994). During the early hours after an ischemic insult, morphological changes in the core include acute shrinkage, angularity, and homogeneous eosinophilia of the cytoplasm. The nucleus becomes shriveled, pyknotic and hyperchromatic (Y. Li, Chopp, & Powers, 1997; Little, Sundt, & Kerr, 1974; Steinberg et al., 1986). Many of these morphological features are inconsistent with necrosis, but rather suggestive of apoptosis. There have been a few reports that observed apoptotic cell death in the ischemic core (Manabat et al., 2003; Nakashima, Yamashita, Uesugi, & Ito, 1999).

In recent years, it has become evident that ischemia-induced cell death in the brain does not occur by typical necrosis or apoptosis as observed *in vitro*. In fact, cell death after cerebral ischemia *in vivo* is more likely a result from combinations of multiple injury mechanisms. Thus, mixed or hybrid features of both necrosis and apoptosis may be seen in a single cell (C. L. Liu, Siesjo, & Hu, 2004; L. Wei et al., 2006; L. Wei et al., 2004; A. Y. Xiao, L. Wei, S. Xia, S. Rothman, & S. P. Yu, 2002). For example, a dying neuron often has a swollen cytoplasm, deteriorated cellular membrane/organelles while its nucleus is highly condensed with suggesting involvement of caspase activation (L. Wei et al., 2006; L. Wei et al., 2004; A. Y. Xiao et al., 2002). These mixed cellular, molecular, and ultrastructural features observed in single cells has been termed hybrid cell death

by us {Yu, 2003 #37}, and has adopted a few different names with similar or various definitions by others (Benchoua et al., 2001; Cho, 2014; Fayaz, Suvanish Kumar, & Rajanikant, 2014; C. L. Liu et al., 2004; J. Puyal, V. Ginet, & P. G. Clarke, 2013; L. Wei et al., 2006; L. Wei et al., 2004; A. Y. Xiao et al., 2002). Autophagy may contribute to ischemia-induced neuronal cell death (Fayaz et al., 2014; W. L. Li et al., 2013; J. Puyal et al., 2013; N. Wei et al., 2013). Whether or not this may happen in the ischemic core has not been shown before. Our report now provides evidence that neurons in the ischemic core bear the morphological and molecular features of necrosis, apoptosis as well as autophagy, leading to hybrid cell death.

GFAP-positive astrocytes accumulated in the peri-infarct area, but did not move into the ischemic core. These cells formed the glial scar around the infarcted zone 7 days after stroke which isolated the ischemic core from the surrounding area. Starting hours after cerebral ischemia, activated microglial/microphages emerged and increased in the ischemic core (Claus et al., 2013; H. Li et al., 2014; Z. Zhang, Chopp, & Powers, 1997). These cells likely invade from the circulation and proliferate in the core (Felger et al., 2010; J. H. Garcia et al., 1994; Matsumoto et al., 2008; Moxon-Emre & Schlichter, 2010). We noticed observed NeuN+ puncta inside the Iba-1+ cells. These are likely the debris of neurons subjected to phagocytosis by microglia and microphages.

A recent study showed that the expression of VEGF receptor 3 (VEGFR-3) could be detected in perivascular cells in the ischemic core 3-7 days after stroke (Shin, Park, et al., 2013). The existence of neurovascular structures and the

remaining levels of neurotrophic factors in the core chronically after stroke should play significant roles in regenerative stroke therapies. Nestin expression within the ischemic tissue increased as early as 6 hours and peaking at 7 days. This expression persisted for at least 4 weeks after transient ischemic stroke (Y. Li & Chopp, 1999; Shin, Kim, et al., 2013). The nestin expression was observed in neuronal and non-neuronal cells. In a microarray analysis of gene expression after permanent MCA occlusion in rats, there were 2,882 genes upregulated and 2,835 genes downregulated in the ischemic core at 3 days after stroke (Ramos-Cejudo et al., 2012). The affected genes were involved with inflammation, cell death/growth/proliferation/migration, DNA replication/recombination/repair, cellular assembly/organization, cell signaling, and neurovascular unit development. This activity suggests controlled activity and regulated cellular activity. In addition, nestin expression and the broad spectrum gene regulation in the core also imply active regenerative/repair processes occurring inside the core even after the severe permanent ischemia.

Using pharmacological hypothermia therapy that provides global brain protection, we demonstrate that cell death in the core region can be significantly decreased by a therapeutic intervention. Cell death in the core has not been commonly examined in previous and current investigations. We recommend that protective effects aimed at reducing death of neuronal and endothelial cell populations in the ischemic core should be specifically evaluated in experimental stroke treatments. Promoting the endogenous regenerative mechanism inside the core should be included as a therapeutic target in future investigations.

Chapter Six: Direct reprogramming of astrocytes to enhance recovery after stroke

Article title in preparation

In Vivo Direct Reprogramming of Reactive Astrocytes into Neurons after Ischemic Stroke

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6.1 Introduction

Human neurons have limited regenerative ability with the only known sources of neural progenitor cells stemming from the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus (SGZ). Consequently, neurodegeneration resulting from chronic or acute diseases including ischemic stroke remains a major health concern with limited curative treatments. With the discovery of transcription factor-mediated redirection of cell fate by Yamanaka et al., neuroregenerative therapy gained a spotlight for its potential in ameliorating the neuronal loss characteristic of many neurological diseases including but not limited to stroke, Alzheimer's disease, spinal cord injury, and traumatic brain injury (Gwak et al., 2012, Pekny and Nilsson, 2005, Sofroniew and Vinters, 2010, Verkhratsky et al., 2010 and Verkhratsky et al., 2012).

Unlike neurons, resident populations of glia in the brain remain mitotic and are able to rapidly replicate in response to injury. Additionally, their rapid reactive proliferation and migration in response to injury provides an abundant source of cells. Gliosis is a complex, time-dependent interplay between various specialized populations of cells with an acute and chronic phase. Astrocytes, which are normally quiescent and distributed throughout the cortex, engage in both hypertrophy and proliferation in response to injury. (Pekny and Nilsson, 2005, Robel et al., 2011 and Sofroniew and Vinters, 2010). Initially, this population of reactive glia confer neuroprotective effects and limit the severity of an insult by forming a barrier to limit the intrusion of toxic cytokines and invasive organisms (Pekny and Nilsson, 2005, Robel et al., 2011 and Sofroniew

and Vinters, 2010). However, after 7-14 days, astrocytes form a glial scar that releases inhibitory paracrine signals that reduce neuroregeneration and axon outgrowth into the stroke region (Sofroniew and Vinters, 2010). Consequently gliosis remains a major challenge faced by transplantation therapies as the growth and development of ectopically introduced cells are limited by these inhibitory factors from astrocytes in the glial scar. Reactive astrocytes in glial scars can be restricted in cases of focal injuries, but can also be widely distributed across brain regions in the case of a diffuse injuries.

Both endoderm-derived fibroblasts and ectoderm-derived glia are able to be reprogrammed directly into neurons by inter-lineage and intra-lineage conversion which, in some cases, involve single transcription factors (Ambasudhan et al., 2011, Caiazzo et al., 2011, Kim et al., 2011, Ladewig et al., 2012, Liu et al., 2012, Liu et al., 2013, Meng et al., 2012, Pang et al., 2011,Pfisterer et al., 2011, Qiang et al., 2011, Son et al., 2011, Torper et al., 2013, Vierbuchen et al., 2010 and Yoo et al., 2011). Intra-lineage cell fate us controlled by a limited number of master switch transcription factors that control the expression of a set of cell type specific proteins.

Here we demonstrate that endogenous glial substrates can be directly reprogrammed into neurons via transcription factor-mediated conversion in vitro and in an in vivo model of ischemic stroke. Astrocytes dissected from mouse pups can be cultured in vitro, activated into GFAP-containing reactive astrocytes with the TLR4 activator bacterial lipopolysaccharide (LPS), and directly reprogrammed into neurons. In vivo, ectopic expression of the transcription

factor NeuroD1 can also redirect reactive astrocytes into neurons. Efficient targeting and delivery of NeuroD1 to these astroglia can be achieved in a temporally specific manner using a lentiviral vector with the potential to decrease scar formation, while replacing lost neurons.

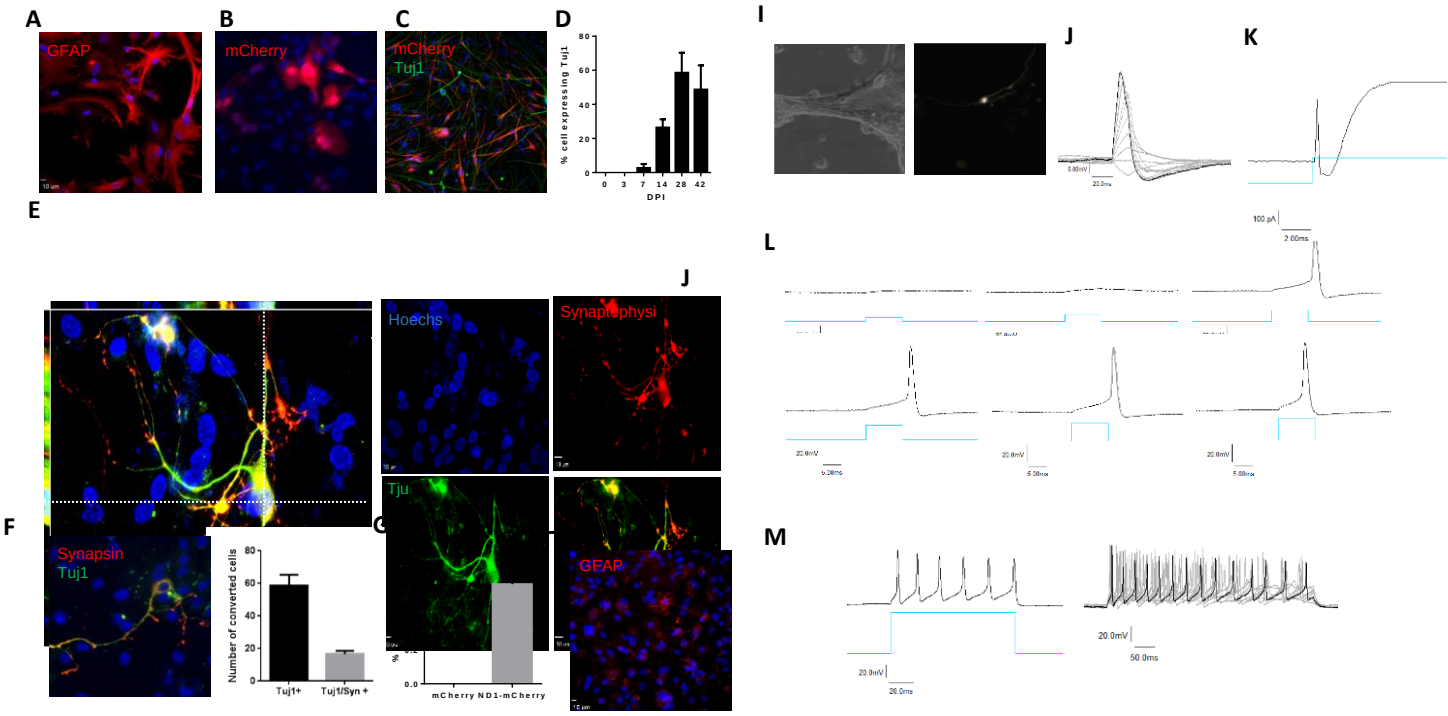
Reprogrammed neurons in the peri-infarct area fulfill two main roles in promoting functional recovery. Firstly, they can passively act in a similar fashion as transplanted stem cells by supporting surrounding cells with paracrine survival factors such as BDNF (J. Chen et al., 2001; L.-R. Zhao et al., 2002). Secondly, these neurons can also play an active role in functional synaptic network reorganization by extending neurites and axons to surrounding brain tissue (Bliss et al., 2007). Previous neural progenitor cell (NPC) transplantation studies in our lab of the somatosensory cortex have shown that NPC projections are random and unordered without stimulation of afferent inputs to the cortex (in press). However, activation of sensory inputs in the form of physical whisker stimulation may direct the development of reprogrammed neurons in vivo. We applied the same stimulation principles in the present study and determined if the stimulation increased survival of reprogrammed neurons and improved functional recovery.

6.2 Results

in vitro direct reprogramming

Dissected astrocytes (from P1 mouse pups) were plated in vitro in DMEM + FBS over a period of 10 days with four days of shaking to remove microglia and neurons. By the end of this regime, GFAP expression was induced uniformly

across the culture by the addition of lipopolysaccharide (LPS) (Figure 1A). Using this method, we were able to confirm that 98.43% of the cells were positive for GFAP at this time (Figure 1A). Expression of GFAP in these astrocytes was critical to the expression of the lentiviral vector we applied as the expression of NeuroD1 was contained a GFAP promoter. Viral titer of GFAP-ND1 tested using a 10^7 dilution yielded approximately 92% infection translating to a titer of approximately $4.8x10^7$ units/uL. After lentiviral infection, successfully infected cells could be monitored over time by the co-expression of a fluorescent mCherry reporter (Figure 1B). Infected cells begin to adopt neuronal features as soon as 2 weeks following infection including the extension of one or more long processes that are uncharacteristic of astrocytes (Figure 1C). Staining using the immature neuronal market Tuj1 revealed that the majority of these cells were unipolar or bipolar (Figure 1C). By 4 weeks, 58.6% of astrocytes were positive for the immature neuronal marker Tuj1 (Figure 1D). Cells infected by an empty vector without NeuroD1 were not observed to develop these processes (Figure 1H). An additional observed effect were that ND1 infected astrocyte cultures did not proliferate at the same rate as control mCherry virus-infected cultures (Figure 2B). As it is characteristic for neurons to exit the cell cycle, it should be expected that at any given time after infection, cellular density is lower in NeuroD1-infected cultures compared to controls. Indeed we observed that by 4 weeks after infection, average cell counts in direct reprogramming cultures were significantly lower than mCherry-infected cultures (Figure 2B).



In vitro direct reprograming of astrocytes to functional neurons using NeuroD1 7 weeks post infection.

(A) P1 mouse astrocytes cultured for 10 days in vitro with GFAP expression induced in 98.43% of cells by the addition of lipopolysaccharide.

(B) GFAP-NeuroD1-IRES-ubi-mCherry cells (red) indicating successful translation of reporter gene in a portion of cells in vitro 7 days post infection.

(C) At14 DPI, cells express the neuronal lineage marker Tuj1 (green) as well as adopt either a unipolar or bipolar morphology that is uncharacteristic of astrocytes.

(D) Timecourse of expression of the neuronal lineage marker Tuj1 in infected astrocytes over time. Expression of Tju1 increases over time and decrease as cells mature.

(E) Confocal microscopy of cells 6 weeks post infection that begin to express complex processes that are positive for synaptophysin.

(F) Astrocytes undergoing direct reprogramming extend processes throughout cell culture and develop synapsin positive terminals throughout. At 6 weeks post infection one third of Tuj1 positive cells also expressed synapsin.

(G) At 6 weeks post infection, 58.7% of cells expressing the mCherry reporter also express neuronal proteins.

(H) Infection by a negative control does not result in the production of any Tuj1 positive cells. Furthermore, no alterations to the morphology of the astrocytes were observed.

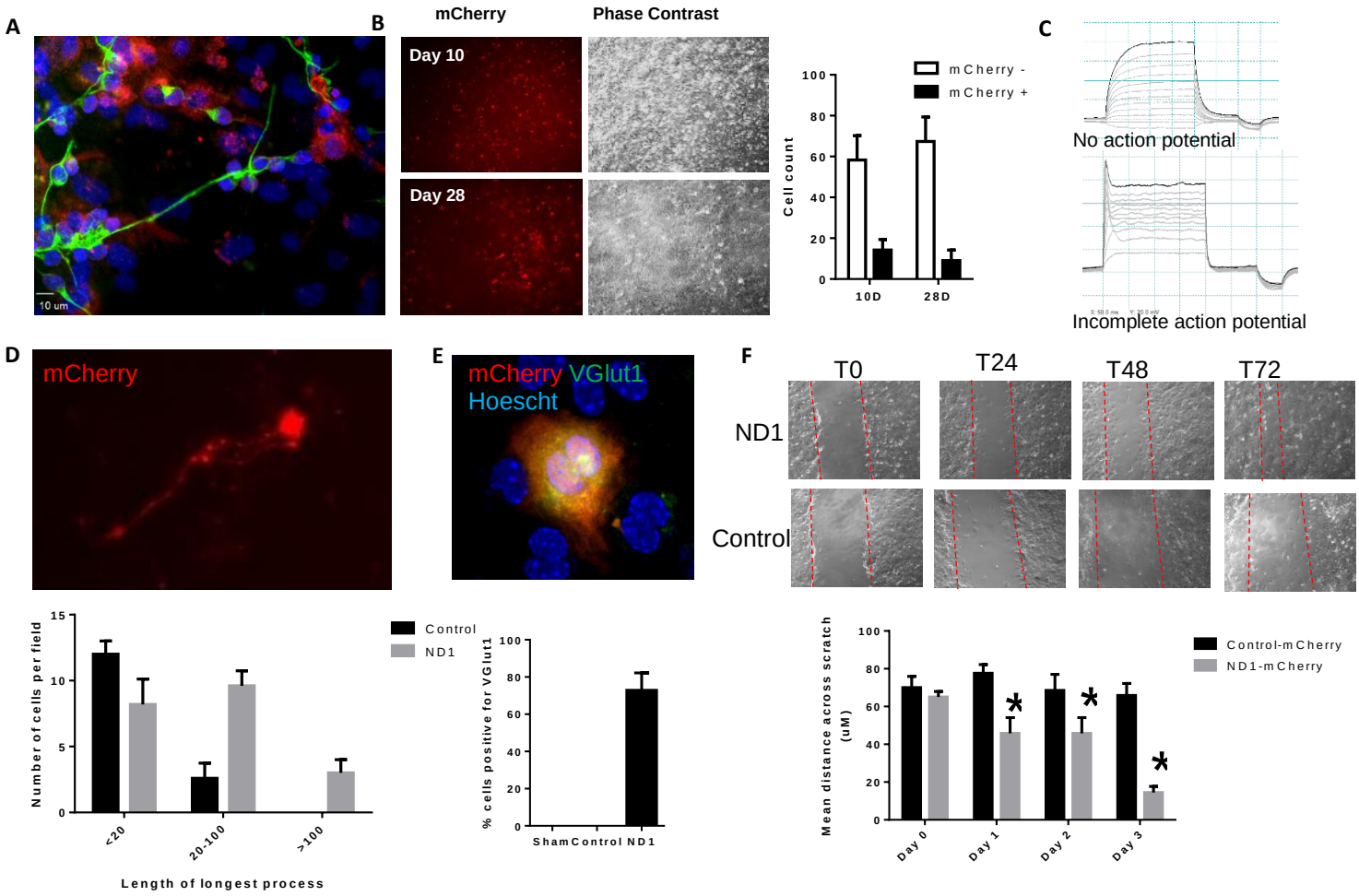
(I) Infected cells could be patched directly in vitro by the presence of a fluorescent reporter gene.

(J) Directly reprogrammed neurons exhibited the ability to fire standard action potentials including hyperpolarization.

(K) Current clamp recordings indicate large inward and outward currents using artificial CSF.

(L) Reprogrammed cell action potentials exhibit voltage dependent threshold effects.

(M) Reprogrammed cells are capable of sustained firing of chains of action potentials without exhaustion. (Figure 1 J-K technical support provided by Dr. Ken Berglund, n=3)



In vitro direct reprogramming of astrocytes to neurons is a continuous process

(A) Premature neurons and infected astrocytes can be observed at intermediate stages throughout the reprogramming process. These cells often feature either positive staining for mCherry (red), Tuj1 (green), or both.

(B) Expression of NeuroD1 in astrocyte cell cultures significantly halts the rate of cell division in astrocytes expressing the mCherry reporter but not uninfected astrocytes.

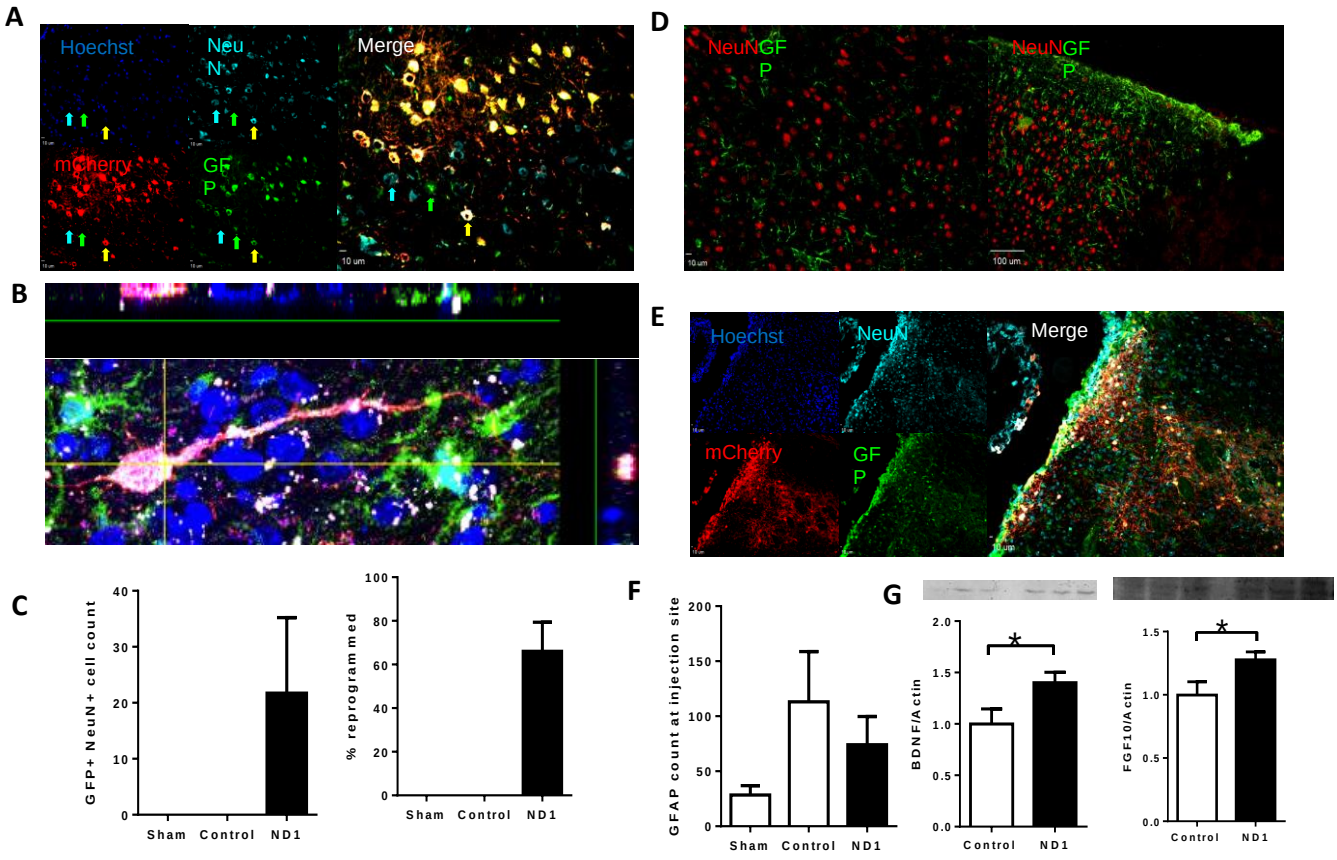
(C) Single cell patch clamp of infected astrocytes indicates that intermediate electrophysiological phenotypes can be observed throughout the reprogramming period including the absence of and presence of incomplete action potentials (technical support provided by Dr. Ken Berglund).

(D) 6 weeks post infection, reprogrammed cells extend longer processes (>20uM) significantly more frequently than cells infected by a control lentivirus

(E) Majority of reprogrammed cells in vitro are positive for vGlut1 but not GAD67 (data not shown).

(F) Astrocyte cell cultures challenged by the scratch test recover significantly faster when infected by NeuroD1 but not an mCherry control.

NeuroD1 directly reprograms astrocytes into mature neurons in vitro 7 weeks post infection.



Direct reprogramming of astrocytes to neurons in vivo using NeuroD1 lentivirus.

(A) 6 weeks post infection of cortical astrocytes in GFAP-Cre x Rosa-YFP mice using NeuroD1-mCherry. Infected GFP positive astrocytes are express the mature neuronal marker NeuN and adopt a neuronal phenotype. Green arrow indicates GFP positive astrocyte which has not been infected and consequently does not express NeuN. Teal arrows indicate existing mature NeuN positive neurons that are neither expressing mCherry nor GFP. Yellow arrows indicate successfully infected astrocytes which now express both mCherry and NeuN.

(B) Confocal microscopy indicates reprogrammed cells express GFP, mCherry, Hoescht, and NeuN (1000x).

(C) At the injection location, a lentiviral titer of 4.8^107 units/mL infects approximately 192,000 cells. This translated to approximately 22 cells per field and at the 6 week time point, 66% of infected neurons express the mature neuronal marker NeuN (n=8 per group, $p<0.05$).

(D) Injection of an empty vector results in segregation of GFP and NeuN immunohistochemistry. GFP+ astrocytes maintain a glial morphology and do not adopt a neuronal phenotype.

(E) Sub-cortical astrocytes in GFAP-Cre x Rosa-YFP mice infected at 7 days post stroke by NeuroD1-mCherry. These infected cells were found in the same location 6 weeks later also expressing the mature neuronal marker NeuN. Reprogrammed neurons infected near the SVZ were observed at a maximum distance of 300uM from the injection site and do not migrate to the peri-infarcted region.

(F) Reprogrammed astrocytes in the peri-infarcted zone are not significantly reduced upon NeuroD1-mCherry infection. The total number of infected astrocytes comprises approximately 10% of total astrocytes in the medial peri-infarcted zone.

(G) Western blot assay for growth factors in the peri-infarcted region following stoke in control vs ND1 animals indicates significantly higher levels of both

BNDF and FGF10 with direct reprogramming but not with an empty vector.

In order to confirm the rate of maturity of in vitro reprogramming of astrocytes to neurons, we co-infected GFAP-NeuroD1 with a Synapsin-GFP reporter. We monitored the cells and found that GFP expression could be induced as soon as 14 days post infection. We could also at the same time observed infected cells expressing mCherry but not GFP implying that the rate of reprogramming for infected cells may vary greatly with some cells maturing at different times.

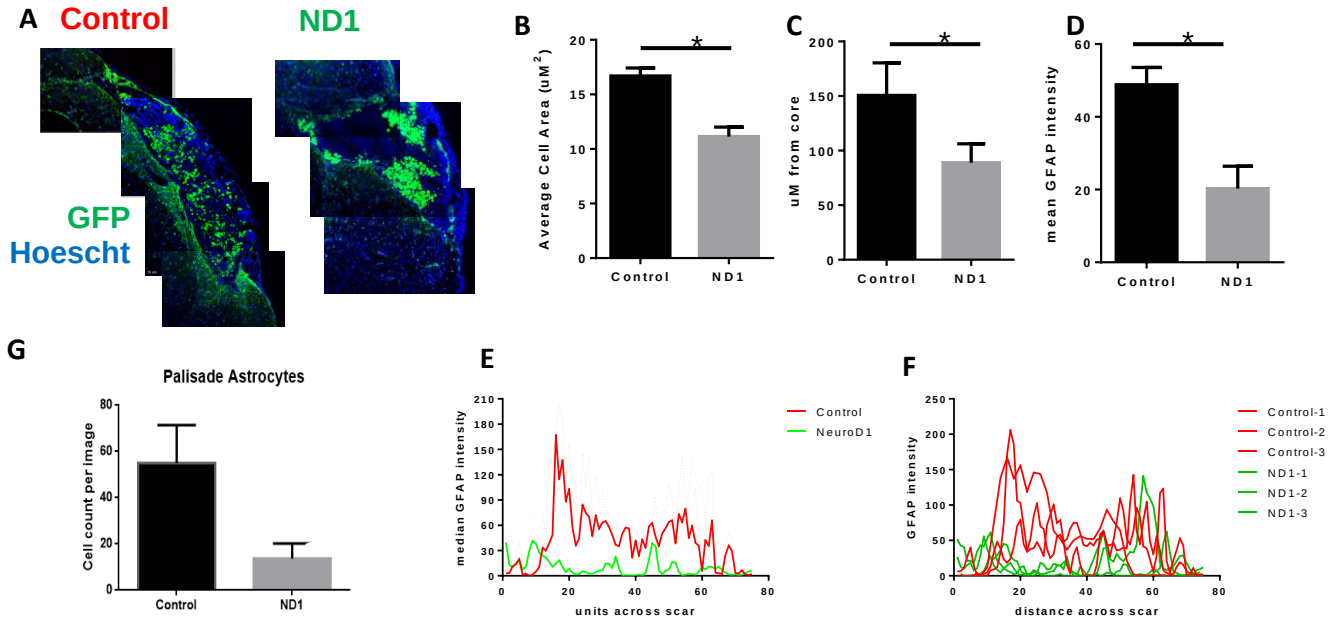
To determine if these reprogrammed astrocytes in vitro expressed functional activity, we applied whole cell patch clamp to elicit electrophysiological activity. NeuroD1 reprogrammed neurons were able to fire both single as well as repeated action potentials by 7 weeks post infection. We also probed these cells for sodium and potassium currents and found evidence of both. Before 7 weeks, patched cells exhibited sodium currents and partial action potentials (Figure 2C).

Animal model of direct conversion Cre-lox system

Astrocytes from GFAP-Cre-Rosa-YFP mice remain YFP positive regardless of conversion fate. To confirm direct conversion of astrocytes to neurons in vivo, we make use of this property to track the conversation fate of astrocytes in the peri-infarcted region following focal barrel ischemic stroke. Virus infected astrocytes express mCherry and only express ND1 so long as GFAP is also expressed. We then used immunohistochemistry to identify neurons using the mature neuronal maker NeuN. Six weeks after injection of GFAP-ND1 lentivirus

to the peri-infarct region, we identified cells labelled for YFP (previously an astrocyte), mCherry (infected by GFAP-ND1), and NeuN (mature neuron) (Figure 3A). The combination of these three markers in a single cell indicates successful viral-mediated reprogramming of endogenous astrocytes to neurons. Co-labeling was confirmed using confocal microscopy and we observed triple labelled neurons in all animals examined (Figure 3B). When cut at the right plane, some converted neurons are observed adopt a pyramidal neuronal phenotype with a single axon and dendritic arbors (Figure 3B).

Direct reprogramming of astrocytes alters gliosis following ischemic stroke.



Direct reprogramming of astrocytes alters gliosis following ischemic stroke.

A) Glial scarring 6 weeks after infection (7 weeks following ischemia) in mice with control mCherry or NeuroD1-mCherry lentivirus administration. Immunohistochemistry using GFAP and Hoechst.

B) Astrocyte hypertrophy measured by total cell area in cross section within the glial scar. Mean control astrocyte cross section averaged from 20 cells was 16.70 uM and mean NeuroD1 was 11.14 uM (n=6 per group, p=0.0011, 20 cells per animal). This indicates greater astrocyte hypertrophy in controls compared to ND1.

C) Average glial scar depth in uM measured by GFAP immunoreactivity from ischemic core to boundary of GFAP reactivity (n=6 per group p=0.0359).

D) Mean GFAP intensity across entire cross section of scar. Gliosis is significantly more visible in control compared to ND1.

E) Gliosis profile analysis by mean gray value across scar transection (n=3 per group). Graph plots median value and range.

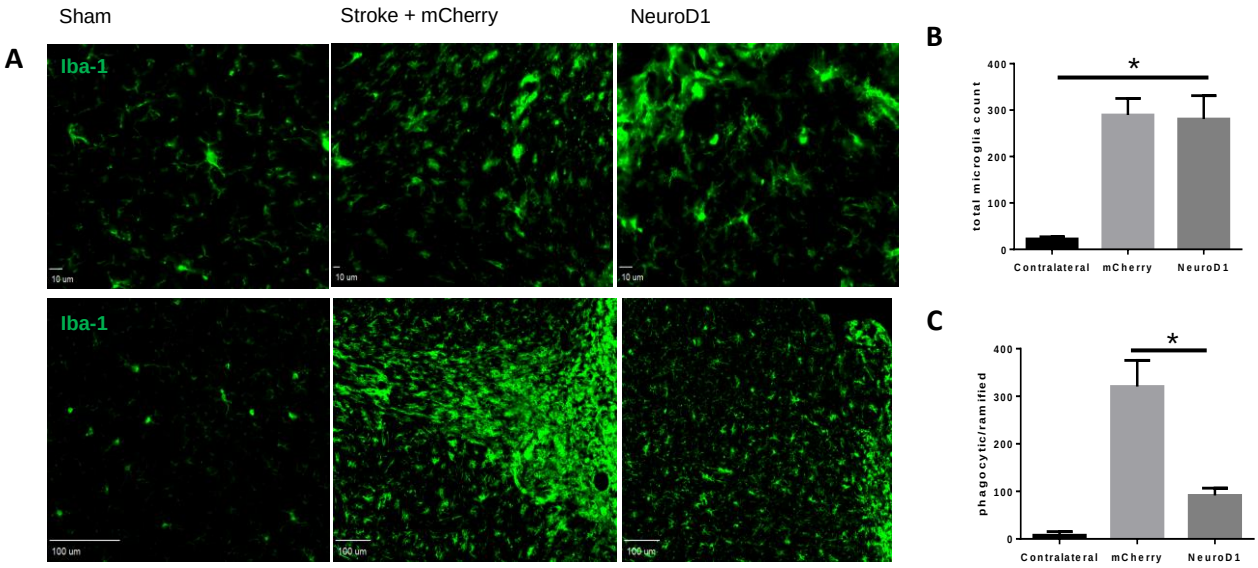
F) Individual scar profiles plotted separately. G. Direct reprogramming reduces number of scar forming palisade astrocytes in the gilal scar.

Gliosis is a well characterized response to ischemic injury in the brain. Immediately after injury both astrocytes and microglia transition from a resting to an active/reactive state. We observe robust astrogliosis involving the migration of astrocytes to the peri-infarcted tissue contiguous with the area of massive cell loss known as the ischemic core. We quantified astrogliosis by quantifying both

the number of reactive, hypertrophied astrocytes migrating to the ischemic injury and the relative thickness of the glial scar formed 6 weeks after stroke with and without direct conversion. When ND1 is introduced to migrating astrocytes just 3 days after ischemia, GFAP reactivity in the peri-infarcted tissue is significantly reduced. Likewise, the relative size and thickness of the glial scar is also ameliorated. Both of these metrics indicate that GFAP-ND1 interferes with astrocytes and alters their fate towards gliosis. These data also suggest that there is no endogenous compensatory reaction by astrocytes to counter the effect of GFAP-ND1.

Reduction in the severity of the relatively mature glial scar at 6 weeks after injury allowed for directly converted cells to extend axons measuring up to 120 um in close proximity to a normally inhibitive environment for axonogenesis.

Activation of microglia after stroke and direct programming.



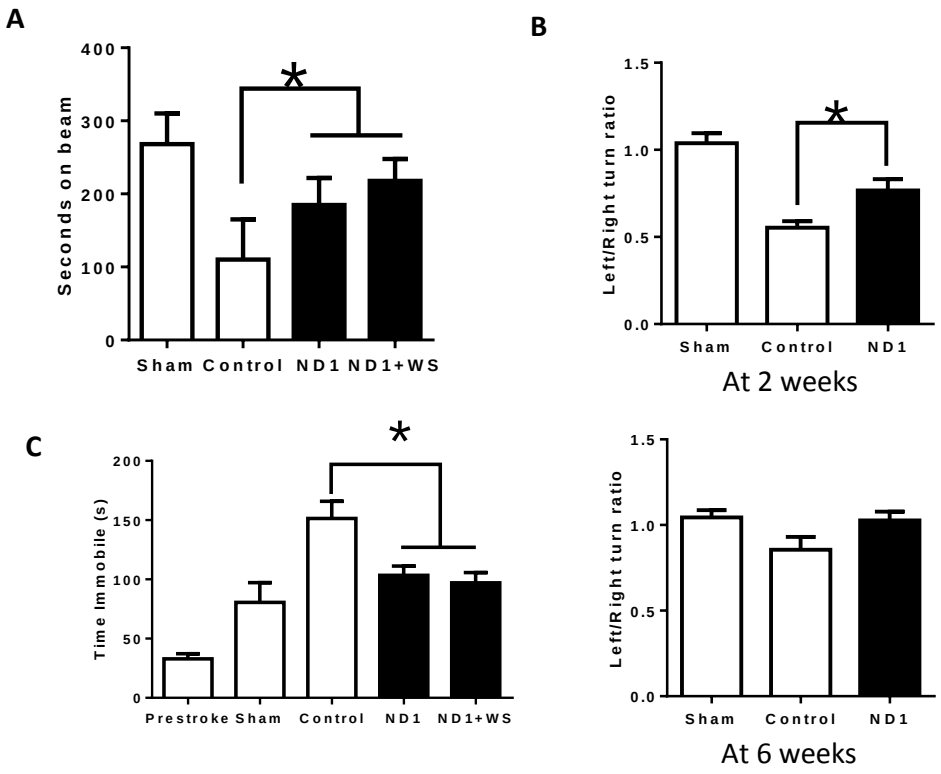
Activation of microglia after stroke and direct programming. IHC using Iba-1 6 weeks post infection of CRE dependent GFAP-YFP (GFAP-Cre x Rosa-YFP) mice using NeuroD1-mCherry.

A) Top: high magnification, bottom: low magnification: after ischemia, microglia are present in significantly greater numbers in the ipsilateral cortex compared to contralateral or non/ischemic brain. Additionally there appears to be milder glial scar formation with NeuroD1 administration.

B) The total number of microglia present in the stroke cortex is significantly greater than in cortex unaffected by stroke. No significant differences observed in the number of microglia between mCherry control virus injections and NeuroD1 injection ($p<0.05$, $n=5$ per group).

C) While the number of microglia did not significantly differ, the ratio of phagocytic:ramified microglia was significantly reduced by NeuroD1 administration measured at the penumbra ($p<0.05$, $n=5$ per group).

Functional recovery and activity dependent repair



Functional recovery after direct reprogramming of astrocytes to neurons in vivo.

(A) 3 weeks after direct conversion and ministroke, ND1 injected mice exhibit significantly longer time balancing on the Rotarod beam but whisker stimulation did not increase time spent balance on beam significantly compared to ND1 alone at a later time point in the same animals.

(B) Corner test turning results show significant increase in right turning preference in stroke animals administered a control compared to NeuroD1. This difference was not observed 6 weeks following treatment.

(C) 4 months after ministroke, GFAP-YFP mice exhibit significantly longer time idle time in forced swim test, potentially indicative of long lasting post stroke depressing outcomes. These are reversed in animals given whisker stimulation

Direct conversion of astroctyes to immature neurons in vitro

GFAP-ND1 expression is reliant on astrocytes with positive immunoreactivity to GFAP indicating a reactive state. We extracted astrocytes from p0 and p5 mice pups. To reliably infect these cells, we applied lipopolysaccharide after plating which induces astrocytes to enter a reactive GFAP positive state. To confirm this, we measured the number of activated astrocytes by immunocytochemistry staining of GFAP. The number of GFAP positive cells was greater than 95% indicating a relatively pure starting culture. Viral titer of GFAP-ND1 tested using a 10^-8 dilution yielded approximately 92% infection translating to a titer of approximately 1.26x10^8 units/uL. An additional observed effect was that ND1 infected astrocyte cultures did not proliferate at the same rate as control mCherry virus infected cultures. As direct reprogramming forces astrocytes to exit the cell cycle, it should be expected that at any given time after infection, cellular density is lower when compare to controls. Indeed we observed that by 4 weeks after infection, average cell counts in direct reprogramming cultures were significantly lower than mCherry infected cultures.

Mouse astrocytes were infected at 7 days after dissection and followed for up to 8 weeks to examine the rate and efficiency of direct conversion. By 2 weeks, 60.5% of astrocytes are positive for the immature neuronal marker Tuj1. By 4 weeks, we observed a subset of astrocytes expressing mature neuronal markers NeuN and Map2.

Synapsin GFP reporter in vitro

We co-infected in vitro astrocytes with the direct conversion reporter Synapsin-GFP. We first observed GFP expression indicating synapsin expression in astrocytes at 28 days after infection (35 days after dissection). After patching these GFP positive cells, we did not observe any action potentials. In direct conversion cell cultures without the synapsin-GFP reporter we patched directly reprogrammed astrocytes using morphological features observed using a microscope and found some sodium channel activity at 4 and 6 weeks but we were unable to elicit any action potentials at 2,4,6, and 8 weeks after the application of ND1 virus.

Direct conversion reduces the glial scar formation 6 weeks after injury

Gliosis is a well characterized response to ischemic injury in the brain. Immediately after injury both astrocytes and microglia transition from a resting to an active/reactive state. We observe robust astrogliosis involving the migration of astrocytes to the peri-infarcted tissue contiguous with the area of massive cell loss known as the ischemic core (Figure 4A). We quantified astrogliosis by quantifying both the number of reactive, hypertrophied astrocytes migrating to

the ischemic injury and the relative thickness of the glial scar formed 6 weeks after stroke with and without direct conversion (Figure 4B-D). When ND1 is introduced to migrating astrocytes just 3 days after ischemia, GFAP reactivity in the peri-infarcted tissue is significantly reduced (Figure 4D). Likewise, the relative size and thickness of the glial scar is also ameliorated (Figure 4B). These metrics indicate that GFAP-ND1 interferes with astrocytes and alters their fate with respect to gliosis. These data also suggest that endogenous compensatory reactions by astrocytes to counter the effect of GFAP-ND1 cannot be observed within 6 weeks of infection.

Reduction in the severity of the relatively mature glial scar at 6 weeks after injury allowed for directly converted cells to extend axons measuring up to 120 um in close proximity to a normally inhibitive environment for axonogenesis (Figure 3B).

Tracking Direct Conversion of Astrocytes to Neurons in vivo

Astrocytes from GFAP-Cre-Rosa-YFP mice remain YFP positive regardless of conversion fate. To confirm direct conversion in vivo of astrocytes to neurons, we make use of this property to track the conversation fate of astrocytes in the peri-infarcted region following focal barrel ischemic stroke. Virus infected astrocytes express mCherry and only express ND1 so long as GFAP is also expressed. We then used immunohistochemistry to identify neurons using the mature neuronal maker NeuN. 6 weeks after injection of GFAP-ND1 lentivirus to the peri-infarct region, we identified cells labelled positive for YFP (previously an astrocyte),

mCherry (infected by GFAP-ND1), and NeuN (mature neuron). The combination of these three markers in a single cell indicates successful viral mediated reprogramming of endogenous astrocytes to neurons. Colabeling was confirmed using confocal microscopy and we observed triple labelled neurons in all animals examined. When cut at the right plane, some converted neurons are observed adopt a pyramidal neuronal phenotype with a single axon and dendritic arbors. These cells also express synaptic markers synapsin indicating potential to form new local neuronal circuits (data incoming).

Direct conversion reduces activation and fate of microglia after stroke

After ischemic stroke, microglia transition from a quiescent state to a reactive, hypertrophied state and eventually adopt a phagocytic phenotype whereby processes retract and the cells become round. We quantified both the number and reactive state of microglia following ischemic stroke in the peri-infarcted tissue at 6 weeks following GFAP-ND1 infection. While the number of microglia did not significantly differ, their phenotype was significantly altered in favor of less hypertrophy and phagocytic stage.

Direct reprogramming improves functional outcomes in mice after stroke

Our stroke model induces specific motor and sensorimotor deficits and we used a combination of the rotarod and corner tests to compare treatment groups. We also provided whisker stimulation on a daily basis as an additional treatment to provide afferent input to the direct reprogramming injection location. 3 weeks after stroke, ND1 injected mice exhibit significantly longer time balancing on the

Rotarod beam but whisker stimulation did not increase time spent balance on beam significantly compared to ND1 alone at a later time point in the same animals (Figure 6A). Corner test turning results show significant increase in right turning preference in stroke animals administered a control compared to NeuroD1 at 2 weeks but not 6 weeks after stroke (Figure 6B). Examination of behavior at 4 months after ministroke, GFAP-YFP mice exhibit significantly longer time idle time in forced swim test, potentially indicative of long lasting post stroke depression outcomes. This phenotype was rescued in animals given NeuroD1 (Figure 6C).

6.3 Discussion

Ischemic stroke is a debilitating disease with a chronic phase characterized by permanent loss of cognitive and behavioral function resulting from the loss of neurons. Host regenerative abilities are insufficient to recover these functions and therapeutic approaches to restoring the lost tissues are an ongoing basic science and clinical endeavor. In this study we present an alternative approach that utilizes the robust regenerative and proliferative phenotype of reactive astrocytes as a cellular substrate for the generation of neurons both in vitro and in vivo.

Part of chronic stroke pathophysiology involves the formation of a glial scar comprised primarily of hypertrophied astrocytes that are known to exert an antagonistic effect on the extension of neurite outgrowth. Direct reprogramming of the glial scar reactive astrocytes to neurons in stroke provides a multifaceted

strategy for functional recovery after stroke. We demonstrate that ectopic expression of the transcription factor NeuroD1 is able to directly reprogram reactive GFAP-positive astrocytes into functional neurons in vitro. With the use of a lentiviral vector, we also demonstrate the ability of NeuroD1 to reprogram reactive astrocytes in the peri-infarcted region following ischemic stroke in vivo. Redirecting the fate of astrocytes in the peri-infarcted region is able to alter the characteristics of glial scar formation as well as the phenotype of microglia. Finally, we also observed significant improvements in the functional recovery of animals administered NeuroD1 suggesting the replacement of neurons in the host cyto-architecture play a role in the functional recovery of animals from ischemic stroke.

Reprogramming reactive astrocytes after ischemic stroke

Ischemic stroke remains a challenging disease with limited acute and chronic treatments available for patients. In the chronic phase, stroke patients suffer long term disability and cognitive impairment. This is due to the degeneration of neuronal cell populations which have very limited regenerative capacity in adult mammals. Gliosis involving the hypertrophy and proliferation of astrocytes to form a glial scar in the peri-infarcted region represent a plentiful cellular substrate for direct reprogramming into neurons. Redirecting astrocyte cell fate to neurons using NeuroD1 provides a multifaceted approach to cell replacement.

Neurogenesis in the adult mammalian brain is limited to just two specific niches, the SVZ and the SDG. However, this regenerative capacity is not sufficient

to ameliorate or repair the damage caused by ischemic stroke. Therapeutic approaches to treatment of chronic stroke utilized several strategies to cell replacement through the use of stem cells. Direct reprogramming of the host's own astrocytes provides cell replacement that is autologous. Furthermore, these cells are already integrated into the brain's extracellular matrix. Neurons are also post mitotic and do not have concerns regarding tumorigenesis that comes with certain types of stem cells.

Using astrocytes as a substrate for direct reprogramming is challenging to study in the context of basic research as tracking and characterizing the resulting neurons can be hard to distinguish from native neurons. To this end we employed the use of a cre lox system to permanently switch on the expression of a fluorescent protein so that astrocytes could be followed throughout the reprogramming process. We could not depend on promoter based expression of a tracking element as astrocytes and neurons are segregated cell populations. Proteins that are specific to astrocytes are not expressed in neurons and likewise. Thus cell specific reporters would be unable to be observed once cells had reprogrammed into their target cell type.

Direct reprogramming of astrocytes alters stroke pathophysiology

Astrocytes play a complex role in stroke pathophysiology (Sofroniew, 2009). It is currently thought that in the subacute and acute phases, astrocytes play an important role in limiting damage to surrounding healthy tissues(Nowicka, Rogozinska, Aleksy, Witte, & Skangiel-Kramska, 2008). It would therefore be detrimental to redirect the cell fate of astrocytes in this phase of stroke. In the

chronic phase of stroke, the glial scar which is comprised primarily of astrocytes forms and acts as a barrier to regeneration by secreting proteoglycan factors that inhibit neurite outgrowth(Huang et al., 2014; Karimi-Abdolrezaee & Billakanti, 2012). We examined the process of scar formation to time our reprogramming experiments to intercept astrocytes after the acute phase of stroke but before a mature glial scar could form(Jiang et al., 2017). In this way, we were able to redirect a portion of reactive astrocytes away from a scar cell fate and towards a neuronal cell fate instead. Our intervention altered the profile of scar formation in the chronic phase in mice following stroke. The reprogrammed cells in the peri-infarcted region are able to extend neurites and can be tracked by the expression of cre-lox dependent YFP. We also observed fewer phagocytic microglia implying that direct reprogramming can alter the function of microglia near the site of injury. Importantly we found that direct reprogramming of astrocytes to neurons after ischemic stroke .

Conclusions

Direct reprogramming of astrocytes to neurons is a viable and efficient means of cell replacement therapy(Guo et al., 2014). It appears to be an attractive therapeutic approach in the context of ischemic stroke which currently has limited available treatment options in the chronic phase and is associated with permanent long term cognitive and physical impairment. Replacing lost brain via direct reprogramming that utilizes an enriched cellular substrate following injury provides an autologous source of cells already integrated into the host tissue extracellular matrix. Direct reprogramming also alters the pathophysiology of

stroke in potentially favorable ways by redirecting the fate of glial scar bound astrocytes and altering microglia phenotypes.

Our study provides evidence for the adoption of direct reprogramming of tissues in the peri-infarction as a substrate for cell replacement for ischemic stroke. Works remains to optimize this approach and further elucidate the complex mechanisms and interplay between direct reprogramming and stroke pathophysiology. We also provide evidence that direct reprogramming of astrocytes can rescue behavioral deficits but work remains to be done regarding additional functional outcomes of stroke involving motor coordination, sensory ability, and cognitive ability. Altering cell fate by the administration of custom transcriptional factors is emerging as a therapeutic approach with promise of a multifaceted means to ameliorate the burden of neurodegenerative disease.

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