

**Preconditioning of Human Neural Stem Cells with Metformin to Promote
Post-Stroke Recovery**

Fares Ould-Brahim

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Department of Cellular and Molecular Medicine, Neuroscience
Faculty of Medicine
University of Ottawa

Abstract

The generation of human induced pluripotent stem cells (hiPSCs) from human fibroblasts has revolutionized cell therapy by providing a source of autologous cells for transplantation. Several studies have demonstrated that transplantation of hiPSC-derived neural stem cells (hiPSC-NSCs) increases regeneration and recovery following stroke, supporting their therapeutic potential. However, major concerns for translating hiPSC transplantation therapy to the clinic are efficacy and safety. Therefore, there is demand to develop an optimal strategy to enhance the engraftment and regenerative capacity of transplanted hiPSC-NSCs. The recent published work shows that metformin, an FDA approved drug, is an optimal neuroregenerative agent that not only promotes the proliferation of neural stem cells but also enhances their neuronal differentiation. In this regard, we hypothesize that preconditioning of hiPSC-NSCs with metformin before transplantation into the stroke-damaged brain will improve engraftment and regenerative capabilities of hiPSC-NSCs, further enhancing cell-mediated functional recovery. Here we show that treatment of hiPSC-NSCs with metformin enhances the proliferation and differentiation of hiPSC-NSCs in culture even after withdrawal of metformin treatment, showing its promise as a novel preconditioning strategy. Furthermore, transplantation of preconditioned hiPSC-NSCs into a rat endothelin-1 ischemic stroke model showed an improved engraftment capability 1-week post-transplant. In addition, metformin preconditioned grafts survived longer compared to naïve grafts and were detectable at 8 weeks post-stroke. However, cell transplantation did not result in improve functional recovery when compared to sham group in this model. These studies represent a vital step in the optimization of hiPSC-NSC based transplantation to promote post-stroke recovery.

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List of Abbreviations

2-AG	2-Arachidonoylglycerol
AMD	Age-related macular degeneration
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP	Anterior-Posterior
aPKC	Atypical protein kinase C
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BMSC	Bone marrow derived stromal cell
BrdU	Bromodeoxyuridine (5-bromo-2'-deoxyuridine)
BSA	Bovine serum albumin
CAMKK β	Calmodulin-dependent protein kinase kinase beta
CBP	CREB Binding protein
c-Myc	avian myelocytomatosis viral oncogene homologue
CREB	Cyclic AMP response element-binding protein
CsA	Cyclosporin A
DARP-32	Dopamine- and cAMP-regulated neuronal phosphoprotein
DCX	Doublecortin
DMEM	Dulbecco's Modified Eagle's Medium
DV	Dorsal-Ventral
EB	Embryoid body
EDTA	Ethylenediaminetetraacetic acid
ET-1	Endothelin-1
GABA	Gamma-Aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
H2B	Histone 2B
HEY2	Hairy/enhancer-of-split related with YRPW motif protein 2
hiPSC	human induced pluripotent stem cell
hiPSC-NSC	Human induced pluripotent stem cell-derived neural stem cell
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
IL-6	Interleukin 6
iNOS	Nitric Oxide Synthase
Klf4	Kruppel-like factor 4
LIF	Leukemia inhibitory factor
LKB1	Liver Kinase B1
MAP2	Microtubule-associated protein 2
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion
MglI	Monoacylglycerol lipase
ML	Medial-Lateral
MSC	Mesenchymal stromal cell
NeuN	Neuronal nuclear antigen
NGF	Nerve growth factor
NGS	Normal goat serum
NPC	Neural progenitor cell
NSC	Neural stem cell
OCT1	Organic Cation Transporter 1
Oct-3/4	Octamer-binding transcription factor 3/4
Pax6	Paired box protein pax-6
PBS	Phosphate buffered saline
PD-MSC	Placenta derived mesenchymal stromal cell

PFA	Paraformaldehyde
PGE-2	Prostaglandin E2
PLO	Poly-L-Ornithin
PTX	Picrotoxin
ROCK	Rho-associated protein kinase
SDF-1 α	Stromal cell-derived factor 1
SMAD	Mothers against decapentaplegic homologue
Sox2	(Sex determining region T)-box 2
SVZ	Sub ventricular Zone
TAp73	Transcriptionally active protein 73
TBS-T	Tris buffered saline-tween
TF	Transcription factor
Tie-2	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TMS	Trimethoprim sulfamethoxazole suspension
TORC2	CREB regulated transcription coactivator 2
tPA	Tissue Plasminogen activator
TSG-6	Tumour necrosis factor stimulated gene 6 protein
UCBC	Umbilical cord Blood cell
VEGF	Vascular endothelial growth factor

1.0 Introduction

1.1 Ischemic Stroke and how it affects Canadians

Approximately 400,000 Canadians suffer from the long-term deficits of stroke and the number of Canadians living with stroke is expected to double by 2038¹. A majority of strokes are caused from a blockage in cerebral blood flow resulting in death of distal brain tissue, termed ischemic strokes. This blockage in cerebral circulation is either characterized as a thrombus, a locally derived blockage, or an embolism, a blockage formed in other vessels in the body that traveled through the blood stream and lodged in a cerebral vessel. Risk factors for ischemic stroke typically contribute to the progression or formation of an embolism or thrombus. These factors include a poor diet, physical inactivity, obesity, hypertension, diabetes mellitus, coronary artery disease, smoking, and excessive alcohol consumption². Brain tissue is highly dependent on oxygen and other nutrients and quickly dies when blood flow is restricted forming a necrotic area termed the infarct core. Adjacent tissue that receives blood supply from both the affected and unaffected circulation, termed the ischemic penumbra, becomes hypoperfused and is especially susceptible to death due to oxidative stress, inflammatory response, and excitotoxicity if sufficient blood flow is not restored³. Rapid treatment of ischemic stroke has thus been encouraged in an effort to minimize expansion of the ischemic core into the penumbra. The Canadian Heart and Stroke Foundation has focused its campaign on awareness and recognition of stroke through the acronym F.A.S.T., Face Arms Speech Time, identifying acute signs and highlighting the importance of fast treatment. Despite this awareness campaign, 43% of Canadians don't know any of the F.A.S.T. signs and only 4.4% of Canadians can name the three hallmark signs of stroke: drooping face, weak arm and slurred or jumbled speech⁴. Furthermore, of patients severe enough to be admitted to hospital, only 67% of patients arrive by ambulance when experiencing a stroke. This

suggests that one third of Canadians arrive by other means including non-urgent transport, thus reducing their odds of a full recovery. At this stage, acute interventions focus on restoring blood flow by a thrombolytic (i.e. tissue plasminogen activator, t-PA) or by removal of the embolism/thrombus by thrombectomy. Despite the rapid onset, 80% of stroke patients survive acute injury but suffer from chronic disability making stroke the leading cause of disability in North America¹. Chronic impairments vary on location and size of stroke but often include motor deficits such as partial or total paralysis and loss of feeling. Cognitive impairments including depression, aphasia, dementia, anxiety, fatigue, and adjustment disorder⁵. In fact, stroke accounts for one third of dementia risk with one third of Canadians developing dementia after having a second stroke⁶. Furthermore, patients that develop dementia with stroke have higher mortality rates and have a higher need for long term and complex care. As such, long-term treatment through physical rehabilitation, counselling, and speech therapy become a reality for many patients and cost Canadians \$2.8 billion in initial treatment of stroke, not accounting for treatment of conditions that stroke may have triggered, including dementia⁷. To effectively treat stroke, emerging treatments must be able to reduce long term disability, potentially contribute to cognitive recovery, yet be practically and financially feasible.

1.2 Stroke Intervention and Treatments

Ischemic stroke treatments can be categorized into two main branches: acute and chronic, both with different goals. Acute ischemic stroke treatments focus on stabilizing the penumbra and limiting the infarct expansion. These treatments are typically not effective after the first 8 hours of symptom onset. As such, acute treatments are thought to be a “damage control” approach and limited in their ability to ameliorate initial stroke damage. Some examples include: thrombectomy, t-PA, neuroprotective agents and hypothermia^{8–10}. Chronic ischemic

stroke treatments focus on regaining lost physical or cognitive function by stimulating remaining neural circuits through rehabilitation. Newer treatments for post-stroke recovery include a wide range of drug interventions, transcranial magnetic stimulation and cell therapies, which are in currently under investigation.

Mechanical thrombectomy, a form of acute ischemic stroke treatment, is a surgical intervention which requires inserting a guidewire followed by an expandable mesh through a proximal artery, typically through a groin puncture to the occluded blood vessel. The stent is then expanded and pulled to extract the clot. Regular blood flow is halted and aspiration in the vessel is applied to prevent the clot from breaking up and relodging in other distal blood vessels, a rare but potential complication of thrombectomy. Thrombectomy is known for its ability to remove larger clots in the middle cerebral artery (MCA) or distal carotid artery, a well-known barrier for t-PA treatment¹¹. Thrombectomy improves functional independence and degree of disability at 3 months if applied within 7 hours of onset of symptoms and most effective within 2 hours of onset¹². Furthermore, thrombectomy treatment can be used alongside intravenous thrombolysis safely and effectively¹³. Complications include a new embolism from the clot breaking apart, stent occlusion, vasospasms, vessel dissection and stent dislocation which occur in about 11% of treated patients and result in poorer clinical outcomes¹⁴. Despite its limitations, mechanical thrombectomy has its place in acute ischemic stroke and will likely remain a critical intervention for the treatment of ischemic stroke.

Stroke results in a plethora of chronic motor and cognitive impairments some of which require a wide range of treatments. Nearly two thirds of patients suffer from some type of motor impairment following stroke. Indeed, there is compelling evidence to support rehabilitation provided in the subacute phase reduces mortality and improves functional outcomes depending on the severity of the stroke¹⁵. Our understanding of how intense and when rehabilitation is best

is not well defined and heavily depends on the time administered and severity of stroke. For example, in the AVERT trial (Efficacy and safety of very early mobilization within 24h of stroke onset) patients mobilized within the first 24 hours showed reduced odds of favourable outcomes at 3 months compared to those mobilized at later timepoints, highlighting the balance required between stabilizing the patient and getting the most out of early rehabilitation¹⁶. Additional barriers to implementing rehabilitation include accommodating for cognitive deficits following stroke and access to rehabilitation facilities, programs and experts¹⁷. As such, only 16% of patients in inpatient acute care receive rehabilitation immediately and only 19% within the first month after leaving the hospital, which is well below the Canadian Stroke Best Practice Recommendations¹. Rehabilitation is not enough on its own to ameliorate deficits after stroke and its implementation is far more complex than originally speculated. As such, there is a demand for more therapies that can be used in conjunction with rehabilitation.

1.3 Rodent Models of Ischemic Stroke

Animal models of ischemic stroke provide the opportunity to preclinically evaluate novel treatments for stroke. Each animal model has its advantages and drawbacks which must be carefully considered on the basis of key translational issues to be addressed. Although differences observed among various animal models, common features for all animal models must include: 1) similar pathophysiological changes to that of human ischemia, 2) reproducible lesions, 3) simple procedure, 4) accessibility and ease of analysis and physiological monitoring¹⁸. For these reasons, mouse and rat models of ischemic stroke are most typically used.

The photothrombosis model uses a photo sensitive dye, typically Rose Bengal, that is injected intravenously or intra peritoneally into the rodent followed by irradiation through the skull overlying the brain region of interest. Once irradiated, permanent occlusion of

microvasculature causes rapid focal ischemia and tissue death¹⁹. This model benefits from the ability to map the location of the infarct by stereotactic repositioning of the light beam, consistent focal stroke lesions and minimal surgical intervention and surgical time. The photothrombosis model has been criticized for its lack of reperfusion and therefore lack of penumbra, critical for studying neuroprotection^{18,20, 21}. Overall this model's consistency and accuracy makes it suitable for studying the neurogenic response to damage of specific structures and electrophysiology of the peri-infarct region and contralateral hemisphere.

The intraluminal middle cerebral artery occlusion model (MCAo) is the most commonly used animal model of ischemic stroke. This model involves introducing a suture through the middle cerebral artery via the external carotid artery or the common carotid artery, thereby occluding all its distal tissues. Reperfusion can occur once the suture is removed and is most commonly done at 60, 90, 120 minutes or left in permanently²¹. This model resembles the mechanism of human ischemia as it directly occludes the most common stroke occlusion in humans, the MCA, and reperfusion creates a large penumbra region. Furthermore, it requires short surgery times and no craniectomy. This model produces infarct regions mainly damaging the striatum with delayed secondary damage to the dorsolateral cortex. In addition, it also damages the hippocampus, hypothalamus, thalamus and substantia nigra, which could potentially interfere with motor function evaluation²². The secondary damaged tissue has up to a 12 hour therapeutic window not observed in humans²³. Furthermore, other complications in surgery include hyperthermia through damage of the hypothalamus, death in older rats, subarachnoid hemorrhage and external carotid artery derived ischemia to the mastication muscles which also associates with reduced post-stroke neurological scores^{24,25}. Although the MCAo model is relatively non-invasive, it may pose challenges when trying to translate research into the clinic.

The endothelin-1 (ET-1) model of focal ischemia uses the potent vasoconstrictive peptide, endothelin-1, which can be applied to specific brain regions of interest through stereotaxic injections. The endothelin-1 model is known for its low mortality rate, ability to reduce blood flow to 30-50% of normal and allow for gradual reperfusion over 16-22 hours, more closely representing stroke compared to the immediate reperfusion in the MCAo model and lack of reperfusion in the photothrombosis model²⁶. Furthermore, this model can be used in aged rats with low mortality which has been a challenge for studying aged rats with the MCAo model^{27,28}. However, the model is limited by the fact that neurons and astrocytes express ET-1 receptors and ET-1 converting enzymes. ET-1 delivery may induce astrogliosis and facilitates axonal sprouting, which may interfere with the interpretation of neural repair experiments²⁰. In addition, the mechanism of ischemia in this model is vasoconstriction rather than obstruction which limits testing of neuroprotective agents that influence vasculature²⁷. As the half-life of ET-1 is very short, all the aforementioned limitations occur in the relatively acute phase of stroke²⁹. Thus, it makes the ET-1 model ideal for studying functional recovery.

Among others, these models make up the backbone of basic stroke research and critical evaluation of their strengths and weaknesses is not only recommended but essential to translational research.

1.4 Stem Cell Based Therapy and the Optimal Cell Source

Cell based therapies have been extensively researched in the last 15 years as a novel treatment for ischemic stroke. Indeed, there are several studies that show great promise for the transplantation of mesenchymal stromal cells and neural stem cells in rodent models of ischemic stroke.

Multipotent Mesenchymal Stromal Cells (MSC) can be isolated from many tissues, most commonly from Bone Marrow (BMSC), Placenta (PD-MSCs) or umbilical cord (UCBCs). They are defined by the International Society for Cellular Therapy by three criteria: 1) ability to adhere to plastic culture dishes, 2) Expression of CD105, CD73 and CD90 but lack expression of CD45, CD34, CD14 or CD11b, CD78 α or CD19 and HLA-DR, and 3) Potential to differentiate into osteoblasts, adipocytes and chondroblasts³⁰. A large body of evidence supports that transplantation of MSCs sub-acutely after stroke promotes recovery in both preclinical research (approximately 44 of 46 studies as of 2013) and clinical trials^{31–33}. As such, there is increasing interest for MSC transplantation with currently 16 clinical trials completed or in progress listed on clinicaltrials.gov for the treatment of ischemic stroke with MSCs. MSCs are known for their immunomodulatory effects but also demonstrate other mechanisms that potentially contribute to stroke recovery including cell replacement and secretion of angiogenic and neurotrophic factors. MSCs act to promote recovery likely through secretion of cytokines (Tumour Necrosis Factor Stimulated Gene 6 protein, Leukemia Inhibitory Factor, Nitric Oxide Synthase and Prostaglandin E2 or TSG-6, LIF, iNOS, PGE-2 respectively) neurotrophic factors (Nerve growth factor, Glial cell line-Derived Neurotrophic Factor, Brain Derived Neurotrophic Factor or NGF, GDNF and BDNF respectively) and angiogenic factors (Basic Fibroblast Growth Factor, Vascular Endothelial Growth Factor , Interleukin 6)³⁴. Furthermore, MSCs promote local growth factor production from astrocytes and endothelial cells^{35,36}. Several studies have also shown that MSCs can be transdifferentiated into neuronal and glial cells following transplantation in the damaged^{37–40}. However, the MSC-derived neural cells exist in small numbers and have not shown the expression of voltage-gated ion channels and there is a lack of evidence suggesting that they become actively integrated into host circuitry^{32,41–43}. As such, MSC transplantation is not considered a cell replacement therapy but rather cell support therapy to modulate inflammation and facilitate endogenous repair.

The generation of human induced pluripotent stem cells (iPSCs) through reprogramming of somatic cells such as skin fibroblasts by a set of core pluripotent transcription factors has revolutionized cell therapy by providing a source of autologous cells for transplantation⁴⁴. To achieve this, human adult fibroblasts were transduced with retroviral vectors containing 4 pluripotent transcription factors Oct3/4, Sox2, c-Myc, and Klf4 (Octamer-binding transcription factor 3/4, (Sex determining region T)-box 2, avian myelocytomatosis viral oncogene homologue and Kruppel-like factor 4 respectively). Following this, recent studies also show that a wide range of small molecules and factors can be used to induce a pluripotent state from fibroblasts and other cell types^{45,46}. To demonstrate their pluripotency, iPSCs were injected into the dorsal flank of immunodeficient mice, arising in tumours of the three germ layers. With the potential to differentiate into all of the neural cell types, iPSCs provide alternative cell resources for autologous cell replacement. Some studies have demonstrated that transplantation of iPSCs promotes functional recovery in animal stroke models and that iPSC can successfully differentiate into cells expressing markers of glial and neuronal lineage *in vivo* following transplantation into stroke-damaged brain^{47,48}. Furthermore, using a non-human primate model, the study shows that iPSCs-derived cells transplanted in an autologous fashion have fewer signs of immune rejection than cells transplanted in an allograft manner⁴⁹. However, due to their pluripotency and high proliferation rate, iPSCs have a high potential for tumorigenesis, which is a consistent concern among studies^{47,48,50,51}. In part by this hurdle, there is only one clinical trial using iPSCs to treat age-related macular degeneration (AMD) which started 2011 in Japan. However, it was stopped in 2015 due to a change in regenerative medicine law in Japan and concerns over spontaneous mutations in their iPSCs⁵². The next clinical trials for iPSC treatments are likely going to focus on AMD and Parkinson's⁵³. iPSCs offers a means to circumvent ethical issues surrounding the use of

fetal/embryonic brain tissues and offer the potential of cell replacement and immune-escape but has practical concerns around tumor formation.

To take advantage of the benefits of iPSCs technology but limit tumorigenesis and maximize differentiation into a neural lineage, iPSCs can be differentiated into neural stem cells *in vitro* using various protocols including dual SMAD (or Mothers against decapentaplegic homologue) inhibition and/or retinoic acid⁵⁴. iPSC-derive neural stem cells (iPSC-NSCs) can be propagated and purified through passages in neurosphere cultures or in monolayer culture. Interestingly several studies have demonstrated that iPSC-NSC transplantation increases functional recovery in rodent stroke models by improving endogenous niche environments^{55–59}.

One of the first transplantation of human iPSC-NSCs into an ischemic stroke model was conducted by Dr. Koichi Oki and colleagues where they injected iPSC-NSCs into the cortex and striatum of mice or rats with transient 30-minute MCAo 48 hours or 1 week post-stroke⁶⁰. Cells survived up to at least 4 months, while a decrease in cell number and population of Ki67 positive cells was observed between the 2-month and 4-month time points. Cells injected into the cortex largely expressed markers of neuronal lineage Neuronal nuclear antigen (NeuN), with a small population of Glial fibrillary acidic protein (GFAP), Dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32), parvalbumin, calbindin and calretinin expressing cells. Furthermore, whole-cell patch clamp recordings showed that grafted cells frequently had spontaneous excitatory postsynaptic currents in the presence of Gamma-Aminobutyric acid (GABA_A) receptor antagonist picrotoxin and postsynaptic currents were blocked in the presence of glutamate receptor antagonists demonstrating functional excitatory synapses. Furthermore, grafted cells in the striatum formed axonal projections to the globus pallidus, one of the dorsal striatum's main projection areas which was traced using retrograde tracer fluorogold⁶⁰. Several other studies also revealed similar findings with a large majority of cells expressing markers of neuronal lineage and

the potential to have functional synapses^{56,59,61}. Furthermore, cell transplantation of iPSC-NSCs increased BDNF and Stromal cell-derived factor 1 α (SDF-1 α) expression, a chemattractant important for directing endogenous neural stem cells toward the stroke lesion, increased number of doublecortin⁺ (DCX) neuroblasts from the ipsilateral subventricular zone (SVZ) and local proliferative neuroblasts cells suggest that iPSC-NSC transplants improves endogenous neurogenic response^{55,56}. There is controversy regarding the effect of iPSC-NSC transplants on endogenous angiogenesis. Increases in VEGF expression and in collagen IV proliferating cells have been reported. However, this did not result in increased vessel density or length^{56,58}. Although iPSC-NSCs have yet to go to clinical trials for the treatment of stroke, they have been shown to promote recovery in motor behavioural tests including staircase, adhesive tape test, rotarod test, stepping task, beam walking task and neurological scores^{22,55,58,59,62–64}. A detailed summary of preclinical studies on iPSC-NSC transplants for stroke has been included in Table 1. As such, iPSC-NSCs have the potential to replace lost tissues and promote endogenous repair resulting in functional recovery.

Table 1. Overview of completed iPSC-NSCs Transplant studies for Ischemic Stroke.

Stroke Model	Injection Time post-stroke / Location	Duration of study	Immunosuppression	Behaviour Difference compared to sham	Earliest improved time point	No Behaviour difference compared to sham	Citation
30min MCAo	1 week, Striatum	5 weeks	CsA 10mg/kg/day SC for 2 days+ drinking water	None	None	Elevated Body Swing, Cylinder Test Adhesive Removal	Jensen et al., 2013 ⁵⁷
30min MCAo	1 week, Cortex+ Striatum	22 weeks	Rats: Nude, mice: 10mg/kg/2days	Staircase, Corridor test	1 week post-transplant	None	Oki et al., 2012 ⁶⁰
30min MCAo	1 week, Striatum	6 weeks	Not reported	Neurological score	5 weeks post-transplant	None	Gomi et al, 2012 ⁶⁵
90min MCAo	1 week, Striatum	8 weeks	CsA 10mg/kg/day SC	Adhesive Removal, Apomorphin-induced rotation test	2 weeks post-transplant	Staircase	Polentes et al, 2012 ⁶³
120min MCAo	Immediately, Striatum	3 weeks	CsA 20mg/kg/day SC	Beam walking task, Rope grab, MWM	3 weeks post-transplant	None	Yuan et al., 2013 ⁶⁶
90min MCAo	1 week, Striatum	8 weeks	CsA 10mg/kg/day IP	Rotarod, Neurological score, apomorphine test Stepping test	1 week post transplant	None	Chang et al., 2013 ⁵⁵
Permanent MCAo	1 week, Striatum	4 weeks	None used	Adhesive Removal	2 weeks post-transplant	None	Mohamad et al., 2013 ⁶⁷
30min MCAo	48 hours, Cortex	20 weeks	Nude and CsA 10mg/kg/day SC for 1 month, CsA 10mg/kg/2days for the second month	Stepping test	8 weeks post transplant	Cylinder	Tornero et al., 2013 ⁵⁹
Permanent MCAo	1 week, peri-infarct	3 weeks	Not reported	None	None	Vibrissae-elicited forelimb placement	Chau et al., 2014 ⁵⁶
Photothrombosis	1 week, Striatum	1 week	TMS Oral/5days	None	None	None	Lam et al., 2014 ⁶⁸
60min MCAo	24 hours, Striatum	4 weeks	Not reported	Adhesive Removal, Beam walking, Rotarod	2 weeks post-transplant	None	Eckert et al., 2015 ⁶²
40min MCAo	72 hours, Cortex	4 weeks	Not reported	None	None	Corner test, Cylinder	Wu et al., 2015 ⁶⁹
Photothrombosis	1 week, Cortex	9 weeks	Tacrolimus IP for 4 days, 3mg/kg/day SC	None	None	None	Moshayedi et al., 2016 ⁶¹
Photothrombosis	1 week, Cortex	6 weeks	Tacrolimus IP for 4 days, 3mg/kg/day SC	None	None	None	Nih et al., 2016 ⁷⁰
30min MCAo	48 hours, Cortex	8 weeks	CsA 10mg/kg/day SC for 1 month, CsA 10mg/kg/2days for the second month	None	None	None	Tornero et al., 2017 ⁷¹

1.5 Investigating Mechanisms of Action for Neural Stem Cell Therapy

There are many proposed mechanisms that may contribute to functional recovery following neural stem cell transplantation for stroke however the details and time line of these mechanisms is not well understood. Briefly, these mechanisms include support of endogenous neurogenesis and angiogenesis, reduction of inflammation, reduced neural cell death and integration of exogenous cells. These processes happen in parallel and likely interact and support each other as summarized by Horie and colleagues⁷². Briefly, early recovery occurs with a decrease in Blood Brain Barrier (BBB) leakage, increased VEGF signaling and a reduction in immune response reflected in reduced allograft inflammatory factor 1 (Iba-1) positive microglia and monocytes. Delayed recovery is accompanied by reduced cortical atrophy and neovascularization reflected in an increase in blood vessel density (BVD) and increase in β subunit of dystroglycan (β DG) expression which is required for astrocytes to bind to the endothelial cell matrix⁷². Due to the multiple complex mechanisms following transplantation, there is a need for more fundamental studies investigating their mechanisms.

The bystander effect refers to the process of transplanted cells to directly secrete regenerative factors or to promote secretion of factors by endogenous tissues resulting in recovery. Neural stem cells secrete multiple angiogenic, neurogenic and immunomodulatory factors that could explain functional and cellular recovery observed in neural stem cell transplant models. Several studies have demonstrated that neural stem cell transplants promote angiogenesis in the subacute phase of stroke^{73,74}. For example Dr. Zhang and colleagues show an increase of 65% in proliferative endothelial cells in the peri-infarct 14 days post-stroke⁷³. Furthermore, over-expression of VEGF following neural stem cell transplants promoted functional recovery suggesting secretions of VEGF from transplanted cells can contribute to functional recovery^{75,76}. In addition to promoting angiogenesis, transplanted neural stem cells (NSCs) secrete

neurotrophins and chemokines which promote the recruitment of endogenous NSCs from the SVZ to injured sites^{77,78}. Transplant-dependent increases in endogenous axonal sprouting and dendritic branching are also correlated with improved functional recovery⁷⁹. Since both angiogenesis and neurogenesis occur over similar time frames it is possible that migration of neuroblasts from the SVZ not only benefits from secretion of neurotrophins such as BDNF but use newly formed vessels as a scaffold to migrate towards the lesion site contributing to a neurovascular niche⁸⁰. In the absence of transplantation, treatment of mice with endostatin, an inhibitor of angiogenesis, reduces DCX⁺ population in the peri-infarct region by 10-fold, suggesting that angiogenesis is required and facilitates recruitment of neuroblasts from the SVZ⁸¹. Additionally, in a similar experiment with MSCs transplants the reduced Nestin⁺ and NeuN⁺ populations in the peri-infarct following endostatin administration correlated with a decrease in functional recovery⁸¹. This is evidence that MSC transplant improves recovery through the support of a neurovascular niche and that a similar mechanism may be involved in neural stem cell transplants. In summary, the bystander effect of cell-based therapies on angiogenesis and neurogenesis accounts for a major contributor to functional recovery.

Cell replacement has been the goal for neural stem cell transplants with these cells having the potential to differentiate into neurons, astrocyte and oligodendrocytes *in vivo*. A multitude of studies have shown that NSCs transplanted into the ischemic core differentiate and demonstrate phenotypic characteristics of mature neurons. Furthermore, electron microscopy studies have shown that transplanted NSCs can form synapses with endogenous neurons and express ion channels and other synaptic proteins^{82–84}. In addition, at least a sub-set of these cells are capable of firing action potentials^{56,59,61,85,86}. Interestingly, transplanted optogenetically competent neurons elicited high-frequency oscillations in hippocampal slice tissue demonstrating integration and participation in endogenous neural network activity⁸⁵. Furthermore, optogenetic stimulation

of neural grafts can promote functional recovery in the cylinder task compared to non-stimulated grafts⁸⁷. However, the number of cells that are capable of maturing into neurons, synapsing with host circuitry and successfully fire action potentials is very low. This raises the question whether this process of integration contributes to functional recovery. In fact, long-term survival of cell grafts is not required for functional recovery 4 months post-stroke⁵⁸. Although it seems that bystander effects of NSC transplants play a major role in functional recovery, cell replacement therapy is an ultimate goal of the exogenous cell transplantation strategy to maximize stroke function recovery by replacing lost neural cells in the stroke-related brain damage. To achieve the ultimate goal, developing optimized approaches for neural stem cell transplantation is in demand.

1.6 The Effect of Metformin on Neural Stem Cells and its Mechanisms

The barriers faced with NSC transplants includes low survival of acute and chronic grafts, disorganized transplanted cells and minimal differentiation even after several weeks post-transplant⁸⁸. This minimal differentiation is thought to be a major hurdle in facilitating functional graft-host interactions. Several different methods to optimize NSC transplants have been explored with varying success including transplantation with hydrogels, hypoxic preconditioning and genetic modifications^{61,89}. These treatments focus on increasing initial engraftment with limited capacity to promote their differentiation into mature neurons. As such, there is a demand to develop an optimal strategy to enhance the engraftment and regenerative capacity of exogenous stem cells, ultimately improving stroke functional recovery.

Our recent published work shows that metformin, an FDA approved diabetes drug, is an optimal neuroregenerative agent by acting on multiple stages of adult neural precursor development including proliferation, differentiation and cell survival⁹⁰⁻⁹². Metformin greatly increases primary murine adult SVZ-derived neurosphere size and number^{90,93}. Intriguingly, these

effects were persistently elevated for secondary and tertiary neurospheres in the absence of metformin in culture. This persistent increase of neurosphere number and size demonstrates promise in the application of metformin for preconditioning of NSCs prior to transplantation. Furthermore, metformin treatment significantly increases neuronal differentiation of adult SVZ NSCs, showing the increased the number of β -III tubulin positive neurons produced from the SVZ NSCs. This increased neuronal differentiation by metformin treatment was also observed previously from both human and murine embryonic neural precursor culture⁹².

Metformin is an FDA approved drug for the treatment of diabetes mellitus and is currently being investigated for the treatment of a polycystic ovarian syndrome and a variety of cancers including breast, lung, pancreatic and colorectal cancer⁹⁴. Metformin is a small molecule belonging to the class of drugs biguanides. In diabetes mellitus, metformin lowers blood glucose levels mainly by inhibiting liver gluconeogenesis and increasing insulin-mediated glucose uptake in skeletal muscle⁹⁵. The underlying mechanisms through which metformin acts to reduce gluconeogenesis are not fully understood. Briefly, metformin inhibits the mitochondrial respiratory-chain complex 1 in the mitochondria of hepatocytes which in turn increases the cellular Adenosine monophosphate to Adenosine triphosphate ratio (AMP-to-ATP ratio)^{96,97}. AMP stimulates Liver Kinase B1 (LKB1) and Calmodulin-dependent protein kinase kinase β (CAMKK β) to phosphorylate AMP-activated protein kinase (AMPK) at Thr172. Binding of AMP to AMPK further blocks dephosphorylation of Thr172⁹⁸. Activation of AMPK through Thr172 ultimately reduces gluconeogenic gene expression via phosphorylation of serine436 of CREB Binding Protein (CBP) resulting in dissociation of the CBP - TORC2 transcription complex and increased insulin sensitivity through reduced lipogenesis^{94,99,100}.

Metformin also acts on NSCs. Activation of AMPK stimulated neuronal differentiation of neural stem cells by activating the atypical protein kinase C-mediated CBP phosphorylation at

S436⁹². Phosphorylation of CBP is thought to promote neurogenesis through modulating histone acetylation but the mechanism has yet to be well defined.

Metformin increases expression level of Transcriptionally active protein 73 (TAp73) in NSCs which is required to elicit metformin-induced proliferation independent of CBP ser436 phosphorylation⁹⁰. TAp73, a member of the p53 transcription factor family, is required for NSC self-renewal and proliferation through the increase in the expression of a protein called “Hairy/enhancer of split related with YRPW motif protein 2” (HEY2)¹⁰¹. TAp73 is also implicated in cell survival, neurite outgrowth elongation, and branching of immature neurons¹⁰². Therefore, metformin acts on two different molecular pathways to enhance adult neural stem cell proliferation and differentiation, although the exact mechanisms are not fully understood.

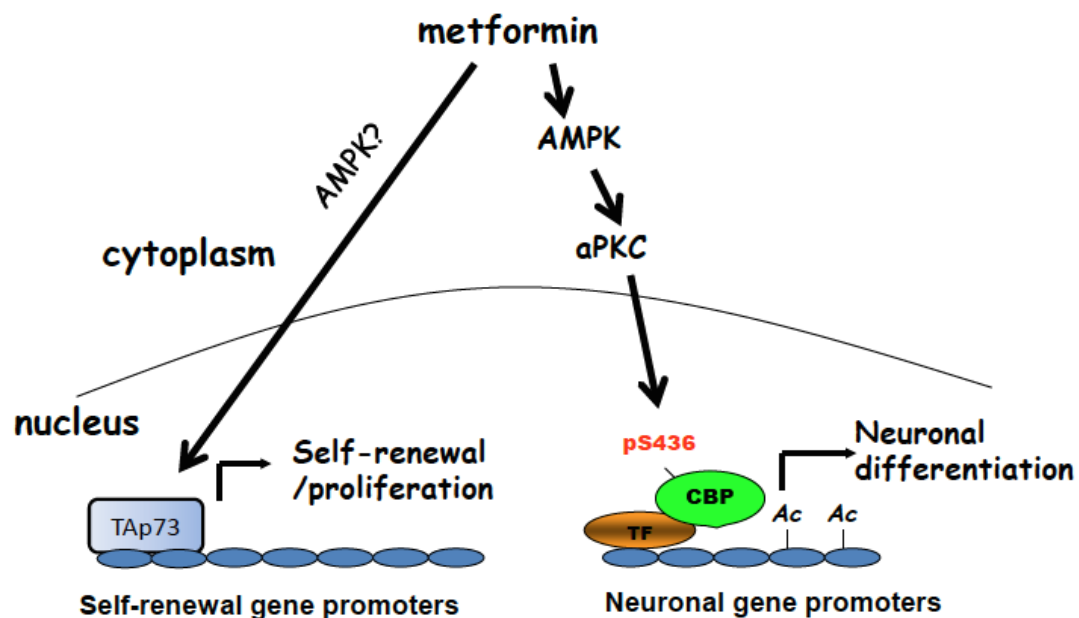


Diagram 2. Model describing two distinct molecular pathways mediating metformin-induced proliferation/self-renewal and neuronal differentiation⁹⁰. Metformin-induced neuronal differentiation is dependent on CBPs436 phosphorylation which may alter histone acetylation by CBP. Metformin-induced proliferation is dependent on a TAp73 expression.

2.0 Hypothesis and Objectives

2.1 Hypothesis

We hypothesize that preconditioning of hiPSC-NSCs with metformin before transplantation into the stroke-damaged brain will improve engraftment and regenerative capabilities of hiPSC-NSCs, ultimately enhancing post-stroke functional recovery.

2.2 Objectives

Aim 1.1

Generate human neural stem cells from a human iPSC line.

Aim 1.2

Determine metformin's ability to enhance proliferation and differentiation of hiPSC-NSCs *in vitro*.

Aim 2.1

Determine the extent to which metformin preconditioning improves cell survival and proliferation 1-week post-transplantation of hiPSC-NSCs into a rat ET-1 stroke model.

Aim 2.2

Determine the extent to which metformin preconditioning improves cell survival, differentiation and functional recovery 8 weeks post-transplantation of hiPSC-NSCs into a rat ET-1 stroke model.

3.0 Materials and Methods

3.1 Animal Handling and Housing

All animals use was approved by the Animal Care Committees of the University of Ottawa in accordance with the Canadian Council of Animal Care policies. Sprague Dawley rats from Charles River (200g-250g) were maintained in reverse light cycle (12h dark/ 12h light) with *ad libitum* access to food and water. Rats were housed in pairs in Sealsafe green line caging. All rats were habituated for 1 week followed by 1 week of handling (approximately 5 minutes/rat/day) before commencing behavioural testing.

3.2 Human iPSC-NSC Induction and Culturing

Human induced Pluripotent stem cell line, WLS1C, was obtained from Dr. William Stanford's Lab at the Ottawa Hospital Research Institute. WLS1Cs were maintained in E8 media containing DMEM/F-12 (Life Technologies, 11330-057), Ascorbic Acid (64mg/L, Sigma, A8960-5G), Sodium Bicarbonate (543mg/L, Sigma, S-5761), Human Insulin Recombinant (20mg/L, Wisten, 511-016-CM), Sodium selenite (4.2µg/L, Sigma, S5261-10G), human holo-transferrin (10.7 mg/L, Sigma, T0665-1G), FGF-Basic (100µg/L, Life technologies, PHG0263), Recombinant human transforming growth factor β 1 (2 µg/L, Life Technologies, PHG9202), and gentamycin (Wisent, 450-135-XL) on matrigel coated plastic plates (Bd Biosciences, 354230) in a 10% CO₂ 5.0% O₂ incubator. To remove differentiated cells, WLS1Cs would be picked under a ventilated stereomicroscope 3 days after passaging and immediately before passages. WLS1Cs were passaged approximately every 5 days or at 80% confluency using EDTA (BioBasic, EB0185). WLS1Cs were washed with PBS (Thermo Fisher Scientific, MT21031CV) and incubated in EDTA for 3.5 minutes. EDTA was removed and replaced with E8 media. WLS1C were then suspended using a cell scraper, broken up using a wide bore pipette and passaged at a 1 in 6 dilution in E8 media.

WLS 1Cs were induced into neural stem cells using the commercially available induction media (Stem cell Technologies, MT21031CV). At 80% confluency, WLS1Cs were picked and suspended using gentle cell dissociation reagent for 10 to 15 minutes. WLS1Cs were mechanically dissociated into a single cell suspension by triturating followed by diluting the suspension with DMEM/F12 at a 1 in 6 dilution. Cells were then centrifuged at 300g for 5 minutes. The supernatant was removed the pellet was resuspended in neural induction media supplemented with 10 μ M ROCK inhibitor Y27632 (Stem cell technologies, 72302). Cells were counted using trypan blue (life technologies, 15250-061) and plated on poly-L-Ornithin (Sigma, 72302) and laminin (Thermo fisher scientific, CB 40232) coated 24-well plates at 200,000 cells/cm² in a 5% CO₂ and room O₂ incubator. Media changes were conducted daily with induction media for 7-9 days or at 95% confluency. At passages, cells were lifted with accutase (Stem cell technologies, 07920) for 8 to 15 minutes and mechanically dissociated until single-celled. The cell suspension was then diluted with DMEM/F12 1 in 3 dilution. Cells were centrifuged at 300g for 5 minutes and resuspended in induction media with 10 μ M ROCK inhibitor onto PLO-Laminin coated 12-well plate at 150,000 cells/cm². Media changes were conducted daily with induction media until the culture reached 95% confluency. Cells were then suspended with accutase as previously described. hiPSC-NSCs were then plated at 80,000cells/cm² on matrigel coated 6-well plates in complete neural progenitor media (Stem cell Technologies, 05833). hiPSC-NSCs were maintained with daily media changes and were passaged every week at 80,000 cells/cm² on matrigel coated plates.

3.3 Metformin and 5-bromo-2'-deoxyuridine *in vitro* treatment

All *in vitro* experiments with metformin treatment were performed on hiPSC-NSCs 5 passages after the start of neural induction or 2 passages after the end of neural induction. To assess proliferation, hiPSC-NSCs were passaged and plated into neural progenitor media at 50,000

cells/cm² and the media was supplemented with 0μM, 50μM or 200μM metformin (Sigma, D150959-5G) diluted in water a day later. Concentrations of metformin were based on previous studies and experiments done in lab (data unpublished)⁹⁰. Daily media changes were performed. After 3 days cells were incubated with 10 μM *5-bromo-2'-deoxyuridine*, BrdU, (Sigma, B9285-1G) suspended in neural progenitor media for 8 hours and fixed.

To assess differentiation, cells were passaged and plated into neural progenitor media at 100,000 cells/cm² and the media was changed to neural differentiation media (NDM) (DMEM/F12 supplemented with 1% N2 supplement (Thermo Fisher, 17502048), 20ng/mL BDNF (PeproTech, 450-02), 1% B27 supplement (Thermo Fisher, 17504-044), 10% Fetal Bovine Serum (Life Technologies, 12484010) and treated with 0μM, 50μM or 200μM metformin a day later. A half media change was performed 2 days later and the culture was fixed at 5 days post-passage.

To determine the effects of metformin preconditioning in culture, cells were treated with metformin at concentrations of 0μM, or 50μM with daily media changes with neural progenitor media for one passage (5-7 days). Following metformin treatment, hiPSC-NSCs were passaged according to the proliferation or differentiation experiments described above.

3.4 ET-1 Surgery

ET-1 (1μg/μL, Abcam, AB120471-100UG) diluted in PBS was sonicated in a 4°C water bath and left on ice for the period of the surgery. After baseline behavioural testing, rats were anesthetized using 4-5% isoflurane and 2% oxygen and mounted to a stereotaxic frame. Total surgery time was approximately 1 hour. Body temperatures were monitored using a rectal thermometer and maintained at 36.5°C on a heating blanket. Bur holes were made at all three injection sites. Injections were performed using a 10μL syringe with a 26G cemented needle (Hamilton, 80366). The needle was positioned at following injection sites:

0.0mm AP, +/-3.0mm ML and -1.7mm DV

+2.3mm AP, +/- 3.0mm ML, and -1.7mm DV

+0.7mm AP, +/-3.8mm ML and -7.0mm DV

AP: Anterior-Posterior, ML: Medial-Lateral, DV: Dorsal-Ventral

The sites selected represent the motor forelimb area and the dorsolateral striatum¹⁰³. Prior to injection of ET-1, 1 μ L/site, the needle was lowered an additional -0.1mm DV for 1 minute and raised back to position to create a pocket to receive the ET-1. The ET-1 was then injected at 0.25 μ L/min over 4 minutes. Incision sites were closed via sutures and treated with bupivacaine immediately after surgery and 4 hours post-surgery. Subcutaneous buprenorphine was administered after rats regained consciousness.

3.5 CM-Dil and Hoechst Cell Labeling

hiPSC-NSCs were labeled with fluorescent lipophilic dye CM-Dil Vybrant (Fisher Scientific, V22888) prior to the transplantation surgery on the same day. HiPSC-NSCs at about 80% confluency were washed with DMEM/F12 and incubated for 20 minutes in 1 mL/ 6-well of neural progenitor media containing 1:1000 dilution of CM-Dil Vybrant or 1:1000 dilution of CM-Dil and Hoechst 33342 (Cell Signalling Technologies, 4082) at room temperature in the dark. Cells were washed with neural progenitor media 4 times for 10 minutes at 37°C, 5% CO₂ and room O₂.

3.6 hiPSC-NSC Transplantation

hiPSC-NSCs were treated with 50 μ M metformin or sterile water for 48 hours before surgery. Cells were labeled with CM-Dil as described above. hiPSC-NSCs were lifted with accutase as described previously and resuspended in 1 mL PBS and counted using trypan blue. Cells were then centrifuged at 300g for 5 minutes and resuspended in PBS at 100,000cell/ μ L. For the sham group, the above protocol was conducted on an empty matrigel coated 6 well-plate and brought

to an equal amount of PBS. CM-Dil staining of the empty matrigel 6-well acted as our negative control for CM-Dil staining. Suspensions were left on ice, away from light, for the duration of the surgery, no more than 6 hours. The surgery was performed over 45 minutes and was conducted similarly to the ET-1 protocol with few adjustments. Immediately before the first injection, cells were resuspended using a pipette and 4 μ L was drawn into a 10 μ L syringe with a 26G cemented needle. Cells were injected into the two cortical sites:

0.0mm AP, +/-3.0mm ML and -1.7mm DV

+2.3mm AP, +/- 3.0mm ML, and -1.7mm DV

Cells were injected into the infarct core (same location as ET-1 sites) to minimize damage to the peri-infarct. Furthermore, cells were only injected to the cortical sites and not the striatal site to match the cell type of our particular hiPSC-NSCs. To limit back flow, the needle was lowered -0.1mm DV and left in place for 1 minute. The needle was then raised to position and the injection was initiated at 200nL/min for 3 minutes. The needle was raised +3.0mm DV and left there for 4.5 more minutes of the injection. The needle was left in place for 2 minutes and removed over a 1 minute period. For sham groups, the same surgery was performed with PBS treated as described above.

3.7 Cyclosporin A and BrdU Injections

Cyclosporin A (BioShop Canada, CYC002.5) was used as an immunosuppressive agent to avoid cell rejection. Cyclosporin A was prepared in cremophor EL (Sigma, C5135) at 100mg/mL. Dissolved cyclosporin A was then mixed with sterile PBS at a 1:5 dilution and administered the same day. Rats received subcutaneous injections of cyclosporin A 20mg/kg daily starting 2 days prior to transplantation and administered every 2 days starting 1 week post-transplantation.

3.8 Tissue Preparation

At 1 week and 8 weeks post-transplant, rats were anesthetized with 1 mL i.p. injection of sodium pentobarbital (65mg/mL). Rats were perfused with 120mL of 4°C PBS (20ml/min for 5 minutes) and 120mL of sterile filtered 4°C 4% paraformaldehyde (Sigma, 159127-500G) in PBS. Brains were then dissected and incubated in 4% PFA in PBS for 6 hours, followed by storage in 30% sucrose solution containing 1% sodium azide (Fisher, 19038-1000) for at least 72 hours. Samples were covered in optimum cutting temperature solution (VWR, 95057-838) and submerged into 2-methylbutane (Thermo Fisher Scientific, O3551-4) at -40°C to -50°C. Serial 30µm sections were obtained using a cryostat (Leica Biosystems, CM1850) to encompass the entire stroke site over 32 gelatin coated slides. Samples were left to dry over an hour and stored at -80°C.

3.9 Western blot analysis

Total lysates from culture was obtained by lysing cells with lysis buffer (Appendix table 2) followed by sonication (3 strokes with 5s length and 1 min interval) and centrifugation at 13,000g for 15 minutes. Total protein amounts were quantified using Pierce BCA Protein assay kit (Thermo Fisher, 23227) and quantified at 562 nm by Fluorstar Galaxy spectrophotometer.

Protein samples were mixed with 5x sample buffer (Appendix table 2), boiled for 5 min at 85°C, and run on a 12% SDS gel in running buffer (Appendix table 2). Gel was then transferred onto a nitrocellulose blotting membrane (GE Healthcare Life Sciences, 10600009) in transfer buffer (Appendix table 2). A 5-minute wash in TBS-T containing 50mM Tris-HCl, 140mM NaCl, 0.1% Tween-20 pH 7.4 was performed followed by a 1 hour block in 3% Bovine Serum Albumin (New England BioLabs, 9998S) in TBS-T. Primary antibodies: anti-acetyl H2B (EMD Millipore, ABE1065), anti-H2B (EMD Millipore, 07-371), anti-Sox2 (EMD Millipore, ABE5603MI), anti-Oct-3/4 (1:2000,

Cell Signaling, 2890S), and anti-GAPDH (1:50,000, EMD Millipore, CB1001) were suspended in 3% BSA in TBS-T. Membranes were incubated overnight at 4°C in primary antibody followed by 5 washes for 5 minutes in TBS-T and a 1 hour incubation in secondary anti-body: anti-rabbit HRP (Cell Signalling technologies, 7074), anti-mouse-HRP (Cell Signalling Technologies, 7076). Exposures were taken between 5 seconds and 30 minutes using Clarity Western ECL kit (Bio-Rad, 1705060), hyblot film (Denville Scientific, DV-E3018) and an automatic X-Ray Film processor (JPI Healthcare, JP-33).

3.10 Immunocytochemistry and Immunohistochemistry

Cells were grown on 24-well sized cover slips coated in matrigel. A 5-minute fixation in 4% PFA was performed followed by three 5 minute washes with PBS. Coverslips were then removed from wells and blocked in 10% normal goat serum (Jackson ImmunoResearch, 008-000-121) or 3% BSA in PBS with 0.3% triton X-100 (Fisher Scientific, 9002-93-1) for at least 1 hour. Samples were incubated in primary and secondary as described below.

Brain sections were removed from -80°C and dried at 37°C for 5 minutes. Sections were rehydrated in PBS for 5 minutes and fixed in 4% PFA in PBS for 5 minutes. Two washes in PBS were performed followed by blocking in 10% NGS in PBS or 3% BSA in PBS with 0.3% triton X-100.

Samples from cultured cells and brain sections were incubated in primary antibody: anti-Ku80 (1:100, Cedarlanes Labs, Y40400), anti- β -III tubulin (1:500, Covance, 802001 and EMD 1:500, Millipore, MAB1637), anti-Ki67 (1:200, BD Pharmingen, 550609), anti-BrdU (1:400, AbD Serotec, OBT0030G), anti-Nestin (1:1000, EMC Millipore, MAB5326) anti-Pax6 (1:500, BioLegend, 901301), anti-Oct-4 (1:1000, Cell Signaling, 2890S), anti-GFAP (1:1000, abcam, ab7260), anti- Sox2 (1:100, EDM Millipore, AB5603MI), anti-Olig2 (1:1000, EMD Millipore, AB9610), or anti-Iba1 (1:1000, Wako, 019-19741) overnight at 4°C. Three 5 minute washes in PBS were performed followed by a

1 hour incubation in alexa fluora conjugated secondary antibody: goat or donkey anti-rabbit Alexa Fluor® 555, (1:500, Cell signaling Technology, 4413S), goat or donkey anti-mouse Alexa Fluor® 555 (1:500, Cell signaling Technology, 4409), donkey anti-rabbit Alex Fuor 488 (1:500, Thermo Fisher Scientific, a31573), goat or donkey anti-mouse Alexa Fluor® 488 (1:500, Cell signaling Technology, 4408S), anti-mouse Alexa Fluor® 647 (1:500, Cell signaling Technology, 4410). Samples were counterstained with Hoechst 33342 (Cell Signaling, 4082) at 1ug/mL in PBS for 5 minutes followed by three 5 minute washes in PBS. Samples were mounted using permafluor solution (Thermo Fisher, TA-030-FM) and imaged once dry.

3.11 Imaging and Quantification

Fluorescent images were taken on a Zeiss Axioplan 2 fluorescent microscope using Zeiss Axiovision software at least 24 hours after mounting. All immunostained samples were kept in 4°C away from light. Images for *in vitro* quantification were taken at random with at least 4 images per treatment with Z-stacking of 10-20 images either 1 µm or 2 µm apart. Counting was performed blinded to experimental groups using FIJI software. For *in vivo* quantification, all slides with positive CM-Dil labelled cells were stained. All ku80 positive cells were imaged. Total ku80+ cells per rat were calculated by summing the number of cells per section and multiplying by the number of slides per series. Orthogonal analysis was performed using FIJI. Images were captured using an LSM800 confocal microscope using Zeiss Zen Pro software.

3.12 Staircase Test

Rats were food restricted to 12g/rat the night before beginning staircase training which continued throughout training and testing days. To habituate rats to the staircase pellets, they received approximately 50 sucrose pellets/ cage/ night for the first week of training. To measure unilateral forelimb coordination, rats were placed in clear Plexiglass staircase boxes with two

descending staircases baited with 45mg sucrose pellets (TestDiet, 1811155-5TUL) in 3mm deep wells for 15 minutes twice a day, once in the morning and once in the afternoon, in a dark room with a fan to provide white noise. The staircases consist of 7 steps with 3 sucrose pellets on each step for a total of 21 pellets per staircase (Figure 13A Right panel). The plexiglass box is narrow enough to prevent the rats from reaching the staircase with opposite paw. Poor sensorimotor function is reflected in the inability to reach, grasp and eat the pellets when food deprived. Rats were habituated for 30 minutes in the staircase room prior to testing. Training began after 1 week of habituation to their cages. The training period spanned 26 trials and rats that did not reach at least 15 pellets with a standard deviation of +/-2 pellets in the last 4 trials were excluded from the staircase analysis. A total of 6 rats were excluded of 29 rats, 2 rats from each group (sham, naïve, and preconditioned). Testing intervals included 3 days of testing where only the last 4 trials would be scored. Rats were scored on the number of pellets eaten, dropped and the lowest step reached with less than 3 pellets at 1, 2, 4, 6, and 8 weeks post-stroke.

3.13 Cylinder Test

Rats were placed in a clear plexiglass cylinder (20cm diameter) under moderate lighting and video recorded for 20 rears (Figure 14). The cylinder task was performed prior to stroke and 1,2,4,6, and 8 weeks post-stroke. Videos were then analyzed using VLC media player to count the number of touches and paw drags against the cylinder with each paw. Paw preference index was calculated as follows:

$$Index = \frac{Ipsilateral\ touches - Contralateral\ Touches}{Sum\ of\ Left\ and\ Right\ touches} \times 100\%$$

3.14 Beam Walking Test

Rats were food deprived as described for staircase and left to habituate for 30 minutes in the testing room under room lighting. One day of training was performed prior to baseline testing, where rats would be incrementally placed at a greater distance from a dark box containing sucrose pellets along a gradually tapered 1m long two-tiered beam (Figure 15). This was performed until rats could run across the beam toward the box without pausing or falling off the beam. On testing days, which took place prior to stroke and 1, 2, 4, 6, and 8 weeks post-stroke, at least 3 trials were recorded and three trials with no or limited pausing were analyzed. Errors were defined as anytime the forelimb paw or hindlimb paw of the rat did not rest on the top of the beam during a step.

3.15 Cresyl Violet Staining

Slides were removed from -80°C and dried at 37°C for 15 minutes. Sections were rehydrated in PBS for 5 minutes and submerged in 0.2% cresyl violet solution dissolves in 0.5% acetic acid (pH 3.5) for 20 - 30 minutes. Samples were then dehydrated in sequential baths of 70%, 95%, and 100% ethanol baths and submerged in clearing agent, citrisolv (Fisher, 22-143-975). Slides were mounted with permount (Fisher, FL-10-0505) and imaged once dried.

3.16 Infarct Volume Measurements

Cresyl violet stained sections were imaged using aperio digital pathology slide scanner (Leica Biosystems) at 20X resolution. Images were analyzed using Imagescope software (Leica Biosystems) and FIJI. Infarct area per section was measured and total infarct volume was calculated using the following equation:

$$\begin{aligned}
\text{Infarct Volume} &= \sum_{k=1}^n (\text{Intact contralateral cortex/striatum} - \text{Intact ipsilateral cortex/striatum})_k \\
&\quad * 0.03\text{mm}(\text{thickness}) * 7(\text{number of series}) \\
&\quad \text{where } n \text{ is the section number}
\end{aligned}$$

3.17 Statistical Analysis

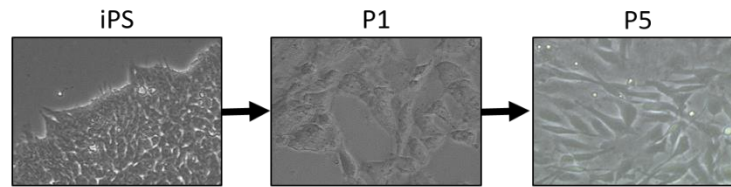
Statistical analysis was conducted using GraphPad Software. Behavioural data was analyzed using a two-way ANOVA and bonferonni post-hoc analysis. Single variable data was analyzed using a One-way ANOVA with tukey multiple comparisons or two-tailed paired t-test. All data was analyzed using a significance level of $\alpha = 0.05$ and all values are expressed as mean $\pm$ standard error of the mean.

4.0 Results

4.1 Generation of human neural stem cells from a human iPSC line

To generate our hiPSC-NSC line we used the stem cell technology neural induction kit, which has been shown to produce Pax6, Emx1 and sox1 positive dorsal cortical neural stem cells for up to ten passages with differentiation potential for the three neural lineages. This method was used instead of the dual SMAD inhibition using Noggin and SB431542 and/or EB methods to limit the need for extensive optimization and to maintain consistency among different batches of neural inductions. Two hiPSC lines were initially tested (WLS-1C and WLS-1D), and only WLS-1C hiPSCs were successfully committed to Pax6+cortical precursors 1 week after neural induction. After plating WLS-1C hiPSCs on poly-L-Ornithine coated plates for 8 days in neural induction media, we performed immunocytochemistry and quantified that 97% of cells had become Pax6 positive cortical precursors while 3-4% of cells remained Oct-3/4 positive (Figure 1B and 1C). Oct-4 positive cells appeared in groups forming 3D structures, reminiscent of the growth of hiPSCs (Figure 1B). Pax6 positive cells often formed groups resembling neural rosettes which persisted until passage 8 and 9 (Figure 1A and 1B). Further immunostaining at later passages identified these cells as largely expressing neural stem cell markers Pax6, Sox2 and Nestin (Figure 1D and 1E). This is consistent with a dorsal neural precursor cell (NPC) identity. Oct-3/4 represented only 1% of the total cell population (Figure 1E). Sox2 was expressed both in neural stem cells and WLS-1C hiPSCs. To discriminate Sox2 expression levels in both hiPSCs and hiPSC-NSCs, we performed a western protein analysis which qualitatively showed that the expression of Sox2 in NSCs was much lower than those in hiPSCs using an overexpose image (Figure 1F). Oct-3/4 protein expression levels were only detected in hiPSCs but not hiPSC-NSCs (Figure 1F).

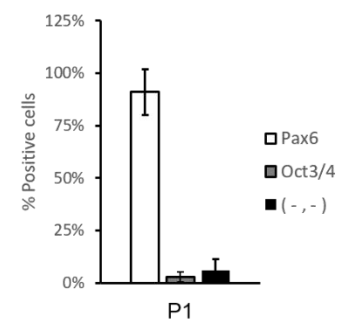
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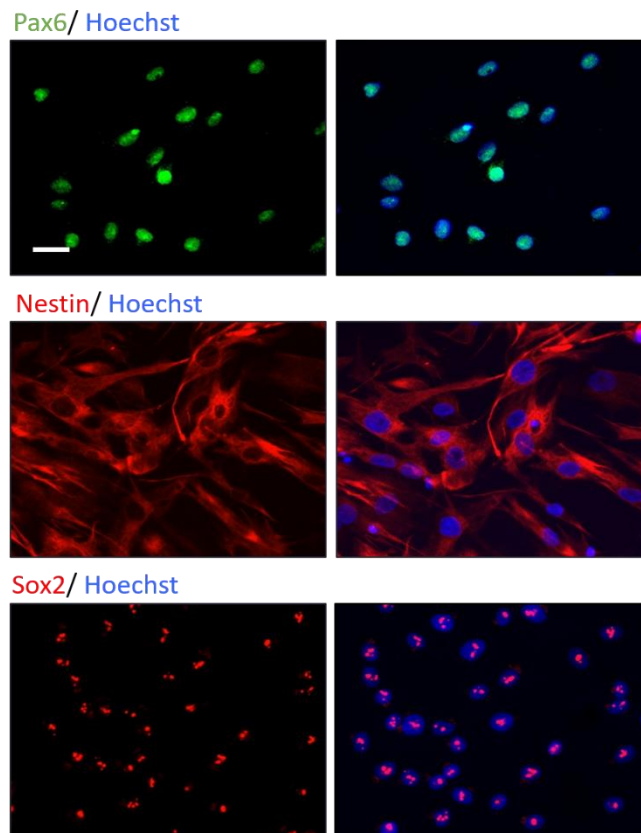
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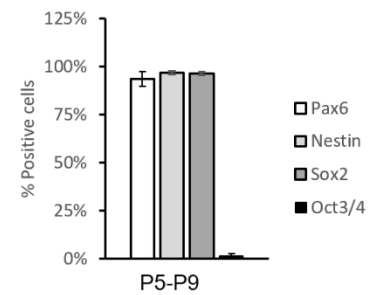
C



D



E



F

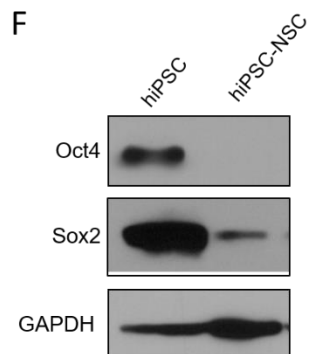


Figure 1. Induction of WLS1C iPSCs was performed using commercially available stem cell technology neural induction media for three passages. (A) Photographs of live iPSCs, iPSCs undergoing induction in P1 (first passage after addition of neural induction media) and iPSC-NSCs after induction in P5 were taken. (B – C) Immunostaining and quantification of iPSC-NSCs at Passage 1 for neural stem markers Pax6 (red) and pluripotent marker Oct3/4 (green), counterstained with Hoechst (blue). Scale bar: 40 μ m (D) Immunostaining of iPSC-NSCs from Passage 5 to 9 for neural stem markers Pax6 (green), Nestin (red) and Sox2 (red) was performed. Scale bar: 40 μ m (E) Quantification of neural and pluripotent markers as a percentage out of total live cells counterstained with Hoechst (blue)(n=3). (F). Western blot analysis of whole protein lysates from cultured WLS1Cs and hiPSC-NSCs shows a drop in pluripotent transcription factors, Oct3/4 and Sox2, when normalized to GAPDH.

4.2 Metformin enhances proliferation and differentiation of hiPSC-NSCs *in vitro*.

Previous studies have already demonstrated that metformin treatment is able to increase the proliferation and differentiation of adult mouse SVZ NPCs^{90,92}. Here we proposed to determine whether similar functional roles of metformin also occur in our hiPSC-NSCs. To assess proliferation, hiPSC-NSCs were plated into neural progenitor media with either water (vehicle control), 50 μ M metformin or 200 μ M metformin followed by BrdU pulse-labeling incubation. Our quantification analysis showed that there is a significant increase in the population of Ki67+/BrdU+ double-labeled proliferating cells in 50 μ M metformin treated group when compared to control (Figure 2B and 2C), but not 200 μ M metformin treated group (n=4, p-value<0.05). This data suggested a dose-dependent effect of metformin in promoting the proliferation of hiPSC-NSCs. To determine whether reduced cell death could contribute to increased proliferation rate caused by metformin treatment, we immunostained hiPSC-NSCs with apoptotic marker CC3 following metformin treatment. Quantification of CC3+ condensed nuclei showed that metformin treatments at both 50 μ M and 200 μ M didn't alter cell survival (Figure 3, n=3).

To assess neuronal differentiation, hiPSC-NSCs were plated into neuronal differentiation media (NDM) and hiPSC-NSCs were treated with water, 50 μ M metformin or 200 μ M metformin. At day 5 d.i.v., hiPSC-NSCs were immunostained for β -III tubulin, marking newborn neurons. Metformin treatment significantly increased the population of β -III tubulin+ neurons in culture when compared to control group for 50 μ M but not 200 μ M (1.6 and 1.7 fold change in 50 μ M and 200 μ M respectively) (Figure 4, n=3, p-value<0.05). These studies support previous findings that metformin enhances proliferation and differentiation of primary adult mouse NPCs^{90,92}.

For preconditioning to be successful *in vivo*, metformin treatments must have persistent effects on hiPSC-NSCs following withdrawal of metformin. To determine the effects of metformin preconditioning in culture, we selected 50 μ M as the optimum concentration to promote both

differentiation and proliferation of hiPSC-NSCs based on the above experiments. hiPSC-NSCs were treated with water or 50 μ M metformin for one passage after plating (5-6 days) and then plated into proliferation assays or differentiation assays as described previously in the absence of metformin. Quantitative analysis showed that hiPSC-NSCs preconditioned with 50uM metformin exhibited a significant increase in the number of Ki67+/BrdU+ co-labeled proliferating cells under proliferation conditions (Figure 5, n=3, p-value<0.05), as well as the population β -III tubulin+ neurons under differentiation conditions, when compared to control (Figure 6, n=3, p-value<0.05). To further determine whether metformin preconditioning treatment would prime hiPSC-NSCs differentiation under proliferation conditions, we immunostained hiPSC-NSCs right after a preconditioning paradigm under proliferation condition with β -III tubulin. The quantification analysis showed that there was no difference in the number of β -III tubulin+ newborn neurons under proliferative conditions between control or 50 μ M metformin (Figure 7, n=3, p-value<0.05).

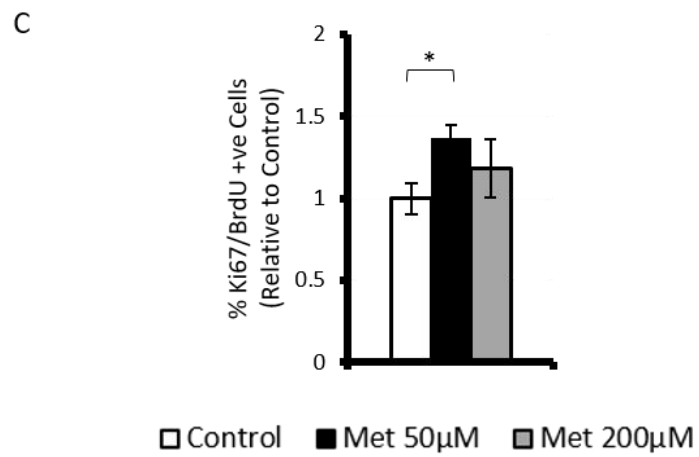
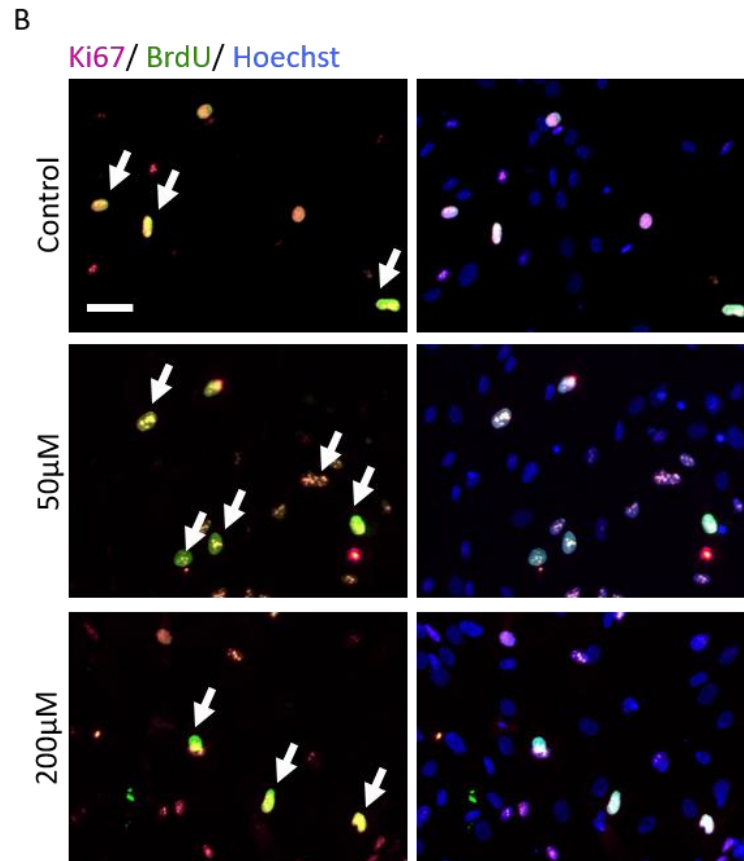
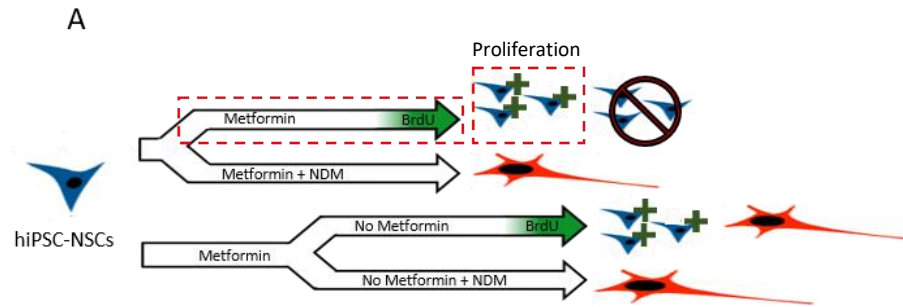
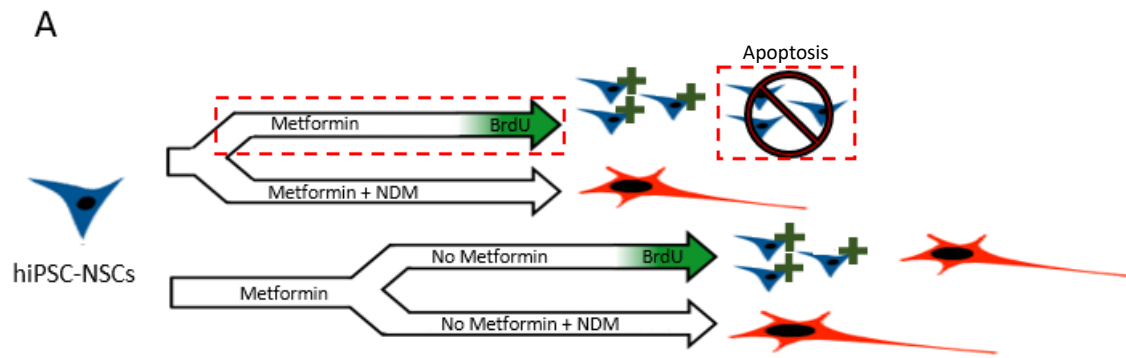
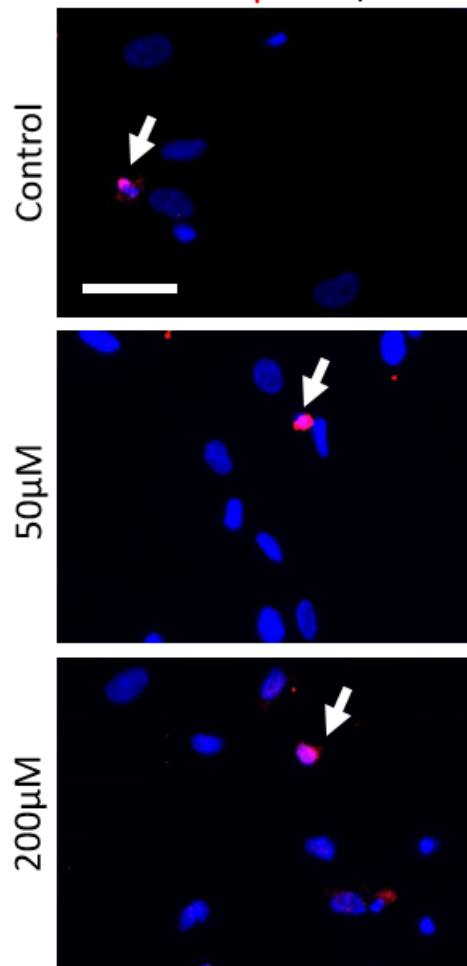


Figure 2. Metformin increases the proliferation of hiPSC-NSCs in a dose-dependent manner in culture. (A) Experimental outline for *in vitro* analysis. Red box represents the specific experiment described. (B) Photographs of hiPSC-NPCs immunostained with Ki-67 and BrdU after 3 days of metformin treatment (Control, 50 μ M and 200 μ M) under proliferation conditions. (C) Quantification of the percentage of co-labeled cells over total live cells in culture, normalized to control group. * $p < 0.05$, Data was quantified from 4 independent experiments. Scale bar: 40 μ m.



B Cleaved Caspase 3/ Hoechst



C

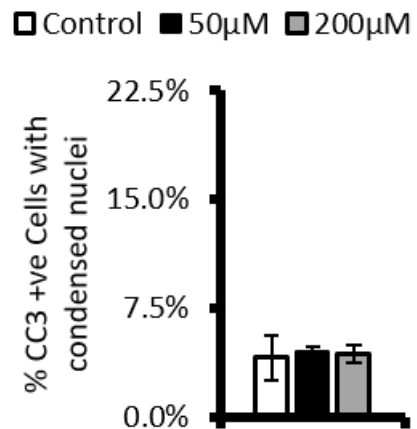


Figure 3. Metformin does not alter cell survival of hiPSC-NSCs in culture (A) Experimental outline for *in vitro* analysis. Red box represents the specific experiment described. (B) Photographs of cultured hiPSC-NSCs immunostained with cleaved caspase 3, an apoptotic marker, 3 days after metformin treatment (Control, 50 μ M and 200 μ M) under proliferation conditions. (C) Quantification of the percentage of cleaved caspase 3+ cells with condensed nuclei over total cells counterstained with Hoechst. Data was quantified from 3 independent experiments. Scale bar: 40 μ m.

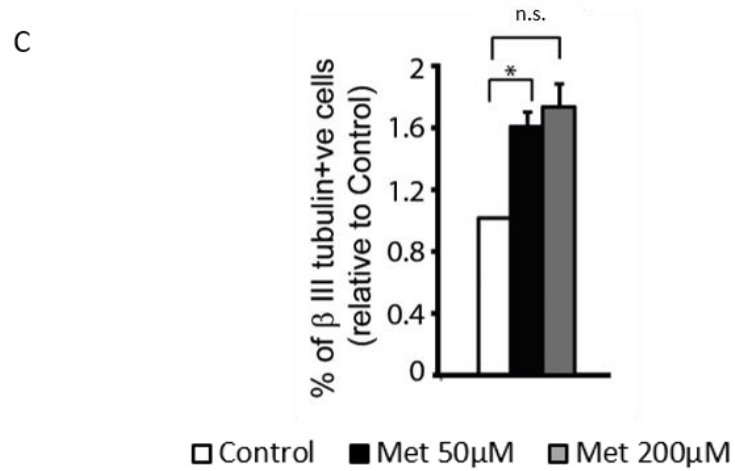
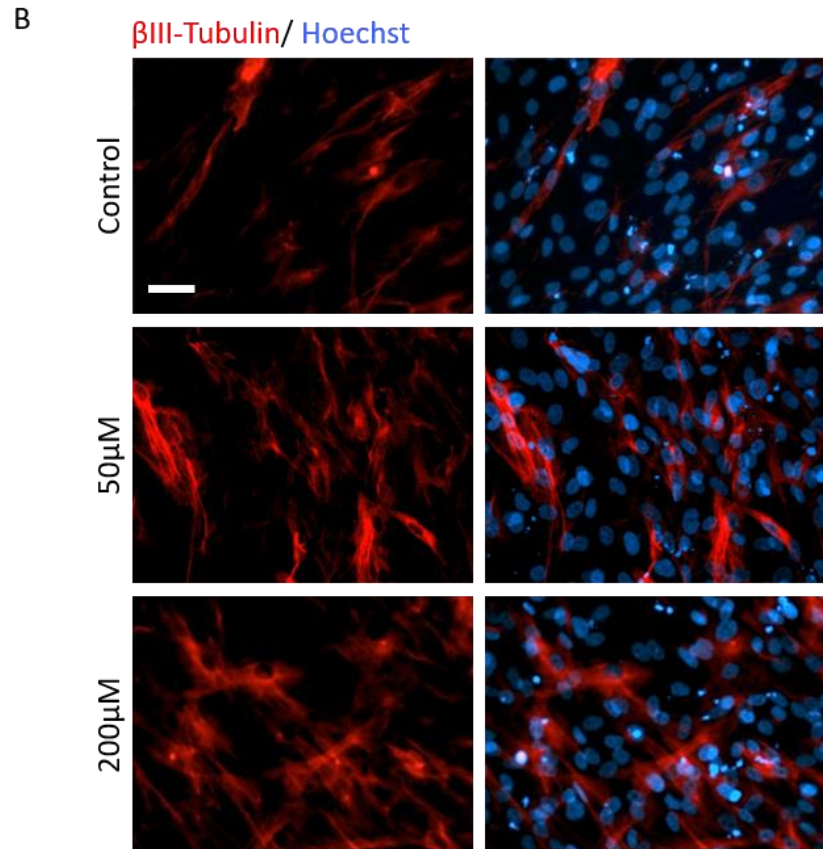
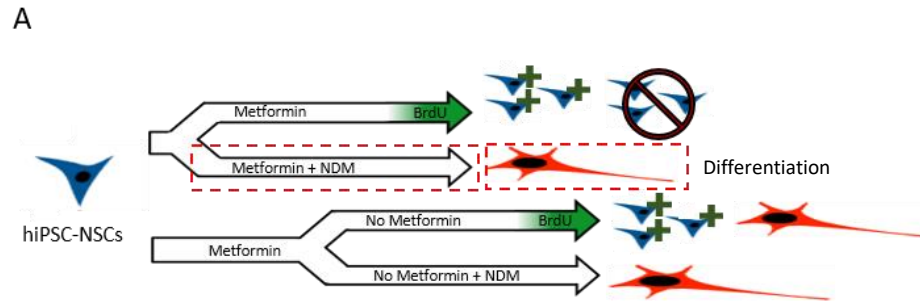
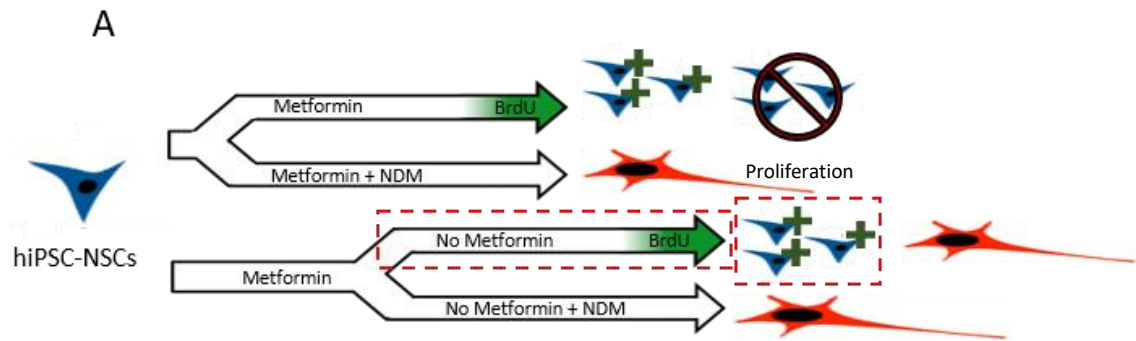
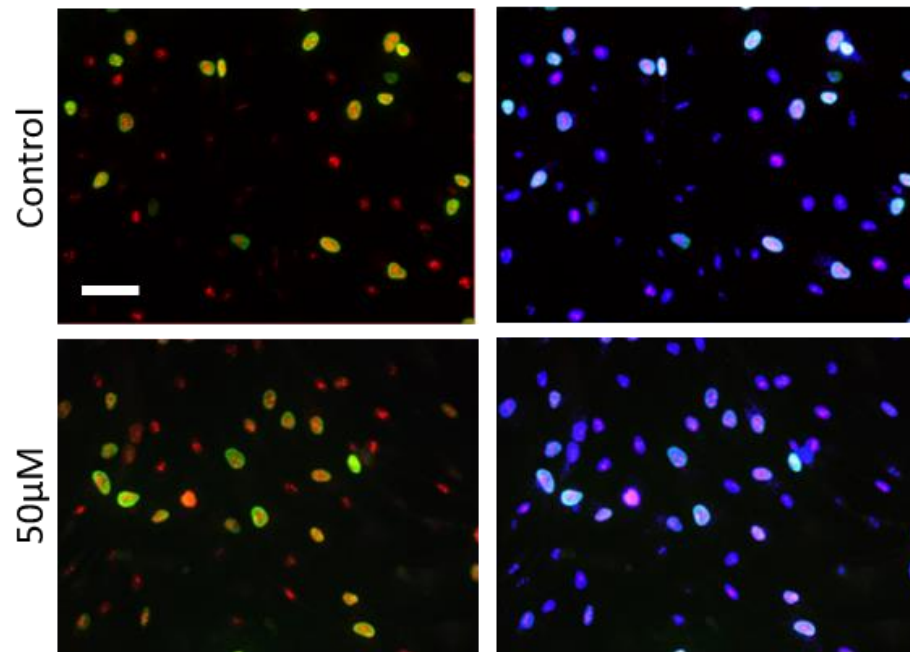


Figure 4. Metformin promotes the differentiation of hiPSC-NSCs in culture. (A) Experimental outline for *in vitro* analysis. Red box represents the specific experiment described. (B) Photographs of cultured hiPSC-NSCs immunostained with β -III tubulin+ 4 days after metformin treatment (Control, 50 μ M and 200 μ M) under neuronal differentiation conditions. (C) Quantification of the percentage of β -III tubulin+ cells over total live cells in culture, normalized to control group. * $p < 0.05$, Data was quantified from 3 independent experiments. Scale bar: 40 μ m.



B Ki67/ BrdU/ Hoechst



C

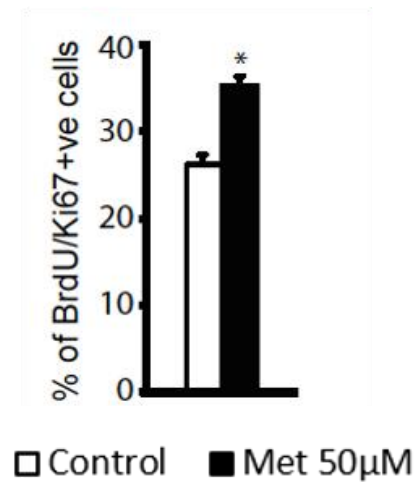


Figure 5. Metformin preconditioning enhances the proliferation of hiPSC-NSCs in culture. (A)

Experimental outline for *in vitro* analysis. Red box represents the specific experiment described.

(B) Photographs of hiPSC-NSCs immunostained with Ki-67 and BrdU, counterstained with Hoechst, under the proliferation conditions 3 days after passaging from metformin/control preconditioning (50 μ M Metformin for 5 d.i.v.) groups. (C) Quantitative analysis of the percentage of Ki-67+/BrdU+ co-labeled proliferating cells out of total live cells. *: $p < 0.05$. Data was quantified from 3 independent experiments. Scale bar: 40 μ m.

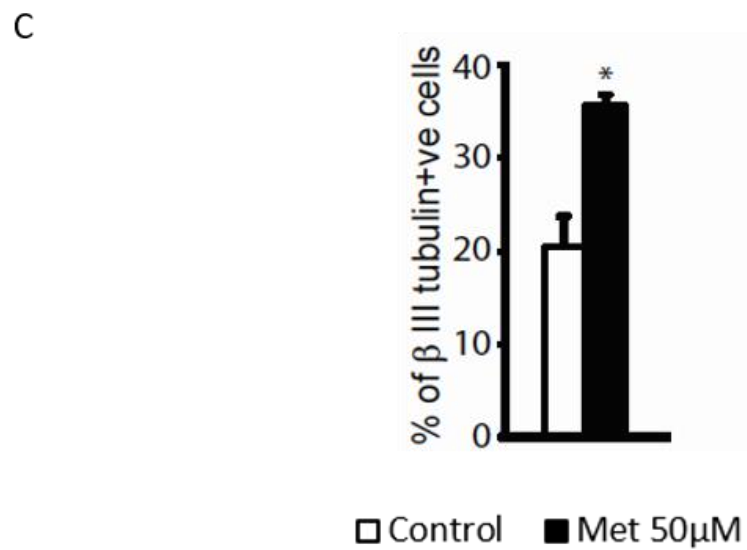
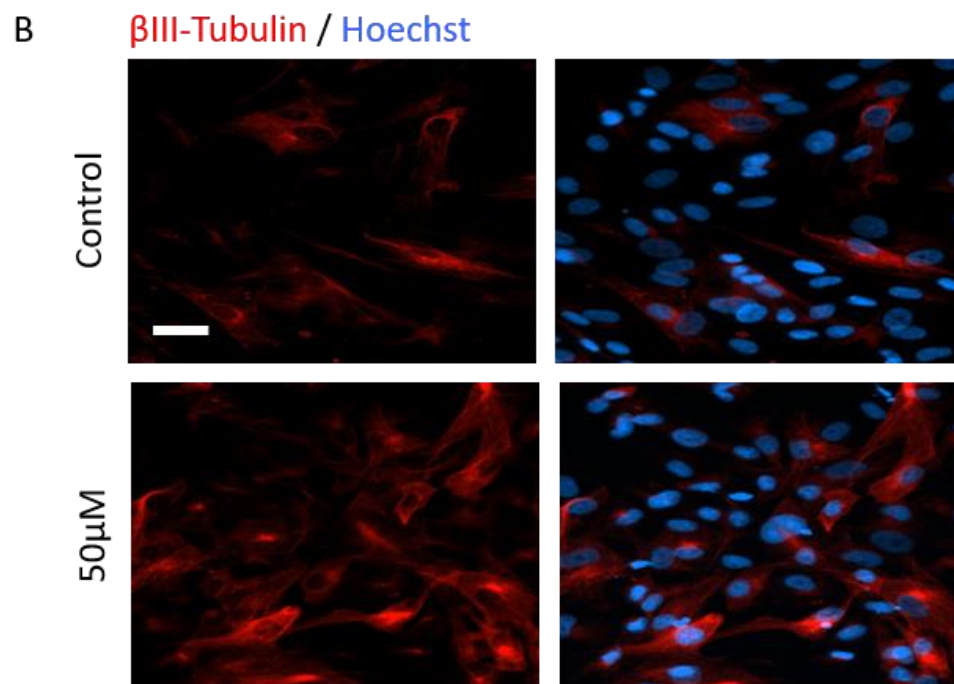
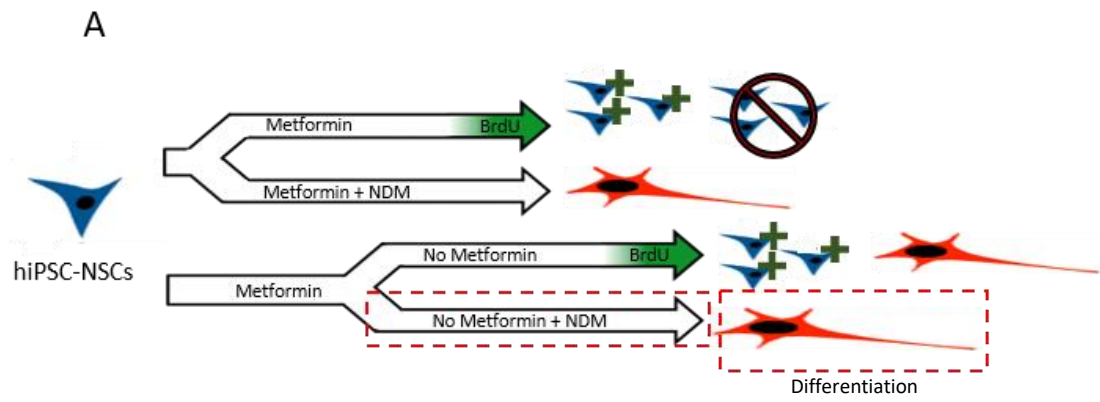


Figure 6. Metformin preconditioning promotes the differentiation of hiPSC-NSCs in culture. (A)

Experimental outline for *in vitro* analysis. Red box represents the specific experiment described

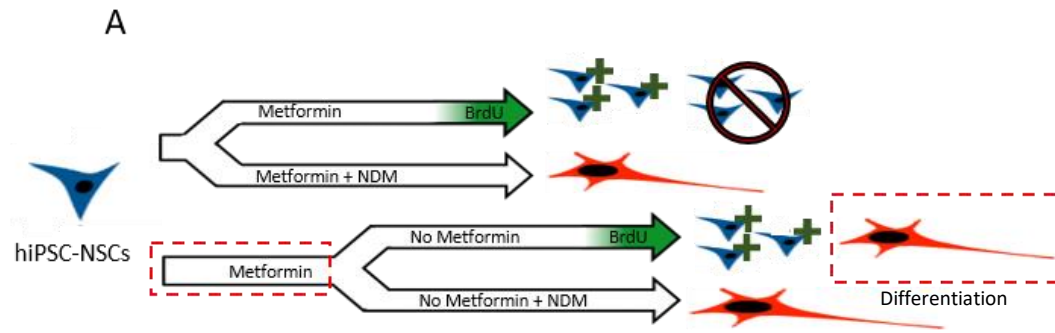
(B) Photographs of cultured hiPSC-NSCs under the neuronal differentiation conditions for 4 days,

immunostained for β -III tubulin and counterstained with Hoechst, after passaging from

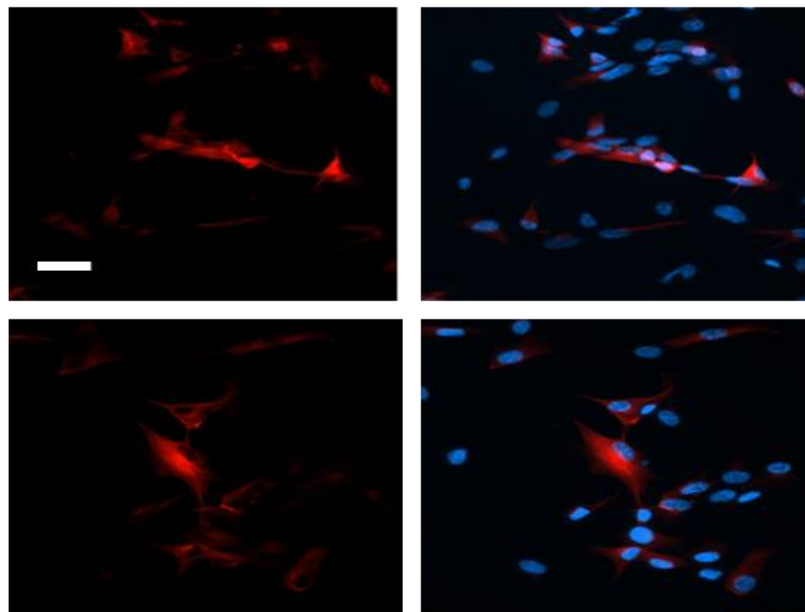
metformin/control preconditioning (50 μ M Metformin for 5 d.i.v.) groups. (C) Quantitative

analysis of the percentage of β -III tubulin+ newborn neurons out of total live cells. * $p < 0.05$. Data

was quantified from 3 independent experiments. Scale bar: 40 μ m.



B β III-Tubulin / Hoechst



C

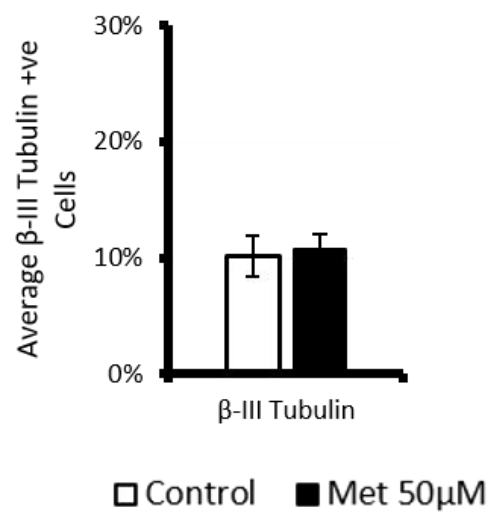


Figure 7. Metformin does not promote the differentiation of hiPSC-NSCs under normal proliferation conditions. (A) Experimental outline for *in vitro* analysis. Red box represents the specific experiment described (B) Photographs of cultured hiPSC-NSCs under the proliferation conditions in the absence and presence of metformin for 4 days, immunostained for β -III tubulin and counterstained with Hoechst. (C) Quantitative analysis of the percentage of β -III tubulin+ newborn neurons out of total live cells. * $p < 0.05$. Data was quantified from 3 independent experiments. Scale bar: 40 μ m.

4.3 Metformin reduces histone acetylation and the expression of Monoacylglycerol lipase in hiPSC-NSCs.

We previously showed that the activation of aPKC-CBP pathway is required for metformin to induce neuronal differentiation of adult murine NPCs⁹⁰. Now our unpublished work in the lab has identified that monoacylglycerol lipase (MglI) is the downstream target of the aPKC-CBP pathway. Work from other lab members showed that MglI repression caused by the activation of the aPKC-CBP pathway mediates metformin-induced neuronal differentiation in primary murine NPCs. To determine whether metformin stimulates the same pathway to induce the neuronal differentiation of hiPSC-NSCs, we performed western blot analysis using metformin treated hiPSC-NSCs to assess histone acetylation and MglI protein expression. Metformin-treated hiPSC-NSCs showed a significant decrease in H2B acetylation at lysine 5 when compared to control (Figure 8A and 8B) suggesting a potential role of reduction of H2B acetylation in metformin-induced differentiation of hiPSC-NSCs. Consistent with reduced H2B acetylation, I observed that MglI protein expression was reduced in metformin-treated group, most likely through reduced CBP binding and histone acetylation on the MglI gene promoter region (n=3, p-value<0.05).

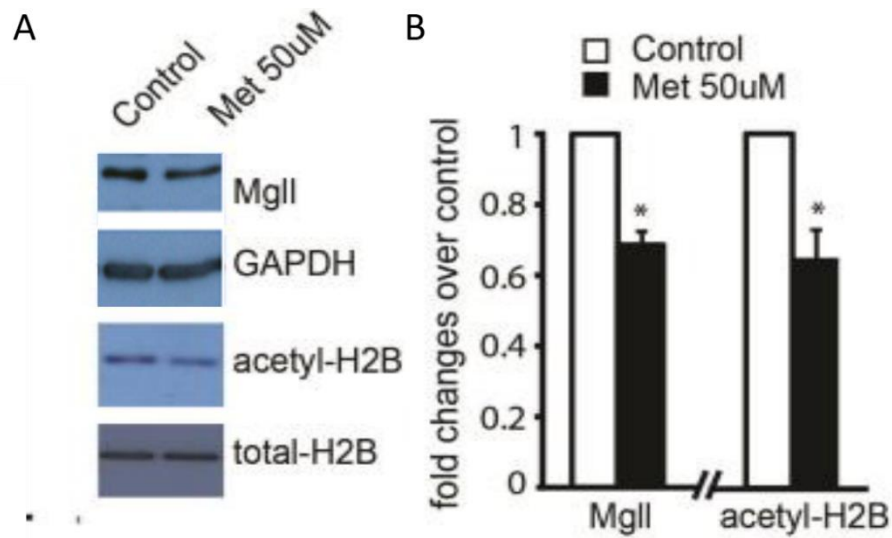


Figure 8. Metformin treatment reduces histone acetylation and MglI protein expression in hiPSC-NSCs. (A) Representative western blot gel images of total lysates from control and metformin preconditioned (50 μ M Metformin, 5 d.i.v. treatment,) hiPSC-NSCs, probed for MglI and acetyl-H2B, using GAPDH and total H2B as loading controls, respectively. (B) Quantitative analysis of monoacylglycerol lipase and acetylated H2B protein expression levels, normalized to control * $p < 0.05$, Data analysis was quantified from 3 independent experiments.

4.4 Metformin preconditioning prior to *in vivo* transplantation improves hiPSC-NSC survival 1-week post-transplantation in a rat ET-1 stroke model

To determine whether metformin preconditioning of hiPSC-NSCs in culture prior to the transplantation improves their initial engraftment in a rat ET-1-induced cortical injury, I transplanted hiPSC-NSCs which were pre-treated with water (naïve group) or 50 μ M metformin (preconditioning group) for 48 hours into a rat ET-1 stroke model. To generate a ET-1 stroke model, rats were subject to 2 cortical and 1 striatal injections of ET-1 to induce focal ischemia in the motor forelimb cortex and dorsolateral striatum. To avoid peak of cell death following ischemia, rats were scheduled for transplantation 1 week post-stroke. Starting from two days prior to transplant, rats were administered daily 20mg/kg/day cyclosporin A until the end of experiment (Figure 9). Rats were sacrificed and perfused with 4% PFA 1 week post-transplant (2 weeks post-stroke). Exogenous human cells were detected using anti-human Ku80 antibody, also known as STEM101. Quantification analysis revealed that metformin preconditioning exhibited a significant increase in the number of grafted cells from 364 $\pm$ 152 (naïve group) to 1037 $\pm$ 228 (preconditioning group) suggesting improved initial cell survival following transplantation (Figure 10 A and B, n=6, p-value<0.05). Human Ku80+ cells were always located within the infarct core. One of 6 rats from Naïve group did not have any ku80+ cells 1 week post-transplant while all of rats from the preconditioning group show detectable engrafts. We further immunostained brain sections with Ki67, a marker for proliferating cells. One rat in the naïve group had no cells engrafted at one week and was removed from the ki67 quantification. Of naïve Ku80+cells, 19 $\pm$ 6% expressed Ki67, while 33 $\pm$ 8% of preconditioned Ku80+cells expressed Ki67. This data showed a trend toward an increase in proliferation although not significant (p-value = 0.27, n=5 naïve, n=6 preconditioned) (Figure 11A and 11B).

Stroke volumes of transplanted rats from 1 week post-transplant time point were

assessed to address potential effects of engrafted cells on infarct volume. The infarct volumes were similar between naïve and preconditioned transplants with no significant difference in both cortical or striatal infarct volumes (n=6 and 7 for naïve and preconditioned groups respectively, p-value=0.30 and p-value=0.38 respectively) (Figure 12A and 12B).

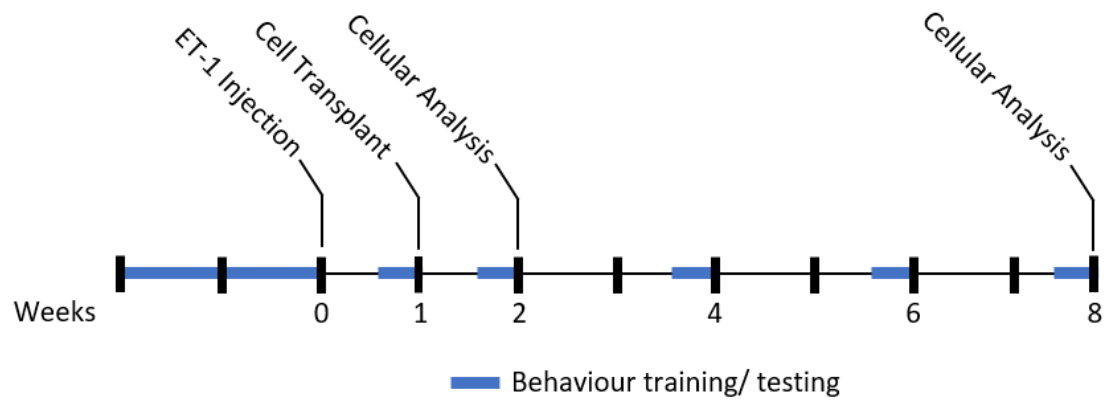


Figure 9. Time line for *in vivo* transplantation of hiPSC-NSCs into rat focal ischemic ET-1 stroke model. Rats were acclimatized for 1 week and handle for 1 week before commencing behavioral training. Figures 10, 11, 12 and 18 assess tissues isolated from 2 weeks post-stroke. Figures 16 and 17 assess tissue isolated from 8 weeks post-stroke.

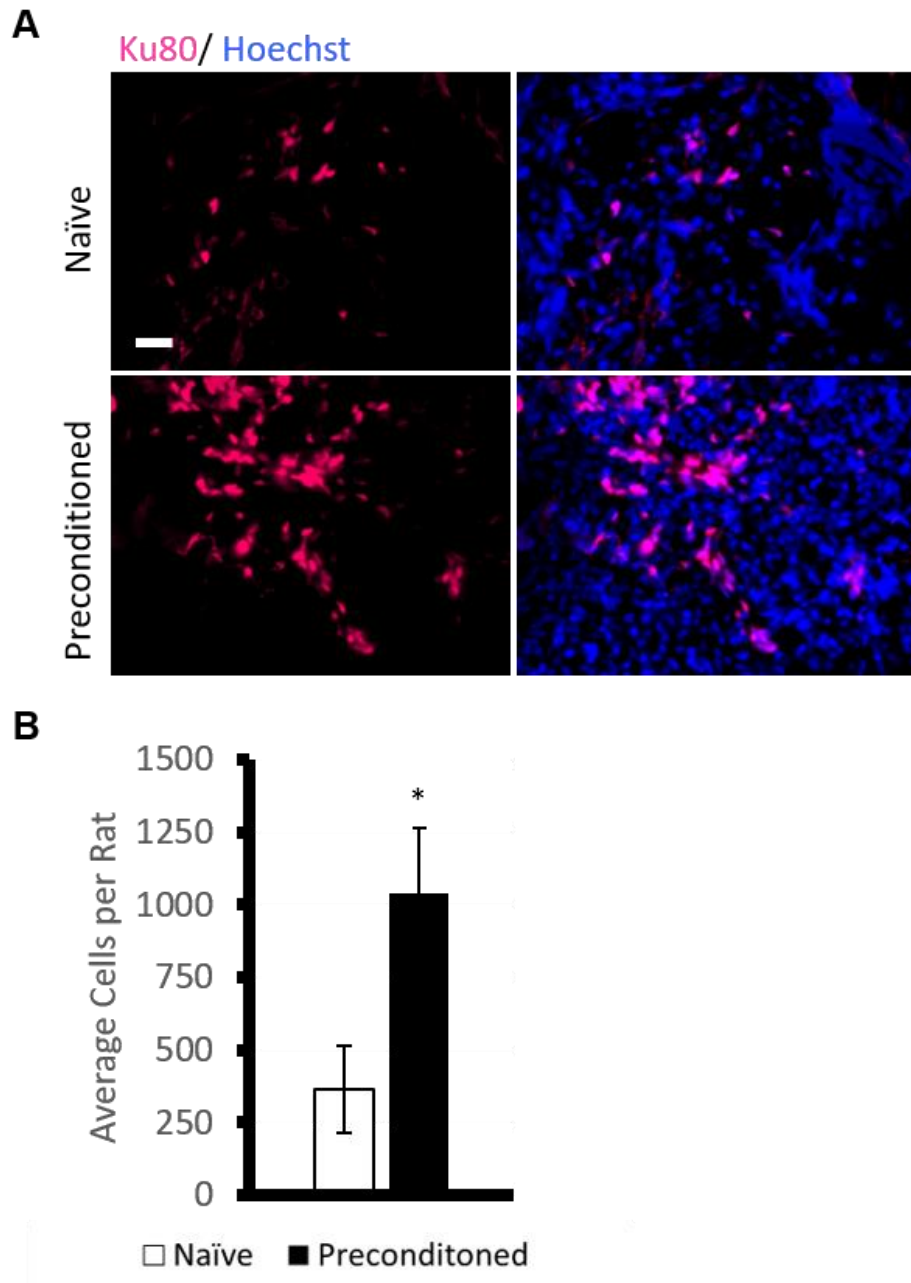


Figure 10. Metformin preconditioning enhances the hiPSC-NSCs engraftment 1 week post-transplantation (A) Photographs of brain sections immunostained for Ku80, a human nuclear marker, in both the naïve and preconditioning groups 1 week post-transplant. (B) Quantitative analysis of average Ku80+ cells per rat in naïve and preconditioning groups 1 week post-transplant (n=6, p-value<0.05). Scale bar: 40µm

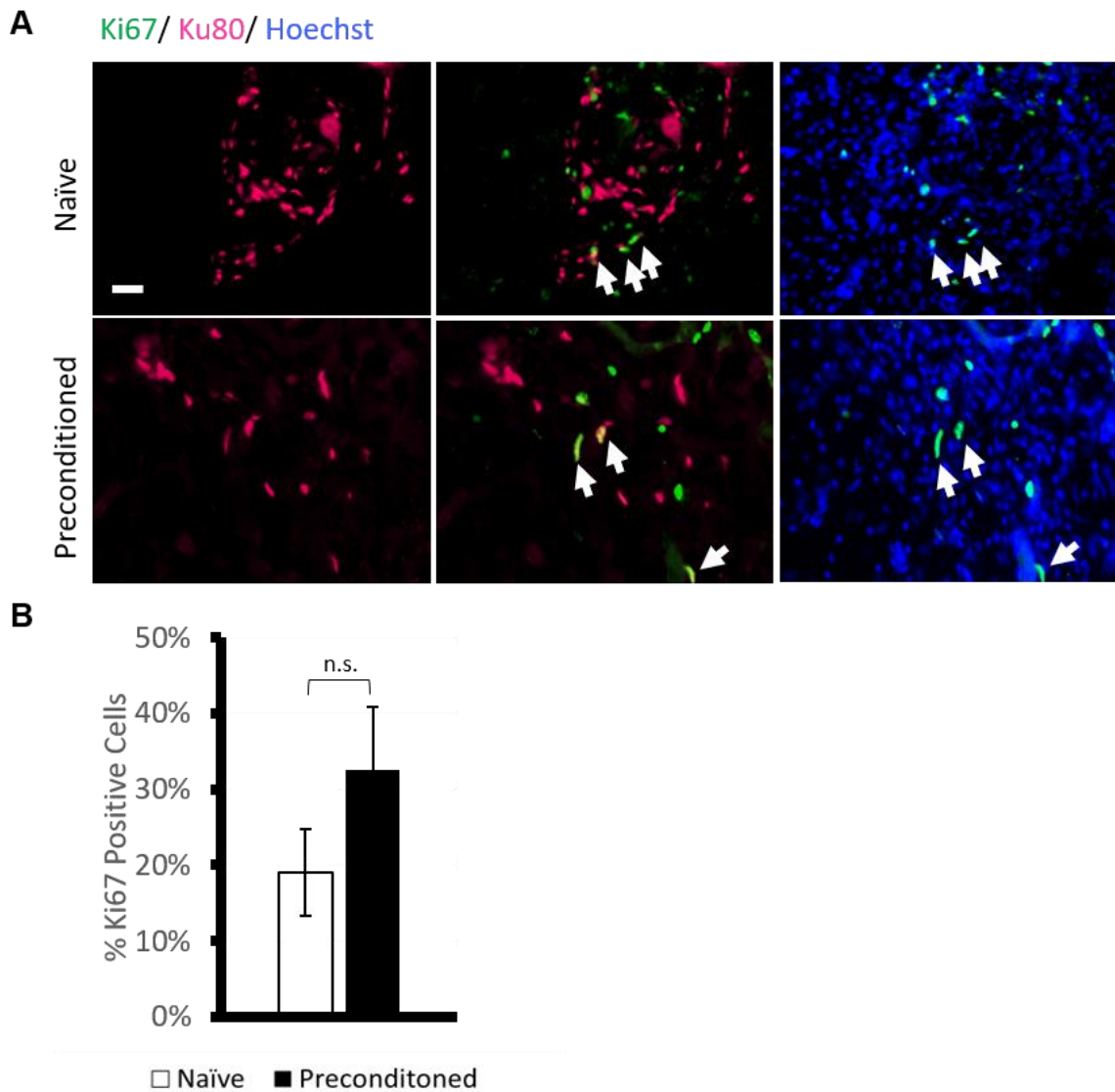


Figure 11. Metformin preconditioning effect on proliferation of hiPSC-NSCs engraft 1 week post-transplantation (A) Photographs of brain sections immunostained for Ku80, a human nuclear marker, and Ki67, a marker for proliferating cells in both the naïve and preconditioning groups 1 week post-transplant. (B) Quantitative analysis of total Ku80+ cells and % Ki67 out of total Ku80+ cells in naïve and preconditioning groups 1 week post-transplant (p-value = 0.27, n=5 naïve, n=6 preconditioned). Scale bar: 40 μ m.

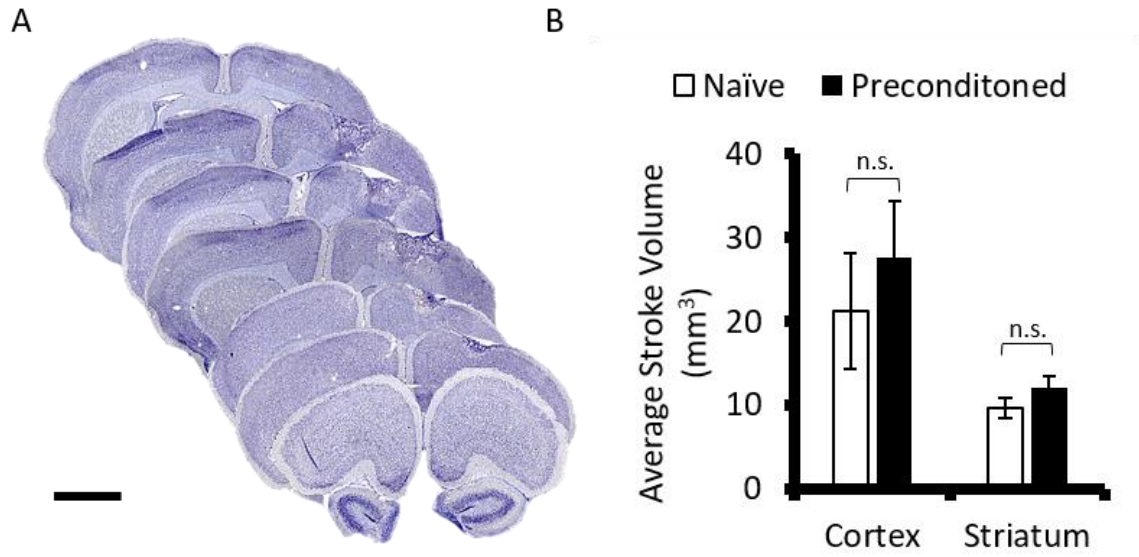


Figure 12. Transplantation of metformin preconditioned hiPSC-NSCs in a rat ET-1 stroke model does not change stroke infarct volume 1 week post-transplant. (A) Serial brain sections stained by cresyl violet and captured using Aperio scanscope. Scale bar: 2mm (B) Quantitative analysis of cortical and striatal infarct volume using cresyl violet staining (Naïve n=6, Preconditioned n=7, $p>0.05$).

4.5 Cell transplantation did not improve functional recovery in forelimb motor behavior tasks.

Several studies have demonstrated that transplantation of iPSC-NSCs into the striatum following MCAo induced ischemia promotes functional recovery in the beam walking and staircase tasks^{60,62,66}. In addition, we observed that metformin preconditioning improved survival of grafts one week post-transplant. To further determine whether metformin preconditioning promote functional recovery during chronic phase of stroke-related brain injury, we treated cell grafts and rats as we described above, followed by behavioural assessment of sensorimotor function up to 8 weeks post-stroke through multiple tests (Figure 9) including the montoya staircase, the beam walking task, and the cylinder test. Furthermore, a group that received ET-1 injections and a sham transplant surgery was included.

In the staircase task, training took a total of 13 days or 26 trials to reach a consistent reaching ability (Figure 13A left panel). No difference in acquisition was observed between groups ($p\text{-value} > 0.05$, $n=7,8,8$ for sham, naïve and preconditioned groups respectively). Time point (1 week) indicates the first test after injection of ET-1 but before cell transplantation. Time point 2 weeks represents the first time point after hiPSC-NSC transplantation. Of rats that successfully learned the staircase task, deficit of approximately 60% in pellets eaten was observed in all groups indicating consistent and significant behavioural deficits among groups (Figure 13B, $p\text{-value} < 0.0001$, $F_{(5,120)}=8.4$). There was no significant difference between groups after cell transplantation in either pellets eaten ($F_{(2,80)}=0.34$), dropped ($F_{(2,80)}=1.4$) or lowest step reached ($F_{(2,80)}=0.47$) (Figure 13B-13D). No spontaneous recovery was observed in pellets eaten between 1 week post-stroke and 8 weeks post-stroke suggesting a major impairment of fine motor function. Furthermore, there is a decreasing trend in average pellets dropped (8 weeks is significantly lower than the 2 week timepoint) and an increasing trend for raised lowest step reaching (6 and 8 weeks are significantly higher than 2 weeks) with no change in pellets eaten.

This suggests that rats gave up on using their contralateral limb at later timepoints potentially because they learned of their limitation and focused on the less-impaired ipsilateral paw.

The cylinder task was used as a measurement of spontaneous forelimb motor function. In the cylinder task, Sprague Dawley rats are placed into a narrow plexiglass cylinder while being video recorded (Figure 14C). The number of paw touches and paw drags is quantified on each forelimb. Deficits are represented by a shift toward increased use of the ipsilateral paw and increase number of paw drags on the contralateral paw. Paw preference index is given by number of touches on the ipsilateral side minus touches on the contralateral side divided by total touches. At one week post-stroke, there was a significant increase in paw drags and paw preference index compared to baseline ($p\text{-value}<0.0001$) with no difference between groups (Figure 14A and 14B). At week 8, there is a significant decrease in paw preference index compared to week 1 reflecting spontaneous recovery for all three groups (Figure 14A, $p\text{-value}<0.05$). Furthermore, at week 6 and 8 there is a significant decrease in paw drags compared to week 1 for all three groups (Figure 14B, $p\text{-value}<0.05$). In summary, cell transplant groups (both naïve and preconditioning groups) gave no beneficial effects on recovery following ET-1 focal stroke, assessed by the cylinder test.

The beam walking task focused on both forelimb and hindlimb motor function. All groups had a significant decrease in successful steps 1 and 2 weeks post-stroke in the contralateral forelimb and hindlimb but not ipsilateral forelimb and hindlimb compared to baseline ($p\text{-value}<0.05$, Figure 15B and 15C). Furthermore, spontaneous recovery was observed after 4 weeks post-stroke in the contralateral hindlimb ($p\text{-value}<0.05$, Figure 15B) and at 8 weeks in the contralateral forelimb compared to 2 weeks post-stroke ($p\text{-value}<0.05$, Figure 15C). Due to the initial deficit difference ($F_{(2,104)}=7.4$, $p\text{-value}<0.01$) in successful steps of the contralateral hindlimb at 1 week post-stroke between preconditioned and sham/naïve groups before the transplant, no improvement in naïve and preconditioned groups was observed compared to sham.

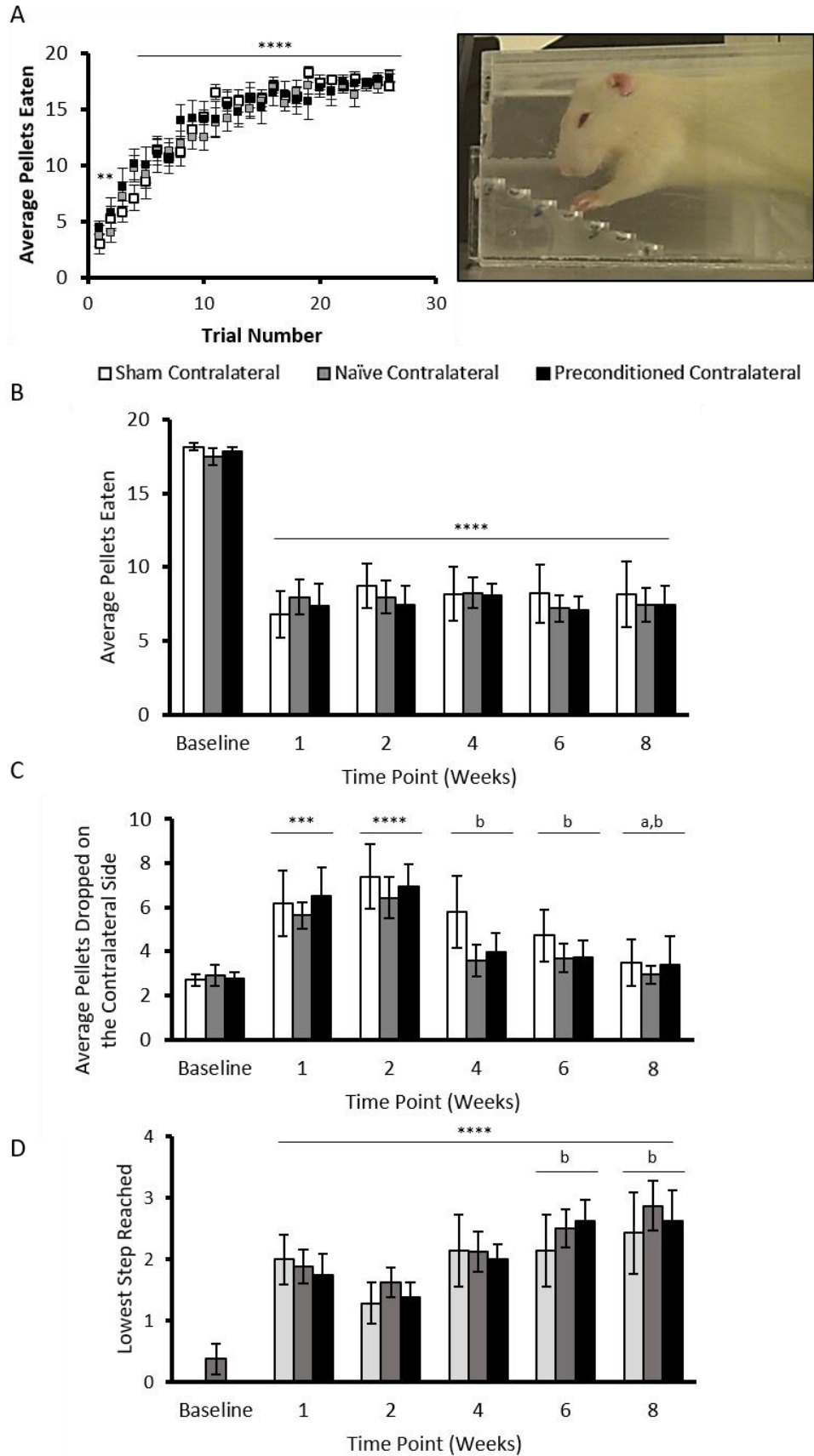


Figure 13. Functional motor assessment of recovery evaluated through the Montoya staircase following transplantation. (A) Left Panel: Staircase acquisition curve organized by treatment group. Average pellets eaten on their highest performing side over 26 trials (2 trials/day). **: p-value<0.01, ****: p-value<0.0001 compared to trial 1. Significant difference by time (p-value<0.0001, $F_{(25,520)}=44$), but not between groups. Right Panel: Image of rat reaching for a pellet in well #4 during staircase testing. (B) Average pellets eaten on their contralateral side. Significant difference by time (p-value<0.0001, $F_{(5,120)}=32$), but not between groups. (C) Quantitative analysis of average pellets dropped on the contralateral side. Significant difference by time (p-value<0.0001, $F_{(5,120)}=8.4$), but not between groups. (D) Quantitative analysis of lowest step reached on the contralateral side. Significant difference by time (p-value<0.0001, $F_{(5,120)}=18$), but not between groups. *: p-value<0.05, **: p-value<0.01, ***: p-value<0.001, ****: p-value<0.0001 compared to baseline. a: p-value<0.05 compared to week 1, b: p-value<0.05 compared to week-2.

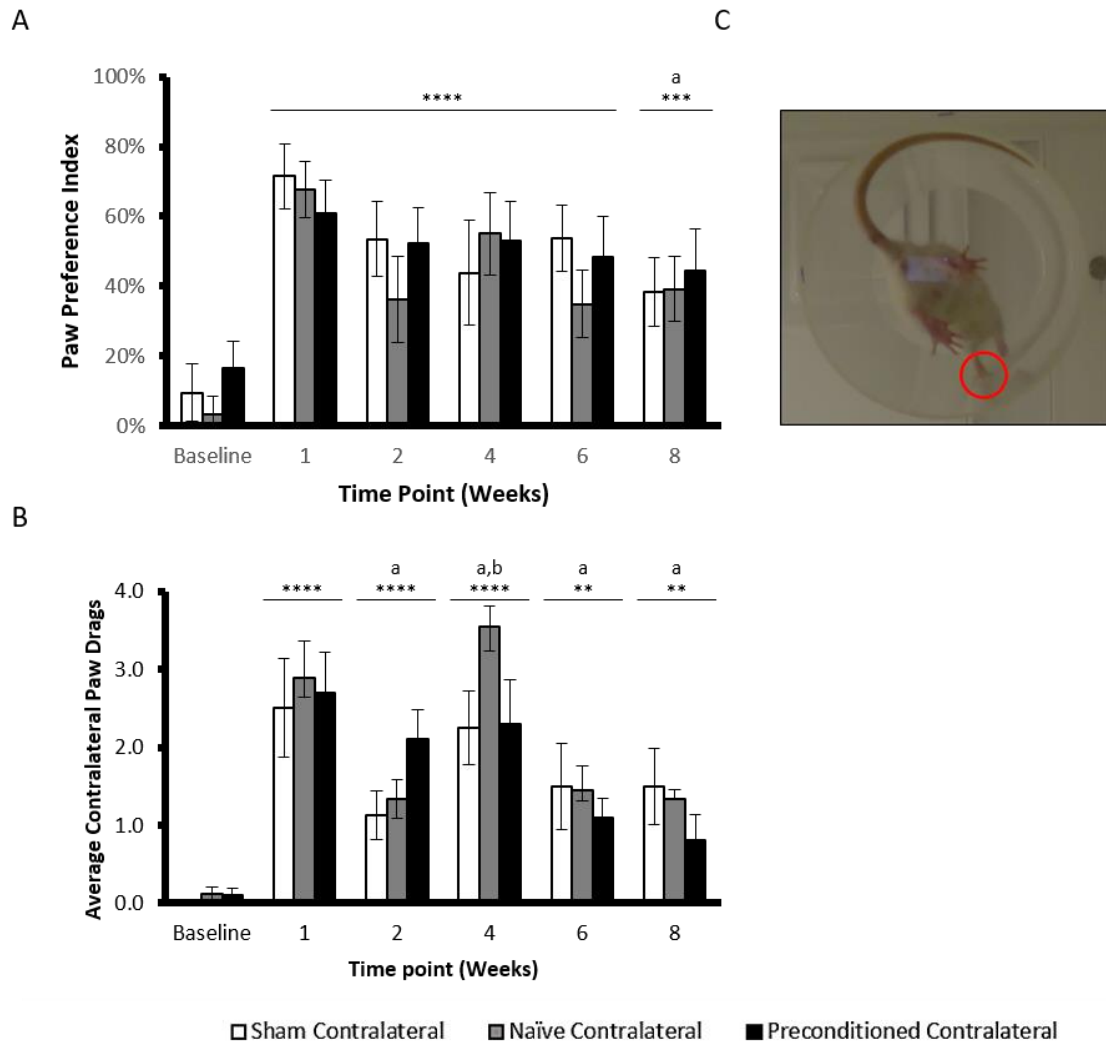


Figure 14. Functional motor assessment of recovery evaluated through the cylinder test following transplantation. (A) Paw preference index calculated as the difference in paw touches between sides divided by their sum. There was a significant difference by time ($p\text{-value} < 0.0001$, $F_{(5,156)}=10$), but not between groups. (B) Quantitative analysis of average paw drags on the contralateral side. There was a significant difference by time ($p\text{-value} < 0.0001$, $F_{(5,156)}=21$), but not between groups. (C) Example image of a left forelimb touch in the cylinder test. *: $p\text{-value} < 0.05$, **: $p\text{-value} < 0.01$, ***: $p\text{-value} < 0.001$, ****: $p\text{-value} < 0.0001$ compared to baseline. a: $p\text{-value} < 0.05$ compared to week 1, b: $p\text{-value} < 0.05$ compared to week 2.

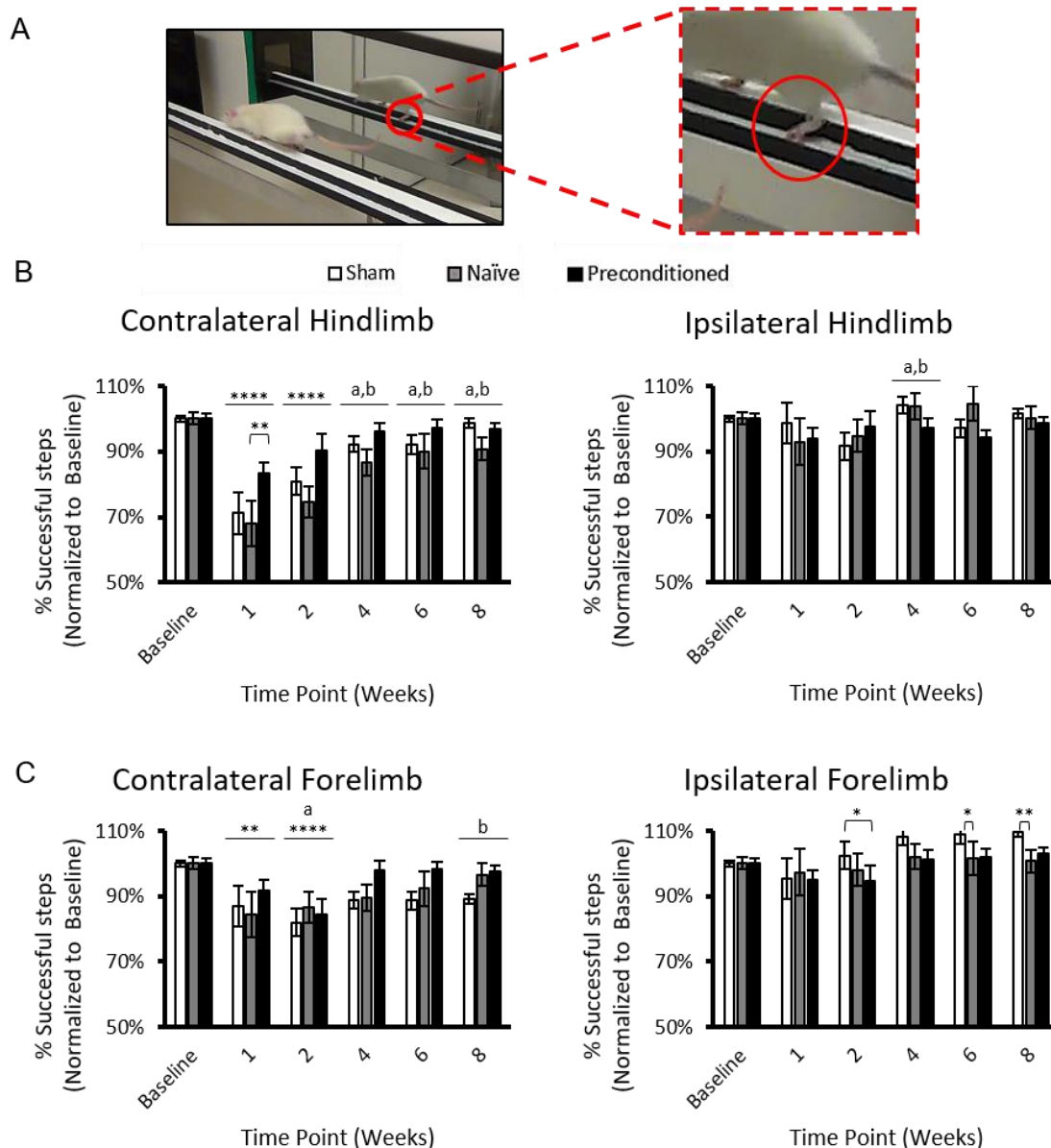


Figure 15. Functional motor assessment of recovery evaluated through the beam walking task following transplantation. (A) Example of a foot fault indicated by paw placement on the 2nd ledge of the beam. (B) Quantitative analysis of percent successful steps normalized to baseline on the contralateral and ipsilateral hindlimbs. There was a significant difference by time (p -value <0.0001 , $F_{(5,156)}=19$) and between groups (p -value <0.01 , $F_{(2,104)}=7.4$) in contralateral steps. (C) Quantitative analysis of percent successful steps normalized to baseline on the contralateral and ipsilateral

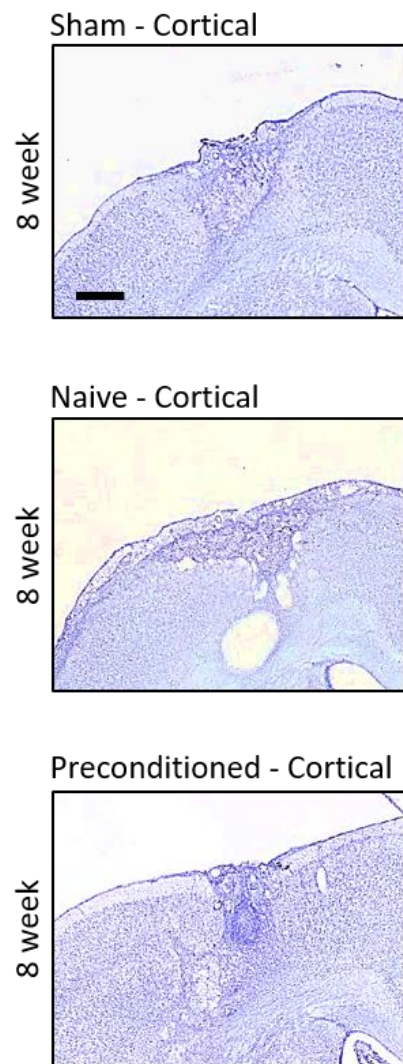
forelimb. There was a significant difference by time ($p\text{-value} < 0.0001$, $F_{(5,156)} = 3.5$), but not between groups in contralateral forelimb steps and no difference in time but a difference between groups in ipsilateral forelimb steps $p\text{-value} < 0.0001$, $F_{(2,104)} = 15$. *: $p\text{-value} < 0.05$, **: $p\text{-value} < 0.01$, ***: $p\text{-value} < 0.001$, ****: $p\text{-value} < 0.0001$ compared to baseline or as indicated. a: $p\text{-value} < 0.05$ compared to week 1, b: $p\text{-value} < 0.05$ compared to week 2.

4.6 Metformin preconditioning improves cell survival 8 weeks post-stroke

Following behavioural testing, rats were perfused with 4% PFA for histological analysis. Cortical and striatal infarct volumes at 8 weeks post-stroke were also determined through cresyl violet staining. A trend towards decreasing cortical infarct size between naïve and preconditioned grafts was observed but not significant with averages of $19 \pm 4 \text{ mm}^3$, $15 \pm 4 \text{ mm}^3$ and $10 \pm 2 \text{ mm}^3$ for sham, naïve and preconditioned groups respectively (p-value > 0.05, $F_{(2,13)}=1.23$, Figure 16A and 16B). No significant difference was observed between striatal infarct sizes (Figure 16B, p-value>0.05). There was no significant difference in naïve cortical and striatal stroke volume between 2 weeks post-stroke and 8 weeks post-stroke (p-value>0.05) but a decrease in cortical stroke volume (p-value<0.01) in the preconditioned group (Figure 16C).

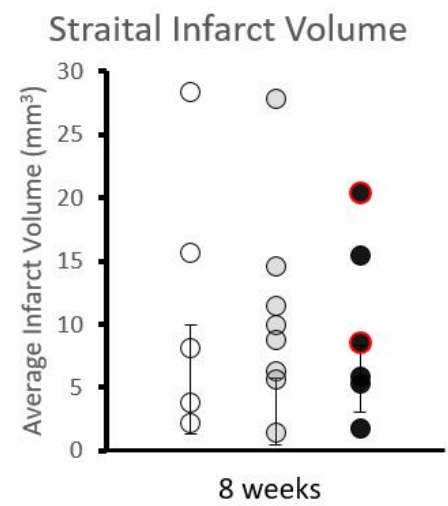
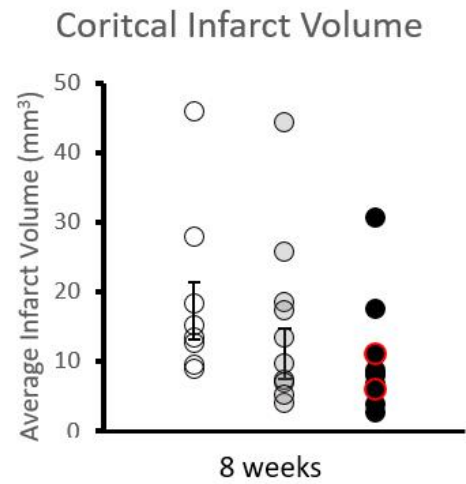
To determine the number of surviving transplants, immunostaining for Ku80 was performed on all sections with spanning the cortical infarct. Two of five preconditioned-treated grafts were detected while none of the naïve rats had detectable grafts. Of these two grafts, both appeared as an aggregate of cells filling the infarct core, bordering the peri-infarct area (Figure 16 and 17A-17D). On average between the two rats there was $23,023 \pm 2514$ cells/ rat. (n=2 preconditioned). To determine the extent of differentiation of these grafts, immunostaining for neuronal and glial markers, β -III tubulin, GFAP and Olig2, was performed. Grafts expressed on average $36 \pm 9\%$ β -III tubulin, $24 \pm 6\%$ GFAP and $7 \pm 1\%$ Olig2 (Figure 17A-17C and 17E). Furthermore, qualitative analysis shows strong Nestin staining adjacent to the infarct core corresponding to the glial scar from endogenous cells and weaker nestin staining within the grafts ball (Figure 17D).

A



B

○ Sham ○ Naïve ● Preconditioned



C

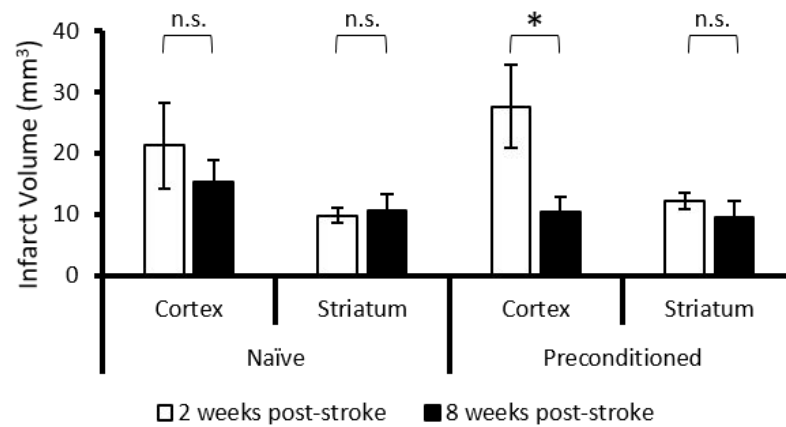


Figure 16. Cresyl Violet staining to analyze infarct volume 8 weeks post-stroke following transplantation. (A) Photographs cortical infarcts of sham, naïve and preconditioned groups stained with cresyl violet and captured using Aperio scanscope. Two preconditioned-graft treated rats show a nuclei dense area corresponding to Ku80+ grafts (lower panel). Scale bar: 0.5mm. (B) Quantitative analysis of cortical and striatal infarct volumes using cresyl violet staining. No difference among groups in cortical (Sham n=9, Naïve n=10, Preconditioned n=10, $p>0.05$) or striatal volumes (Sham n=5, Naïve n=8, Preconditioned n=6, $p\text{-value}>0.05$). Red highlighted markers correspond to rats with grafts at 8 weeks post-stroke. (C) Quantitative analysis of cortical and striatal infarct volume compared between 2 weeks post-stroke and 8 weeks post-stroke. *: $p\text{-value}<0.05$.

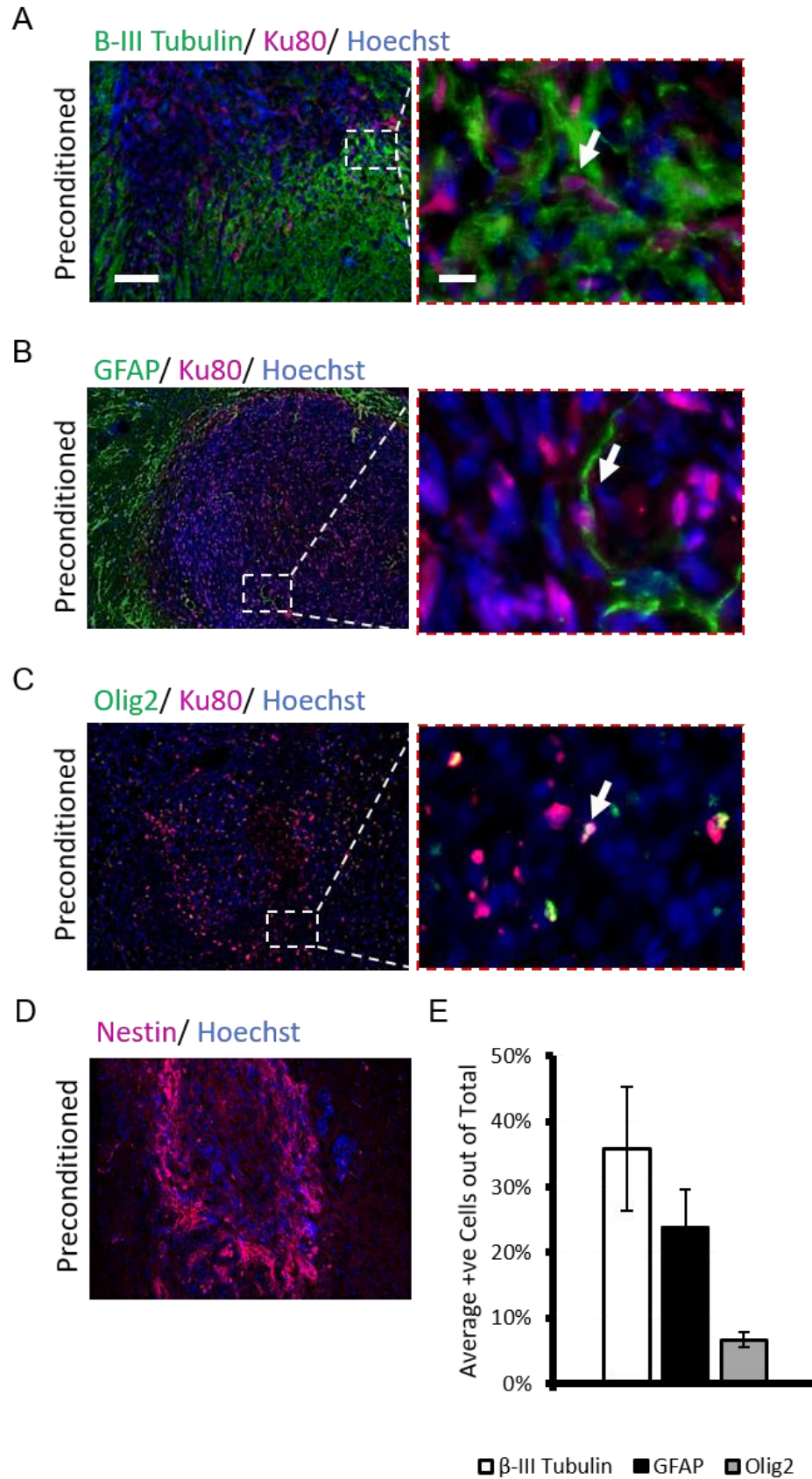


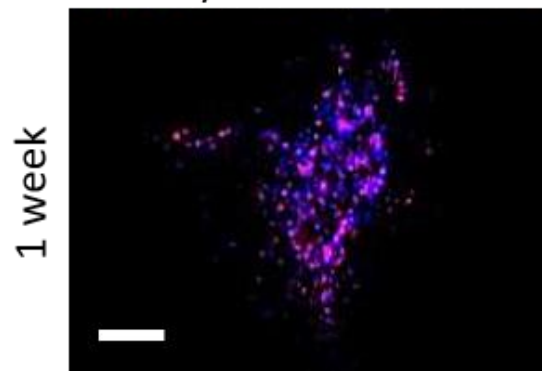
Figure 17. Assessment of cell identity of grafts 8 weeks post-stroke. (A-D) Micrographs of brain sections immunostained for β -III tubulin+, GFAP+, Olig2+, and Nestin+ 8 weeks post-stroke. Left panels scale bar: 100 μ m. Right panels scale bar: 20 μ m (E) Quantitative analysis of the percentage of β -III Tubulin+, GFAP+, and Olig2+, cells over total human Ku80 8 weeks post-transplant (n=2). Two of Five (40%) preconditioning grafts were detected 8 weeks post-transplant compared to 0 of 5 in the naïve treated grafts.

4.7 Fluorescent dyes label endogenous Iba+ cells and are unsuitable for cell tracing in ischemic transplant studies

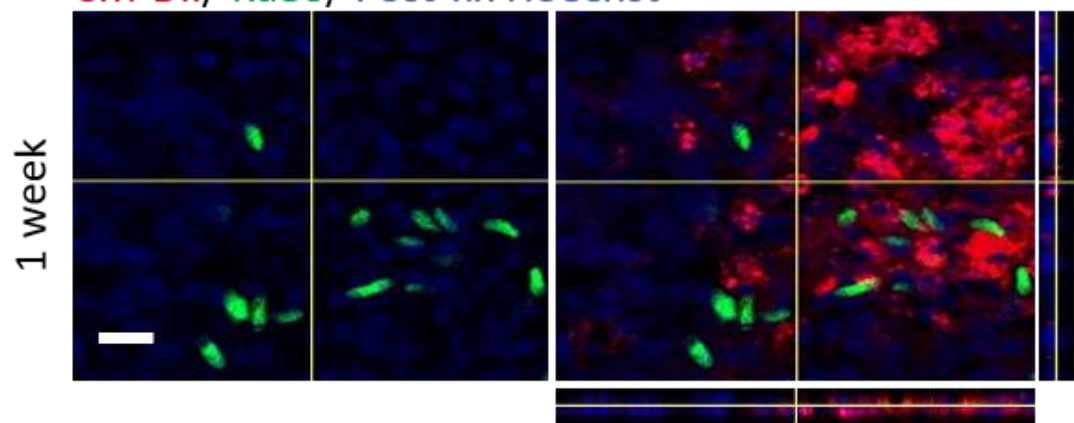
Initial pilot experiments highlighted difficulties in detecting transplanted cells with anti-human specific antibodies including anti-human mitochondrial antigen, anti-HLA Class I, anti- β microglobulin and anti-Human nuclear antigen (Data not shown). As such, the use of pre-labeling with fluorescent dyes was explored as they have been used in other studies of transplantation. Cells were pre-labeled with Hoechst and CM-Dil prior to the transplantation. Initial tissue analysis showed promise with double-labelled cells (CM-Dil+/ Hoechst+) in the cortical infarct regions 1-week post-transplant (Figure 18A). CM-Dil labelled grafted cells for the 8-week experiment were difficult to distinguish from background (data not shown). This was potentially due to a diluting effect following multiple dividing processes, uptake of CM-Dil debris from dying exogenous human cells into endogenous microglia that were recruited into the infarct region, or decrease in dye fluorescence.

As such, we reverted to immunolabeling techniques using a relatively new human marker STEM101 or anti-Ku80. Immunostaining of 1-week samples with anti-Ku80 yielded lower engraftment values as observed by CM-Dil. Furthermore, many cells were labeled with CM-Dil despite being Ku80- (Figure 18B). Indeed, there were Iba+/CM-Dil+ cells in the infarct core suggesting uptake of the dye to endogenous microglia (Figure 18C).

A CM-Dil/ Pre-labeled Hoechst



B CM-Dil/ Ku80/ Post-fix Hoechst



C CM-Dil/ Iba1

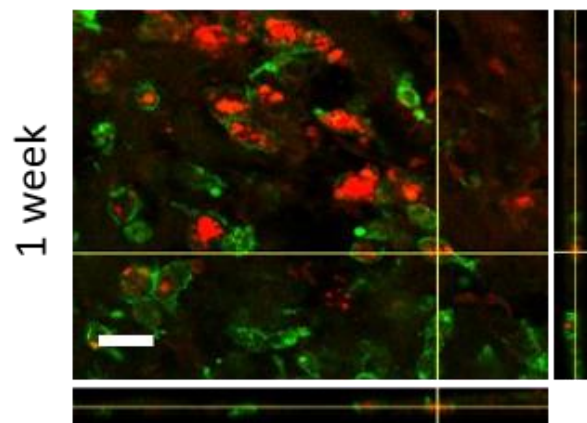


Figure 18. Evaluation of prelabeling grafts with florescent dyes for cell tracing following transplantation into ischemic stroke. (A) Photomicrographs of CM-Dil+ and Hoechst+ cells in the cortical ischemic lesion 1-week post-transplant. Cells were labeled with CM-Dil and Hoechst *ex vivo* prior to transplant. Scale bar: 300 μm . (B) Immunostaining of human marker Ku80 in CM-Dil prelabeled grafts. Orthogonal view of CM-Dil+/Ku80- cell. Scale bar: 50 μm . (C) Orthogonal view of CM-Dil+/Iba+ cell in the ischemic lesion 1-week post-transplant. Scale bar: 50 μm .

5.0 Discussion

To optimize neural stem cell transplant treatment for stroke, I preconditioned hiPSC-NSCs with metformin, an FDA-approved drug, prior to cell transplantation to determine whether preconditioning promotes hiPSC-NSC neuronal differentiation, proliferation and cell survival *in vivo*. I successfully generated hiPSC-NSCs from an iPS line, WLS-1C. In the culture experiment, I observed that metformin treatment promoted proliferation and differentiation of hiPSC-NSCs through both direct stimulation and a preconditioning paradigm. My study also provided a novel potential molecular mechanism of action for metformin-induced differentiation of neural stem cells by repressing Mgl1 expression. Furthermore, metformin preconditioning increased cell engraftment 1-week post-transplant and increased the number of surviving grafts 8 weeks post-stroke compared to naïve grafts. In addition, cell transplantation did not result in improve functional recovery when compared to sham group in this model. In summary, my studies revealed that metformin preconditioning is a vital step to optimizing cell transplantation for stroke.

5.1 Metformin promotes the proliferation and differentiation of human iPSC-NSC in culture

Previous studies in the laboratory have shown that metformin promotes both the differentiation and proliferation of primary murine adult NSCs^{90–92}. To be able to translate our basic research into clinical application in humans, I further confirmed the neuroregenerative capability of metformin in hiPSC-NSCs. This is the first time that metformin has been shown to promote both the neuronal differentiation and proliferation of hiPSC-NSCs, further supporting that hiPSC-derived NSCs resemble phenotypes of other NSCs in culture^{90–92}. In this study, I used a 8-hour BrdU chasing assay to synchronize cultured proliferating cells and assess their proliferation

at the S phase, resulting in the same conclusions as other proliferation assays, such as neurosphere assays, used previously^{90,93}.

The doses used in this experiment are different than those used in other experiments working on different cell types^{90,100,104,105}. In general, nanomolar range of metformin concentration is required for enhancing neurogenesis from primary rodent NSCs⁹⁰, while micromolar and millimolar ranges of metformin concentration are required for promoting the differentiation of hiPSC-NSC, hES-NSC and hiPSCs^{104,105}. This could potentially be due to temporal changes in expression levels of metformin's cell surface transporter organic cation transport 1 (OCT1) throughout neural stem cell differentiation¹⁰⁶. Another explanation that could account for the difference is the different metabolic regulation in both rodent and human cells since the direct target of metformin in the cells is to inhibit the mitochondrial complex 1.

To assess the feasibility of metformin preconditioning strategy to be used for cell transplantation, it is imperative to measure whether metformin-pretreated hiPSC-NSCs have the capability to sustain enhanced neuronal differentiation and proliferation in the absence of metformin. Here, for the first time, I showed that metformin-pretreated hiPSC-NSCs increased neuronal differentiation and proliferation when passaged in normal culture condition in the absence of metformin as compared to control group (PBS-preconditioned group) (Figure 5 and 6). To explain this sustained effect, a better understanding of the underlying mechanisms that mediate metformin-induced neuronal differentiation was required. We previously showed that metformin enhances neuronal differentiation by activating the aPKC-CBP pathway. Our recent unpublished work has identified that monoacylglycerol lipase (MgII) is the downstream target of the aPKC-CBP pathway. The working model supported by our preliminary data suggests that metformin activates the aPKC-CBP pathway, by phosphorylation of CBP at Ser436. The p-

CBPS436, will be subsequently removed from MglI gene promoter regions to reduce MglI gene expression.

To further support the working model, I have shown that metformin preconditioning in hiPSC-NSCs reduced the MglI protein expression, associated with reduced histone H2B acetylation, a dominant substrate of CBP. Future chromatin immunoprecipitated (ChIP) assays are required to link global reduction of H2B acetylation to the specific promoter region of MglI. Since MglI is a lipase that hydrolyses the endocannabinoid (2-AG) into arachidonic acid and glycerol¹⁰⁷, A reduction in MglI expression upon metformin preconditioning may promote the differentiation of neural stem cells by accumulating 2-AG through prevention of its hydrolyses in NSCs. 2-AG can then bind endocannabinoid receptors which have been shown to promote the differentiation of NSCs upon activation¹⁰⁸. As such, I propose that metformin preconditioning activated the epigenetic pathway, the aPKC-CBP pathway that persistently reduces H2B acetylation and MglI expression even in the absence of metformin, contributing to an increase of neuronal differentiation of hiPSC-NSCs. These experiments provide significant insight into the molecular mechanisms of metformin and potential approaches for its manipulation.

5.2 Metformin preconditioning promotes cell engraftment 1-week post-transplant

Using a focal ET-1 stroke model, my study for the first time showed that metformin could be used as a preconditioning agent to enhance cell engraftment shortly following transplants. Since over 90% of cell die four days following transplantation¹⁰⁹, the remaining cell population at 1 week post-transplant best represents earliest time point of the surviving graft. The increase in cell engraftment 1-week post-transplant could be due to a multitude of factors. Firstly, the increase could be attributable to the increase in proliferation rate of transplanted hiPSC-NSCs. Although we could not detect a statistical significance, there is a strong trend in increased

proliferation in metformin-preconditioned group (Figure 11). In addition, metformin does promote cell survival in many disease models^{110–113}. For example, preconditioning of rats with metformin prior to ischemia reduced TUNEL staining in the periinfarct¹¹³. It has been hypothesized that metformin increases cell survival in cytotoxic environments through stimulation of autophagy flux^{111,113}. Metformin likely promotes engraftment through one or a combination of these mechanisms.

Although metformin preconditioning promoted cell engraftment, the engrafted cell number observed in both the naïve and metformin preconditioned groups was much less than other neural stem cell transplants studies^{59,60,68}. There are many potential causes. This is the first hiPSC-NSCs transplantation conducted in a focal ET-1 stroke, while all other hiPSC-NSCs transplantation studies used the MCAo model. One reason for lack of engraftment could be due to the type of injury caused by ET-1 compared to that of the MCAo model. Endothelin-1 intracerebral injections could cause axonal damage and astrogliosis not seen in the MCAo model which could hinder cell engraftment^{20,114}. Furthermore, the additional glial scar caused by the needle during intracerebral injections may contribute to poor survival although the extent of damage is likely not enough to result in such a decrease in cell survival. In addition, cyclosporin A, an immunosuppressant agent, may not reach optimal concentration in rats to fully inhibit the adaptive immune system through inactivation and inhibition of proliferation of T-cells¹¹⁵. Partial inactivation of T-cells could result in extensive rejection of xenografted cells. Although our transplantation model did not provide sufficient cell engraftments as shown in other studies^{59,60,68}, it is a valid model to be implemented for developing optimized approaches that will enhance cell engraftment for brain regeneration following stroke-related brain injury.

Since there is the potential for different size of stroke to cause different cell engraftment rate, I examined stroke volumes between naïve cell group and preconditioned cell group 1-week

post-transplant and found no significant difference between two groups (Figure 12). This data suggests that metformin preconditioning increased cell engraftment despite having similar stroke sizes. On the other hand, it also suggests that increased cell engraftments by metformin preconditioning was not able to change stroke infarct volume within the short term (1-week post-transplant) potentially due to limited time or limited ability to promote endogenous repair.

5.3 Metformin preconditioning promotes cell regeneration 8 weeks post-transplant

Although bystander effects can trigger remodeling and promote early recovery, in order for NSC transplants to reach their full potential as a cellular replacement therapy, long-term survival of grafts, neuronal differentiation and integration is required. In the previous studies with regards to hiPSC-NSCs transplants into ischemic stroke, many reports showed that only a fraction of rats contained graft cells months after transplant^{57,58}. Metformin preconditioning promoted the long-term cell grafts compared to our naïve control which showed no sign of viable cells at 8 weeks. Grafted cells that survived at 8 weeks from metformin preconditioning group represents a 23- fold of increase in cell numbers as compared to those at 1-week post-transplant (1037 ± 228 cells/rat at 1-week post-transplant compared to 23023 ± 2514 cells/rat at 8-weeks post-stroke), suggesting that exogenous hiPSC-NSCs have expanded since 1 week post-transplant possibly through metformin enhanced proliferation. Other studies had higher initial engraftment which likely allowed for the survival of more grafts at later time points. This would explain why our naïve group has notably fewer rats with grafts at later time points since initial engraftment at 1-week number was relatively low. Furthermore, metformin preconditioned cell grafts at 8 weeks included immature neurons, astrocytes and oligodendrocytes and did not express pluripotent marker Oct-3/4 (Figure 17, data not shown). It is difficult to determine if this level of differentiation is higher than naïve transplants due to low engraftment in naïve treated grafts.

However, successful engraftment and differentiation of metformin preconditioned grafts shows promise to develop metformin preconditioning as one strategy to optimize neural stem cell transplantation. Overall, metformin preconditioning promoted long-term cell survival likely through an increase in initial cell survival upon transplant and potentially increased proliferation.

Although not significant, metformin preconditioning showed a trend for reduced cortical infarct volume but not striatal infarct volume when compared to the sham group. Considering only 40% of metformin preconditioned grafts survived to 8 weeks, this is a noticeable effect on endogenous tissue. Furthermore, rats receiving metformin preconditioned cell transplants had a significant decrease in cortical stroke volume from 1 week to 8 weeks post-transplant (Figure 16C). This suggests that metformin preconditioning of hiPSC-NSCs may promote endogenous repair mechanisms to reduce infarct size. Increased cell number in preconditioned grafts may contribute to the secretion of angiogenic and neurotrophic factors that reduce cortical stroke size.

Despite an increase in cell engraftment, metformin preconditioned grafts did not increase functional recovery as assessed by forelimb motor tasks when compared to naïve cell grafts. Furthermore, neither naïve nor preconditioned grafts promoted functional recovery compared to sham, contrary to many other hiPSC-NSC transplant studies. The most likely explanation for the lack of functional recovery is due to low cell engraftment at 1-week post-transplant. Lower cell numbers could account for little early recovery through the bystander effect. Furthermore, the lack of integration of grafted cells into existing healthy brain tissue and neural circuits later time point may result in little potential for cell replacement mechanisms to promote recovery. Future work will be focused on developing an optimized approach to allow exogenous hiPSC-NSCs to migrate and fully differentiate into mature neural cells in the injured brain, ultimately forming neural network with existing neural circuits.

Spontaneous recovery was observed in both the beam walking task and the cylinder task however not in the Montoya staircase among any treatment groups. This lack of spontaneous recovery in our measurement of fine motor function may reflect the severity of our model selected and thus our ability to detect the effect of our treatments. ET-1 induced stroke damage to primary motor areas involved in forelimb function may make it impossible or too difficult to see any functional improvement in this model despite any therapeutic intervention. We injected ET-1 into 2 cortical and 1 striatal location, while only the 2 cortical sites received cell transplants because our hiPSC-NSCs are biased to form dorsal cortical neurons^{116,117}. Treatment of the striatum may be critical for successful cell transplants to improve stroke functional recovery in our ET-1 stroke model. Furthermore, cortical coordinates used in this study were more lateral than those established in previous studies (3.0mm from Bregma compared to 2.5mm from Bregma)¹⁰³. The change was made to more precisely target the cortical motor forelimb area in Sprague Dawley rats. This change may have damaged so much that it precluded any spontaneous recovery from taking place. Indeed, intact cortical motor areas and interconnected remote areas undergo anatomical and physiological changes following stroke that may contribute to spontaneous recovery¹¹⁸. In future studies, efforts to increase grafting size, adding other therapies such as rehabilitation and leaving the striatum intact to assess motor recovery will provide a more suitable model to assess long-term functional recovery.

5.4 Cell Labeling Methods for Transplantation into Ischemic Stroke

In addition to my main objectives in developing an optimized approach for hiPSC-NSC-based therapy to treat ischemic stroke, I also provided strong evidence arguing that prelabeling cells with fluorescent dyes prior to transplantation is not an ideal approach to trace exogenous cells in the rat stroke-damaged brain. Fluorescent dyes including lipophilic dyes (ex. CM-Dil) and

DNA binding dyes (ex. Hoechst 33342) have been used in many transplant models to label cells ex vivo and trace post-intravenous injection or intracerebral injection. Although fluorescent dyes provide a cost efficient, fast, easy method for identifying exogenous cells in the brain sections there is a challenge to use them through intracerebral injections into ischemic areas. Due to the extensive cell death observed in transplantation studies for stroke, the likelihood of leaking is much higher compared to transplantation into intact tissues. Here we show that the number of CM-Dil and Hoechst labelled cells was several fold more than number of cells that are detected by immunohistochemistry staining of human antigen Ku80 more. Furthermore, we show that CM-Dil was taken up by Iba-1 positive cells, marking endogenous microglia. As such, the large exogenous cell death following transplantation in stroke-damaged brain make fluorescent dyes prelabeling strategy not feasible for the stroke study and could confound results as demonstrating in the massive difference in cell quantification between CM-Dil+ cells and ku80+ cells (Figure 18). Thus, there is a need for a simple, cheap mechanism to identify transplanted cells in long-term studies.

5.5 Conclusion

Through the generation of human iPSC-NSCs and use of the ET-1 stroke model, I observed that metformin treatment promoted not only proliferation but also differentiation of hiPSC-NSCs via both direct stimulation and preconditioning paradigms. My study also provided a novel potential underlying mechanism that mediates metformin-induced differentiation of neural stem cells by repressing Mgl1 expression. In the cell transplantation model, I found that metformin preconditioning increased cell engraftment 1-week post-transplant, and further enhanced the number of surviving grafts 8 weeks post-stroke. However, I did not observe any functional recovery. Overall, my studies revealed that metformin preconditioning is an effective approach to enhance cell engraftment in the long term in the ET-1 induced stroke-damaged brain.

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Appendix

Table 2. List of solutions.

Solution	Concentration	Company	Catalog #
Lysis Buffer pH= 7.4			
Tris-HCL	50mM	Fisher	BP153
NaCl	150mM	Fisher	BP358
Nonidet P-40	0.20%	Sigma-Aldrich	NP40S
Sample buffer			
Sodium dodecyl Sulfate	2% w/v	Fisher	BP1311
Glycerol	4% v/v	Fisher	BP229
Tris-HCL	40mM	Fisher	BP153
Bromophenolblue	0.01% w/v	Fisher	BP392
β-mercapto-ethanol	1%	Fisher	ICN19024280
Running buffer			
Tris	25mM	VWR	CADDH4500
Glycine	192mM	Fisher	BP3815
Sodium dodecyl Sulfate	0.1%	Fisher	BP1311
Transfer Buffer			
Tris	192mM	Fisher	BP3815
Glycine	192mM	Fisher	BP3815