Acute cellular responses to oxygen-glucose deprivation/reperfusion in human induced pluripotent stem cell-derived neurons

BY

MARZIEH SEDIGHIPOUR CHAFJIRI

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ABSTRACT

Ischemic stroke occurs when a blood clot obstructs blood flow to the brain, depriving brain cells of oxygen and glucose and causing cell death. In the acute post-stroke period, the ischemic core can expand into the penumbra due to delayed death of injured neurons. There are no treatments in the clinic that specifically rescue these injured neurons. Although strategies to rescue injured neurons have proven effective in preclinical studies, they have failed to translate into clinical treatments due to a combination of factors including species differences. The development of human-induced pluripotent stem cells (hiPSCs) has provided an opportunity to study how human neurons respond to ischemia in real-time. The goal of this project was to establish a cell culture model of hiPSC-derived neurons and assess the cellular response to an ischemic insult. After 2 weeks, hiPSC cultures consisted primarily of neural progenitor cells (NPCs) and neurons. To replicate ischemic conditions of the penumbra, oxygen-glucose-deprivation/reperfusion (OGD/R) was used. Apoptosis significantly increased at 24h post-OGD. Neurons were more susceptible to OGD/R compared to NPCs, exhibiting greater cell death. This study has provided an *in vitro* model for understanding the cellular mechanisms post-stroke, potentially bridging the gap between preclinical findings and clinical treatments.

GENERAL SUMMARY

Stroke can either kill or injure brain cells. Within the core of a stroke injury, brain cells die, whereas brain cells surrounding the core are damaged but salvageable. However, there are no clinical treatments that specifically target injured brain cells. Although research using animal models has identified strategies to rescue these injured brain cells, they have failed to translate into effective clinical treatments. The recent development of transforming skin cells from human donors into brain cells in a culture dish enables real-time investigation of how human brain cells respond to stroke. The goal of this thesis was to: (i) develop a cell culture model of human-derived brain cells and (ii) to replicate 'stroke-in-a-dish' to assess how a stroke injures human brain cells. With this goal, it is hoped that the transition from laboratory findings to patient treatments will be facilitated.

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
AC3	Active caspase-3
AraC	Cytosine Arabinoside
ATP	Adenosine triphosphate
BAK	Bcl-2 Homologous Antagonist/Killer
BAX	Bcl-2 Associated X Protein
BBB	Blood brain barrier
BCL-2	B-cell CLL/lymphoma 2
BDNF	Brain-derived neurotrophic factor
BH3	Bcl-2 Homology Domain 3
BID	BH3 Interacting Domain Death Agonist
Ca^{2+}	Calcium
Cat#	Catalogue number

CBF	Cerebral blood flow
cm	Centimeter
CO_2	Carbon dioxide
CytoC	Cytochrome C
DAPI	4',6-diamidino-2-phenylindole
DISC	Death-inducing signaling complex
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F-12	Dulbecco's Modified Eagle's Medium/Nutrient Ham's Mixture F-12
DNA	Deoxyribonucleic acid
dPBS	Dulbecco's phosphate-buffered saline
H_2O	Dihydrogen monoxide
ET-1	Endothelin-1
EVT	Endovascular thrombectomy
FADD	Fas-Associated Death Domain
Fas-L	Fas Ligand

FDA	Food and drug administration
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
Hif-1α	Hypoxia-inducible factor 1-alpha
hiPSC	Human induced pluripotent stem cell
K^+	Potassium
MAP2	Microtubule-associated protein 2
MCAO	Middle cerebral artery occlusion
MEM	Minimum Essential Medium
mL	Milliliter
mM	Millimolar
MOM	Mitochondrial outer membrane
MOMP	Mitochondrial outer membrane permeabilization
N_2	Nitrogen
Na^+	Sodium
NB	Neurobasal

ng/mL	Nanograms per millilitre
NMDA	N-methyl-d-aspartate
NPC	Neural Progenitor Cell
O_2	Oxygen
OGD/R	Oxygen-glucose deprivation/reperfusion
P	Passaged
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
P-gp	Permeability Glycoprotein
pH	Potential of hydrogen
PI	Propidium iodide
ROCKi	Rho-associated coiled-coil containing protein kinase inhibitor
ROS	Reactive oxygen species
S100β	S100 Calcium Binding Protein Beta
SD	Standard Deviation
tBID	Truncated BH3 Interacting Domain Death Agonist

TNF	Tumor necrosis factor
TNFR1	Type 1 TNF receptor
tPA	Tissue plasminogen activator
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VGCC	Voltage-gate calcium channel
μL	Microliter
μM	Micromolar

Chapter 1 : INTRODUCTION

1.1 Stroke epidemiology

Stroke is a cerebrovascular disease. A stroke happens when a blood vessel experiences either a rupture or a blockage, leading to a disturbance in blood supply to specific areas of the brain. This disruption in blood flow results in long-term morbidity and mortality (American_Stroke_Association., 2024; Hankey et al., 2002).

Stroke is the third leading cause of death and the leading cause long-term disability impacting millions worldwide (World_Health_Organization., 2024, August 7; World_Stroke_Organization., 2024). For every 100 individuals who experience a stroke, 15 die, 10 recover completely, and 75 are left with varying degrees of disability (Heart&Stroke., 2017). Of the 75, 40 of these individuals suffer moderate to severe impairment, significantly affecting their independence and quality of life, 10 face such severe disability that they require long-term care, and 25 of these individuals recover with a minor impairment or disability (Heart&Stroke., 2017). This negative impact on health and well-being highlights the significant financial burden placed on stroke survivors, their families, and healthcare systems, which exceeds $721 billion globally (Feigin et al., 2022). This underscores the urgent need for effective prevention or treatment strategies to support the recovery of stroke survivors (Benjamin et al., 2018).

1.2 Ischemic Stroke

Strokes are classified as either ischemic or hemorrhagic (Joseph R Shiber 2010). Hemorrhagic stroke is the least common of the two forms of stroke occurring in 13% of all strokes. Hemorrhagic stroke is caused by a rupture of a blood vessel resulting in bleeding within the brain. Ischemic stroke is the more common form of stroke occurring in 87% of all stroke cases (Krishnamurthi et al., 2013). Ischemic stroke occurs due to a blood clot obstructing blood

flow to the brain. Ischemia refers to the lack of blood flow and therefore both oxygen and glucose deprivation. Both forms of stroke impair the delivery of oxygen and glucose to the brain causing a failure of energy metabolism and function of the affected brain region (Lakhan et al., 2009). The focus of this thesis is on ischemic stroke.

An ischemic stroke often results in two regions of injury: an ischemic core and an area lying between the ischemic core and the healthy brain, known as the penumbra region (Astrup et al., 1981; Lakhan et al., 2009). Advanced imaging techniques can determine these regions by providing detailed information about brain tissue viability and blood flow (Lin & Liebeskind, 2016). The extent of cerebral blood flow (CBF) reduction depends on the severity of the stroke and the time elapsed since its onset. The average normal CBF in humans is 50-60 mL/100 g/min (Lassen, 1985). CBF in the infarct core can be close to zero or significantly reduced (below 12 mL/100 g/min), whereas in the penumbra region, CBF is between 12 to 22 mL/100 g/min (Figure 1.1) (Heiss, 2000). As time passes without rapid reperfusion, irreversible damage of the core expands into the penumbra region and coalesces with this area (Back et al., 2004; Heiss, 2012).

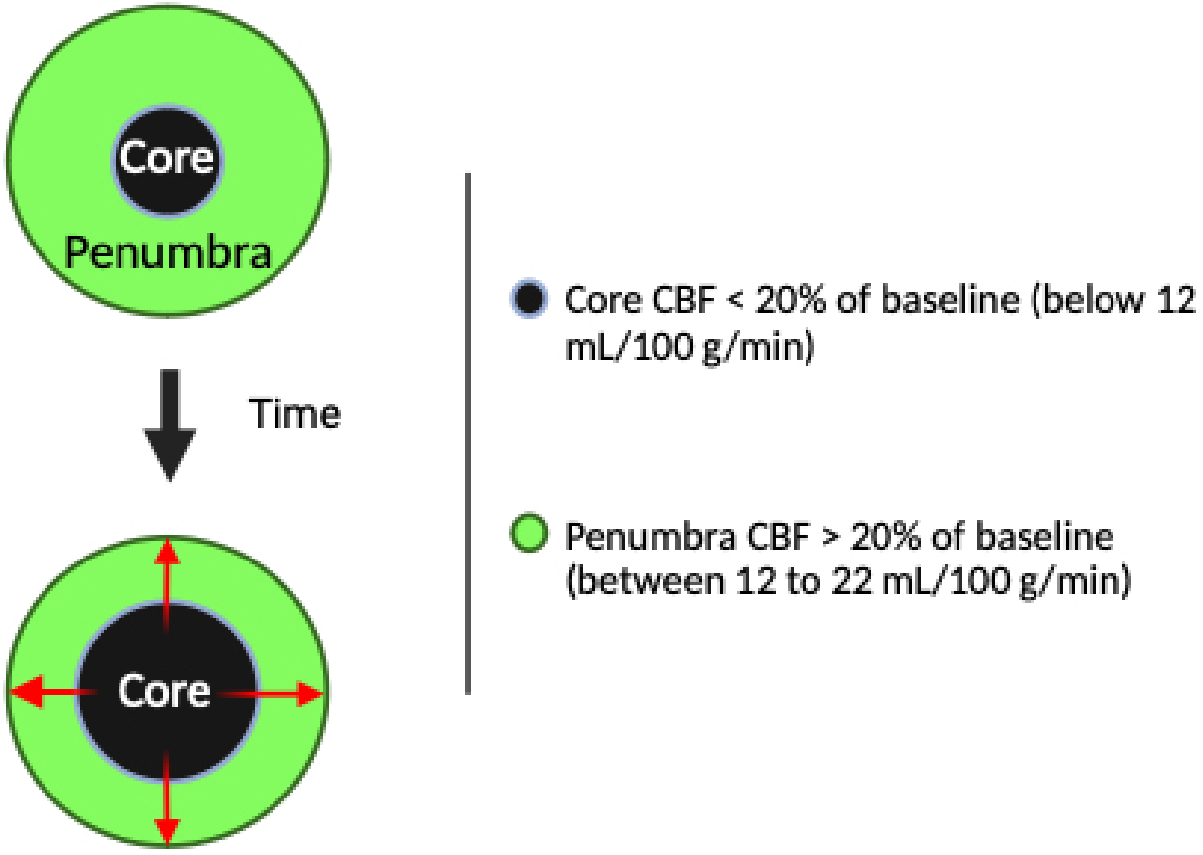

Figure 1.1: Cerebral blood flow within the core and penumbra following ischemic stroke.

Following an ischemic stroke, there's a significant drop in cerebral blood flow creating the ischemic core. Surrounding this area is the penumbra. As time passes, the core expands into the penumbra region. Figure created with Biorender.com. Black= Ischemic core, Green= Penumbra region. Cerebral blood flow (CBF).

1.2.1 Ischemic core

Cells within the infarct core are deprived of blood causing drastic deprivation of oxygen and glucose leading to irreversible injury and death (Woodruff et al., 2011). Within minutes of this deprivation, cells undergo complete bioenergetic failure leading to dysfunction of mitochondrial oxidative phosphorylation and insufficient adenosine triphosphate (ATP) synthesis (Siesjö et al., 1995). Rapid depletion of ATP causes failure of the ATP-dependent ion pumps, allowing ions such as Na^+ and Ca^{2+} and water into the cell (Jiang et al., 1992). This results in swelling of the cell and compromising the plasma membrane resulting in rupture and necrotic cell death (Figure 1.2) (Friedman & Haddad, 1993). The damage in the infarct core is rapid and surpasses the cells' ability to recover (Fisher, 2006). Even if blood flow is restored, the cells have already sustained irreversible damage and their structural integrity is lost.

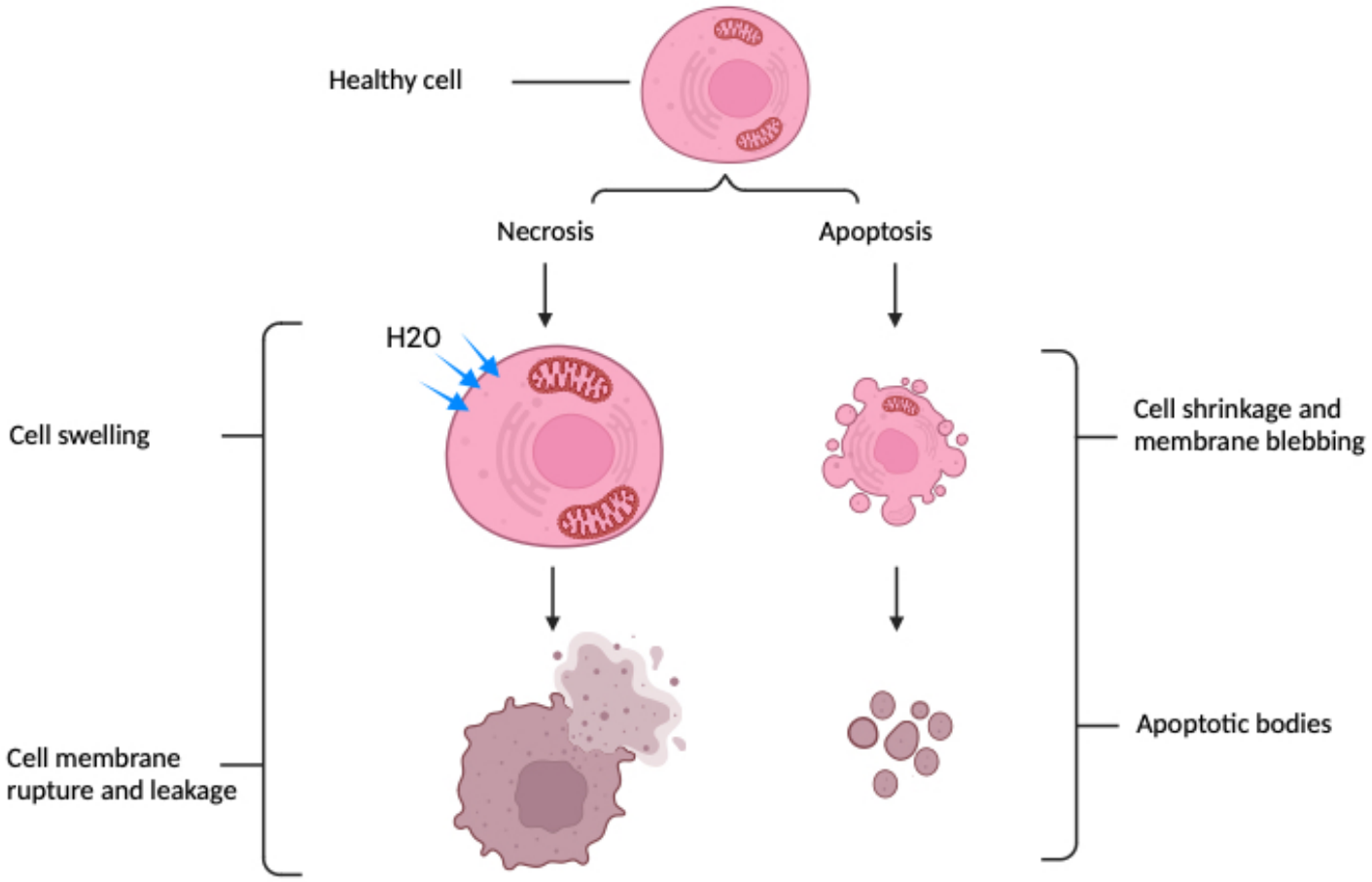

Figure 1.2: Morphological changes of cells undergoing necrosis and apoptosis.

(Left) During necrosis, cells and organelles swell up, resulting in the rupture of the plasma membrane, releasing the cellular contents into the extracellular environment. (Right) Apoptosis is associated with cell shrinkage and chromatin condensation. Fragmentation of DNA and plasma membrane blebbing result in the formation of apoptotic bodies. Figure created with Biorender.com.

1.2.2 Ischemic penumbra

The process of cell death in the ischemic penumbra is more complex. The ischemic penumbra refers to the region of brain tissue with less severely reduced blood flow than the ischemic core. Neurons in this area still receive some blood flow; thus, they are metabolically active but have impaired functional activity (Astrup et al., 1981). Cells in the penumbra region are damaged but salvageable if blood flow is rapidly restored by reperfusion or neuroprotective treatments soon after ischemic onset (Astrup et al., 1981; Ueda & Fujita, 2004).

Oxygen and glucose deprivation disrupts oxidative phosphorylation by the mitochondria causing a drop in ATP production and damages energy-dependent membrane receptors, ion channels, and pumps (Murphy et al., 2008). In the absence of ATP, the sodium-potassium (Na^+/K^+)-ATPase is unable to function, leading to the disruption of ionic gradients and the depolarization of cells resulting in activation of voltage-gated calcium channels (Kumar & Kurup, 2002). Calcium influx triggers the excessive release of excitatory neurotransmitters (primarily glutamate) into the extracellular space (Hirsch & Gibson, 1984). The excess of excitatory neurotransmitters in the extrasynaptic sites leads to prolonged activation of glutamate receptors and excitotoxicity. Glutamate binds and activates glutamate receptors, particularly N-methyl-d-aspartate (NMDA) receptors, which allows calcium influx into the neurons (Choi et al., 1988; Katchman & Hershkowitz, 1993). Calcium overload triggers cascades of excitotoxic events within the neuron, including increased reactive oxygen species (ROS) production, mitochondrial dysfunction, and activation of cell death pathways, including apoptosis and necrosis, depending on the severity of the insult (O'Regan et al., 1997; Siesjö et al., 1995).

1.3 Cell death and neurodegeneration

1.3.1 Necrosis

The restricted blood flow initiates a cascade of neurotoxic events, resulting in tissue damage, cell injury, and cell death (Woodruff et al., 2011). Necrosis and apoptosis are the two main types of cell death caused by ischemic injury (Figure 1.2) (Puyal & Clarke, 2009; Unal-Cevik et al., 2004). Ischemic injury refers to the cellular and tissue damage that occurs as a result of ischemia. Necrosis is the predominant form of cell death within the infarct core, occurring rapidly within minutes of ischemic onset. Membrane depolarization is followed by the influx of water compromises the membrane integrity causes abnormal organelles' morphology and swelling of the cell and its organelles including the endoplasmic reticulum and mitochondria and activation of cytosolic enzymes (Balan et al., 2013; Erecińska & Silver, 1994; Friedman & Haddad, 1993; Hansen, 1985). This event compromises the integrity of both plasma and lysosomal membranes (Bano et al., 2005). Lysosomal membrane breakdown releases proteolytic enzymes, which causes nucleus damage, cytoskeleton degradation, and peroxidation of membrane lipids (Troncoso et al., 2018). Calcium overload and elevated levels of ROS also lead to the oxidation of lipids and proteins within the membranes, along with DNA fragmentation, ultimately resulting in necrotic cell death within the ischemic core (Endres et al., 2000). If this event continues, the cell membrane will rupture, releasing its contents into the extracellular environment and triggering an inflammatory reaction around the dying cell (Denes et al., 2007).

1.3.2 Apoptosis

The term "apoptosis" was introduced by Kerr, Wyllie, and Currie in 1972 to describe a specific type of cell death (Kerr et al., 1972). Due to the morphological changes in the nucleus, apoptosis is also called the nuclear type of cell death. Apoptosis is the main form of neuronal

death in the penumbra (Figure 1.2) (Ferrer, 2006; Linnik et al., 1993). Injured cells in the penumbra undergo cell death within several hours to days following a stroke (Guégan et al., 1998). Apoptosis is a form of programmed cell death regulated by a complex pathway involving two families of proteins, including the family of cysteine aspartic acid proteases known as caspases and the B-cell CLL/lymphoma 2 protein family known as Bcl-2. The Bcl-2 family act at the initiation of the apoptosis pathway whereas caspases act downstream to regulate the enzymatic destruction of the cell and its organelles.

1.3.2.1 The Bcl-2 protein family

The Bcl-2 protein family is composed of three major groups of proteins based on their roles: multi-domain pro-apoptotic proteins, anti-apoptotic proteins, and pro-apoptotic Bcl-2 homology domain 3 (BH3)-only proteins. The anti-apoptotic proteins promote cell survival by inhibiting the pro-apoptotic proteins. The BH3-only proteins act as sensors and initiate apoptosis by directly activating the pro-apoptotic proteins or inhibiting the anti-apoptotic proteins (Cheng et al., 2001; Willis et al., 2003). Once activated, the pro-apoptotic proteins (e.g., Bax and Bak) oligomerize and form pores on the mitochondrial outer membrane (MOM). The permeabilization of MOM causes the loss of membrane potential and the release of proteins such as cytochrome c into the intercellular space and activating the caspase cascade.

1.3.2.2 Caspase protein family

The family of caspase proteins are divided into two functional groups: the initiator caspases and executioner caspases (McIlwain et al., 2013). The initiator caspases (including caspase-2, -8, -9, -10) cleave and activate the downstream executioner caspases. The executioner caspases (including caspase-3, -6, -7) carry out the enzymatic degradation of the cell (Anson et

al., 2021). They cleave various substrates in the cell essential for cellular integrity and function. Caspases are activated by various signaling pathways, ultimately executing apoptotic cell death (Willis et al., 2003). An essential executioner caspase that initiates morphological changes during apoptosis is caspase-3 (Jin & El-Deiry, 2005; Taylor et al., 2008). Specific morphological features include compaction of chromatin and nuclear condensation. These modifications are accompanied by cytoplasm shrinkage and folds in the plasma membrane (membrane blebbing) that break off to form apoptotic bodies (Figure 1.2) (Elmore, 2007; Kerr et al., 1972; Lemasters et al., 1987; Zhang, 2022). The role of apoptosis in cell death following stroke is highlighted by a study demonstrating that the genetic deletion of caspase-3 resulted in more than a 50% reduction in infarct volume compared to wild-type controls at 24 hours post-stroke (Dojo Soeandy et al., 2019). This finding suggests that apoptosis significantly contributes to cell death in the initial hours post stroke.

1.3.3 Apoptosis Pathway

Apoptosis can be activated by two energy-dependent pathways, the intrinsic (or mitochondrial) and the extrinsic (or death receptor) pathway (Puyal et al., 2013) (Figure 1.3). While distinct, both pathways lead to caspase activation and mitochondrial damage, and molecules in one pathway can influence the other. Following an ischemic injury, both intrinsic and extrinsic pathways can be activated.

The intrinsic pathway is activated by intracellular stressors such as an excess of intracellular Ca^{+2}, which leads to loss of integrity of the MOM (Ghavami et al., 2004; Puyal et al., 2013). The MOM permeabilization (MOMP) leads to the release of cytochrome c, a pro-apoptotic factor, from mitochondria into the cytosol and then formation of a complex called the

Apoptosome (Adrain & Martin, 2001; Arnoult et al., 2005). The Apoptosome mediates activation of initiator caspase-9 and subsequently executioner caspases like caspase-3, resulting in cell death (Adrain & Martin, 2001; Li et al., 1997).

The extrinsic pathway is triggered in response to extracellular signals. In response to injury, immune cells secrete death ligands such as TNF (tumor necrosis factor), Fas-L (Fas ligand), and TRAIL (TNF-related apoptosis-inducing ligand). These ligands bind to their homologous death receptors on the cell membrane such as type 1 TNF receptor (TNFR1), Fas (also called CD95/Apo-1) and TRAIL receptors. Upon ligand binding, cytoplasmic adapter proteins such as FADD (Fas-Associated Death Domain) are recruited. Adapter proteins act as an adaptor molecule that bridge the activated death receptor and the initiator caspases (Ranjan et al., 2012). They associate with procaspase-8 and procaspase-10 and form a death-inducing signaling complex (DISC) (Kischkel et al., 1995). The DISC functions to cleave and activate initiator caspases such as caspase-8, similarly to the Apoptosome. Once activated, caspase-8 initiates apoptosis directly by cleaving and activating executioner caspases or by activating the intrinsic apoptosis pathway by cleaving and activating the BH3-only protein BID generating truncated BID (tBID). tBID translocates to the mitochondria where it activates BAX and/or BAK, promoting MOMP, the release of cytochrome c and the formation of the Apoptosome (Figure 1.3) (Luo et al., 1998).

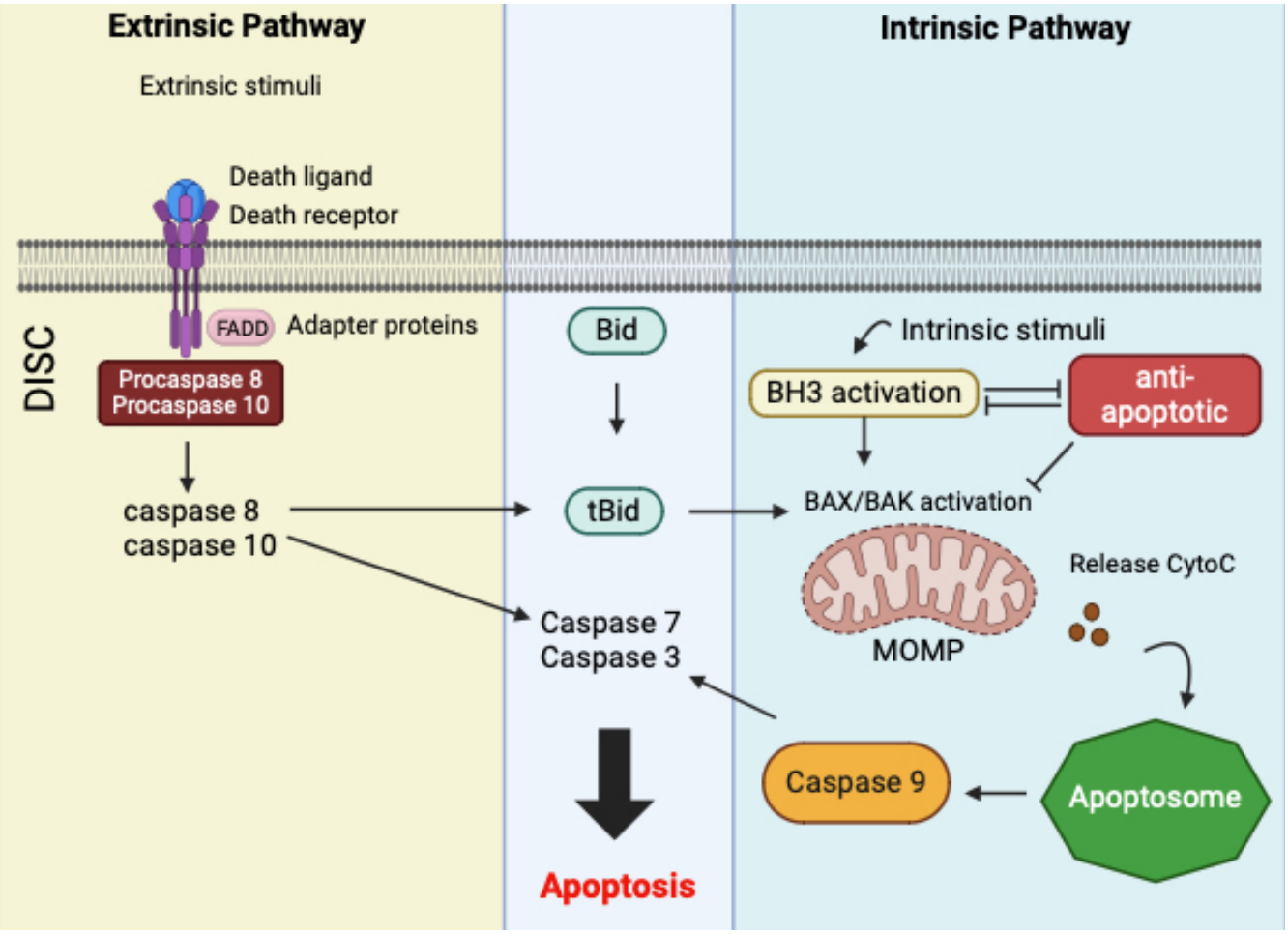

Figure 1.3: Overview of the intrinsic and extrinsic pathways of apoptosis.

The intrinsic pathway is initiated by intracellular stressors, resulting in MOMP and the release of cytochrome c. Cytochrome c facilitates the formation of the apoptosome, which activates initiator caspase-9 and executioner caspases like caspase-3. This ultimately leading to apoptosis. The extrinsic pathway is triggered by extracellular death ligands binding to their respective death receptors. This ligand-receptor interaction recruits adapter proteins, such as FADD, which bridge the death receptor and procaspases-8/-10 to form the DISC. DISC cleaves and activates initiator caspase-8, which can directly activate executioner caspases or cleave the BH3-only protein Bid into tBid. tBid translocates to the mitochondria, triggering MOMP, CytoC release, and apoptosome formation, effectively linking the extrinsic and intrinsic pathways. Figure created with Biorender.com. Mitochondrial outer membrane permeabilization (MOMP); Fas-Associated Death Domain (FADD); Death-inducing signaling complex (DISC); Truncated BH3 Interacting Domain Death Agonist (tBID), Bcl-2 Associated X Protein (BAX), Bcl-2 Homologous Antagonist/Killer (BAK), Bcl-2 Homology Domain 3 (BH3), Cytochrome C (CytoC).

1.3.4 Neurodegeneration

Neurodegeneration is a multifaceted process that leads to the progressive loss of neuronal structure or function and eventually, the death of neurons in the brain causing brain damage and impaired function (Polster et al., 2017). Following cerebral ischemia, a cascade of inflammatory and degenerative mechanisms such as neuroinflammation, excitotoxicity, oxidative stress, and apoptosis are initiated. This influences the extent and severity of the injury, the progression of secondary neurodegeneration, and the level of disability or recovery experienced (Polster et al., 2017).

Within hours of stroke, microglia and astrocytes are activated resulting in the production and secretion of cytokines and chemokines causing an infiltration of leukocytes across the blood brain barrier (BBB) (Iadecola & Anrather, 2011; Jin et al., 2010). Oxidative stress, an imbalance between ROS production and antioxidant defenses is also triggered during cerebral ischemia, mainly due to inflammation and reperfusion, leading to increased free radicals and degeneration (Sinning et al., 2017).

Ischemic stroke also causes the disruption of the neuron's cytoskeletal structure (Gutiérrez-Vargas et al., 2022). In the penumbra region, surviving neurons experience pathological alterations including degeneration of dendritic processes, spines, and axonal damage that can reduce postsynaptic potentials (Brown et al., 2008; Gresle et al., 2006). These events are associated with accumulating protein aggregates and releasing cytoplasmic proteins into the extracellular space, indicating neurodegeneration or cell death (Cao et al., 2017; McMurray, 2000). Reduction in Tau protein, a microtubule-associated protein, and microtubule-associated

protein 2 (MAP2) is indicative of neurodegeneration, reflecting the fragmentation of neuronal processes following cerebral ischemia (Guo et al., 2017; Kosik et al., 1986; Pluta et al., 2018).

Neurons that survive in the penumbra region show signs of neurodegeneration in a time-dependent manner. Following an endothelin-1 (ET-1) stroke in mice, a reduction in intact MAP2+ dendrites and SMI312+ axons was observed by 4 hours post-stroke, which became more extensive by 24 hours (Fifield & Vanderluit, 2020). Neuronal degeneration continues for several days within the ischemic region after ET-1 induced stroke (Fifield & Vanderluit, 2020; Nguemeni et al., 2015). These studies indicate that the survival of injured neurons in the penumbra region is precarious due to the ongoing degeneration of neurons days after the initial insult. Previous studies show that structural damage to neurites and the soma can be partially reversed, contingent upon the duration of ischemia (Zhou et al., 2017). Thus, injured neurons within the penumbra have a limited time window for rescue during the acute post-stroke period. Preventing neurite loss or promoting neurite regeneration and plasticity are potential therapeutic targets for promoting neurological recovery after a stroke.

1.4 Therapies

Despite significant advancements in medical research, current therapies for ischemic stroke remain limited to reperfusion strategies (Seyedsaadat & F Kallmes, 2019). These include tissue plasminogen activator (tPA), which dissolves the blood clot and endovascular thrombectomy (EVT) (Jauch et al., 2013; Rehani et al., 2020; Tymianski, 2017). After testing more than 1000 therapeutic agents in animal models and testing 114 of these agents in acute stroke patients, tPA remains the only drug approved by the US Food and Drug Administration (FDA) for enhancing outcomes in specific populations during acute ischemic stroke

(National_Institute_of_Neurological_Disorders_and_Stroke_(NINDS)_rt-PA_Stroke_Study_Group., 1995; O'Collins et al., 2006). Although tPA is an effective treatment for ischemic stroke, many patients do not receive it, because they arrive at the hospital too late or are not eligible due to comorbidities (Go et al., 2014; Smith et al., 1998). The therapeutic time window for tPA is short and limited to 4.5 hours after the symptom onset. Moreover, it can be associated with severe side effects such as increased risk of hemorrhagic transformation (Elijovich & Chong, 2010; Emberson et al., 2014; Hacke et al., 2008; Kleindorfer et al., 2004; S. Liu et al., 2018; Tobin et al., 2014). Among stroke patients who seek medical attention on time after stroke onset, some are excluded from tPA intervention due to their age (over 80 years old) or medical history that increases the risk of brain hemorrhage. Timely imaging and proper diagnosis in the hospital are also factors for a successful outcome.

EVT involves the mechanical removal of a blood clot from a vessel in the brain. This treatment option offers a broader time window of effectiveness extending up to 6-24 hours from the onset of stroke symptoms for eligible patients (Nogueira et al., 2018; Y. Yang et al., 2022). While both treatments can restore blood flow, a considerable number of patients continue to experience significant neurological deficits even after undergoing either intervention (Berkhemer et al., 2015; Campbell et al., 2015; Goyal et al., 2015; Seyedsaadat & F Kallmes, 2019). No other treatment exists to rescue injured neurons within the penumbra. Since the pathophysiology of the penumbra develops over hours and days after ischemic onset, rescuing injured cells in this region can be expected to reduce damage and improve outcomes (Green, 2008; Kim et al., 2014). Given the limited treatment options for stroke, it is necessary to understand the early cellular response to stroke to develop strategies to slow down the progression of neurodegeneration and rescue

injured neurons. Blocking or preventing apoptosis in the penumbra may be a reasonable therapeutic goal for limiting cerebral infarct volume after stroke to improve stroke outcomes.

1.5 Challenges in translational research

A variety of strategies have been explored in preclinical experimental stroke studies. Some of these strategies include using neuroprotective agents, such as inhibitors of glutamatergic activity to prevent presynaptic glutamate release or block postsynaptic glutamate receptors or ion channel modulators to block calcium channels (Xiong et al., 2004). Stem cell therapy and novel immunological agents have also been investigated as potential treatments. However, these approaches have yet to achieve success in clinical settings (Borlongan, 2019; Elkins et al., 2017; O'Collins et al., 2006). Animal models are indispensable tools for studying neurodegenerative diseases, as they provide valuable insights into disease pathogenesis, mechanisms, and the development of novel therapeutic regimens. However, most preclinical stroke research has not successfully translated into effective clinical treatments (Ginsberg, 2008; Wahlgren & Ahmed, 2004).

Difficulties with translation to the clinic have been attributed to several factors. One significant problem is that most preclinical stroke studies are performed on young, healthy rodents that differ significantly from humans as they are separated by 70 million years of evolution and lack complex human pathophysiology and comorbidities (Mestas & Hughes, 2004). Humans and rodents differ hugely in size and lifespan (rodents have a lifespan of 2 to 3 years), so they cannot model human aging and associated stroke risk factors (Cheng et al., 2004; Xu & Pan, 2013). Aside from macroscopic structural discrepancies, it is unclear whether human neurons differ from rodent neurons in their response to stroke. Rodents are naturally very

resistant to the development of many of the chronic conditions that are prevalent in human populations, which can significantly influence stroke outcomes and treatment efficacy (Kottaisamy et al., 2021).

Studies show that there are cellular and molecular differences between humans and rodents. Expression levels of BBB transporters and pumps distinguish the human BBB from that of the rodent (Syvänen et al., 2009). In a study conducted by Stina Syvänen, the brain uptake of radiolabeled P-glycoprotein (P-gp) substrates was compared across species using positron emission tomography (Syvänen et al., 2009). P-gp is an ATP-dependent efflux transporter that moves various drug molecules at the BBB and in other organs. The study revealed significant species differences in brain and brain-to-plasma concentrations of P-gp substrates with humans showing higher brain distribution than rats. This suggests that in humans, sufficient BBB permeability may be maintained to allow molecules to act at intracerebral targets (Syvänen et al., 2009). Several *in vitro* studies have also shown that the ability of P-gp to transport substrates differs among cell lines transfected with P-gp from different species. This suggests species differences in substrate recognition (Baltes et al., 2007; Katoh et al., 2006; Ohe et al., 2003; Xia et al., 2006; Yamazaki et al., 2001).

Given the significant inflammatory component in stroke pathology, it is also important to consider differences in immune biology between species. Neutrophils comprise about 10–20% of the white blood cells in rodents, whereas in humans, they constitute 50–70%. Conversely, lymphocytes account for 50–100% of the white blood cells in rodents, compared to 20–40% in humans (Haley, 2003). Also, some important immune signaling molecules are expressed in humans but not in mice and vice versa (Mestas & Hughes, 2004).

In addition to the species differences in physiology, the treatment time window in preclinical studies often does not represent clinical practice. Stroke patients must travel to the hospital to receive medical intervention and there can be a significant delay before medical attention is received (Silvestrelli et al., 2006; H. Yang et al., 2022). Meanwhile, preclinical settings typically administer treatments shortly after stroke onset.

In summary, numerous challenges are associated with translating stroke research from preclinical to clinical application. In order to improve the relevance and translatability of preclinical findings, more representative models should be developed that incorporate human pathology.

1.6 *In vivo* vs *in vitro*

Stroke models used in research have unique strengths and weaknesses that influence the interpretation and application of experimental results. Most *in vivo* stroke models focus on blocking blood flow in large arteries or smaller arterioles. The most frequently used *in vivo* experimental ischemic stroke model is the endovascular filament middle cerebral artery occlusion (MCAO) model (Guan et al., 2012; Koizumi J, 1986; Sommer, 2017). The endovascular filament MCAO involves the insertion of an intraluminal suture to occlude the middle cerebral artery (MCA) temporarily or permanently (Fluri et al., 2015). This model was developed by Koizumi and colleagues in rats and followed with several modifications regarding the type of filament, the coating, and the length, as well as modifications of the access route (Koizumi J, 1986; Longa et al., 1989). It is increasingly common to use this model for mice as well (Hata et al., 1998). A significant advantage of using MCAO as a stroke model is that it allows the timing of reperfusion to be controlled and creates a robust penumbra (Fluri et al.,

2015). This approach closely mimics the conditions of human stroke concerning the location, as the MCA is the most common artery occluded in stroke (Chiang et al., 2011; Orset et al., 2007). Although stroke models that target the MCA better replicate the human condition, MCA strokes are highly invasive and cause massive infarct sizes in both mice and rats, ranging from 21% to 45% of the affected hemisphere (Kumar et al., 2016). However, in humans, MCA strokes are smaller with a range of 4.5 to 14% of the hemisphere being infarcted (Carmichael, 2005). Therefore, the size of an MCAO-induced infarct in mice does not reflect the typical stroke sizes observed in clinical settings. This model also demonstrates a sensitivity to stroke duration. A range of 15 to 30-minute variation in stroke duration in this model can cause a sixfold difference in infarct volume in mice (McColl et al., 2004).

Craniectomy is an alternative stroke model that allows for direct occlusion of the artery, however, it is likely to increase brain damage and a risk of hemorrhage (Kumar et al., 2016). Similarly, thromboembolic models replicate human stroke conditions by introducing microspheres or a thrombin clot into an artery, such as MCA for occlusion. In this model, thrombolytics such as tPA can be evaluated for their ability to break down clots. However, there are several disadvantages to this model. This model can result in infarcts that vary in size depending on the occluded blood vessel (McCabe et al., 2018). Furthermore, the clot may spontaneously break up and occlude other arteries, leading to multiple smaller focal infarcts (McCabe et al., 2018). Local application or injection of ET-1, a 21-amino acid peptide with potent vasoconstrictor properties, is another stroke model that has gradual reperfusion 16 to 48 hours post-injection, which represents clinical stroke (Biernaskie et al., 2001). This model produces focal infarcts with a notable penumbra region that holds potential for rescue (McCabe et al., 2018). However, it has been reported that the intra-cortical injection of ET-1 directly into

the brain causes mechanical damage and induces astrocytosis and axonal sprouting, which may complicate the interpretation of results (Uesugi et al., 1998). The photothrombosis stroke model also causes early vasogenic edema and BBB breakdown, which is uncharacteristic for human stroke, and it cannot be used to investigate neuroprotective agents (Dietrich et al., 1987; Lee et al., 1996).

In vitro models are commonly used in preclinical stroke studies. The complexity of ischemic stroke cannot be accurately modeled *in vitro*, as these systems lack intact blood vessels, blood flow, and leukocyte infiltration (Kuriakose & Xiao, 2020; Sommer, 2017). However, ischemic conditions can be simulated in cell culture by depriving the cells of both oxygen and glucose to mimic the environment they experience during ischemia. The *in vitro* models enable the study of specific cellular and molecular mechanisms under stroke-like conditions (Sommer, 2017; Woodruff et al., 2011). Oxygen and glucose deprivation (OGD) is the most common and physiologically relevant way to induce ischemia-like conditions in culture (Tasca et al., 2015). In this approach, cell or tissue cultures are placed in a hypoxic chamber and incubated in a glucose-free medium under a deoxygenated atmosphere (Tasca et al., 2015). Depending on the cell type and the required level of ischemic damage, the cultures are maintained in the chamber for various durations between 30 minutes and 24 hours (Van Breedam & Ponsaerts, 2022).

Monocultures of rodent primary neurons is the most common cellular platform used for *in vitro* stroke research. This approach is favored for studying cell-specific responses to OGD and/or for assessing the effects of neuroprotective compounds on specific cell types (Van Breedam & Ponsaerts, 2022). Moreover, researchers have utilized rodent primary neurons to investigate the impact of hypoxia (oxygen deprivation) on neuronal activity, using multi-electrode arrays to monitor changes in activity during varying durations of hypoxia exposure (le

Feber et al., 2016). Many *in vitro* models incorporate co-cultures of different cell types, such as neurons, astrocytes, and endothelial cells. This approach mimics certain properties of the BBB and examines cell-cell interactions under ischemic conditions (Yang et al., 2012).

In vitro stroke models are simplified, affordable, and provide precise control over experimental conditions, which is preferred when investigating the cell-cell interactions and cell type-specific responses following oxygen-glucose-deprivation/reperfusion (OGD/R) (Ashammakhi et al., 2019; Sommer, 2017; Woodruff et al., 2011). They mimic the conditions of the ischemic penumbra, the target tissue for therapeutic intervention. *In vitro* models allow high-throughput preclinical screenings of neuroprotective compounds, even on a human-based background (Sommer, 2017).

Neuroblastoma cell lines, such as SH-SY5Y, have been predominantly used in human-based studies (Y. Liu et al., 2018; Lorenz et al., 2009). However, the physiology of primary neurons is not always accurately reflected by these cells, as they have cancer-like properties (Hoffmann et al., 2023). The primary human brain slice is another model that is highly physiologically relevant, but access to human brain tissue is extremely limited (Marcoli, Bonfanti, et al., 2004; Marcoli, Cervetto, et al., 2004; Werth et al., 1998). Due to these limitations, the use of rodent-based systems has been preferred over human-based systems. However, species differences in cell properties are a significant challenge. The recent development of induced pluripotent stem cells (iPSCs) from human somatic cells has offered a new cell source that can overcome the outlined limitations. Considering the importance of species differentiation in translating preclinical findings to clinical treatments, utilizing human-based *in vitro* models can provide clinically relevant, accessible option to gain insights into cellular responses to stroke (Barthels & Das, 2020).

1.7 iPSCs

Induced pluripotent stem cells (iPSCs) have allowed researchers to study how human neurons respond to an ischemic condition in real-time. The iPSCs are classified as pluripotent stem cells that can differentiate into any cell in the human body. However, unlike other pluripotent stem cells, iPSCs are derived from adult somatic cells (such as skin fibroblasts) through genetic reprogramming. This technique was first introduced by Shinya Yamanaka in 2006 (Takahashi & Yamanaka, 2006). By introducing a combination of specific transcription factors, Oct4, Sox2, Klf4, and c-Myc (the Yamanaka factors), differentiated mouse skin cells can be induced into a pluripotent embryonic-stem cell-like state (Takahashi & Yamanaka, 2006). In 2007, Yamanaka and his team generated human iPSCs using the same reprogramming approach to revert human fibroblast cells to induced pluripotent stem cells (Takahashi et al., 2007).

The first step in producing neural lineages from human induced pluripotent stem cells (hiPSCs) is the generation of neural progenitor cells (NPCs) by exposing them to neural induction media containing specific growth factors, followed by differentiation into functional neurons (Figure 1.4). NPCs are classified as multipotent stem cells meaning they can self-renew and differentiate into various neural cell types including neurons, astrocytes, and oligodendrocytes (Abud et al., 2017; Baker et al., 2017; Tcw et al., 2017; Yan et al., 2013). Human iPSCs-derived neurons can be used for various experimental purposes, including disease modeling and drug screening (Villa et al., 2021). Using this approach, scientists can observe disease progression *in vitro*, study the molecular and cellular responses, and identify potential therapeutic targets.

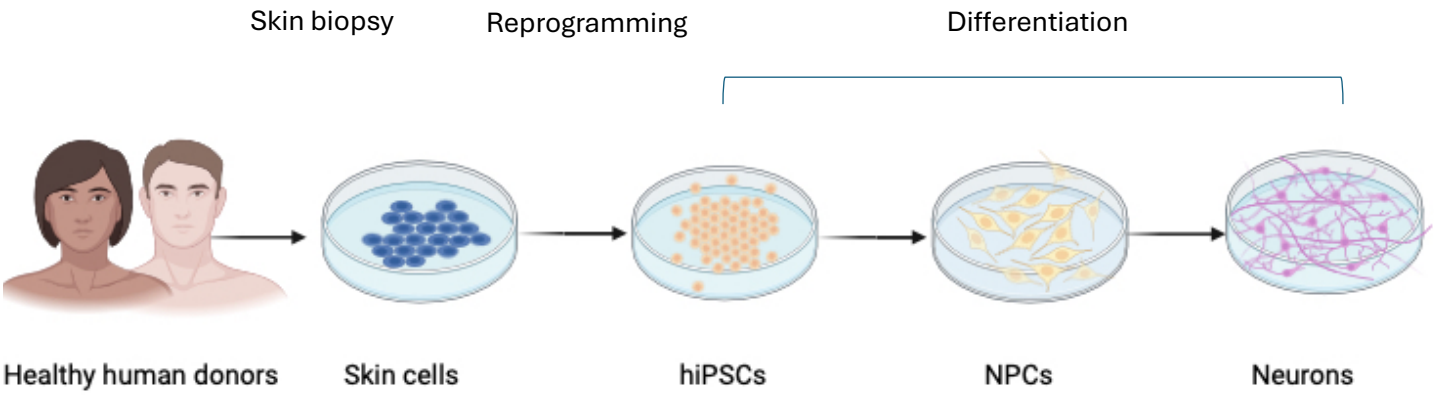

Figure 1.4 : Generation of neural lineages from human skin cell.

This schematic illustrates the process of deriving neurons from human skin cells through reprogramming and differentiation. Skin cells are obtained from healthy human donors via a biopsy. These cells are then reprogrammed into hiPSCs using specific reprogramming factors that revert them to a pluripotent state. hiPSCs possess the ability to differentiate into various cell types, including NPCs. NPCs are multipotent and serve as precursors to neural lineages. Under appropriate culture conditions and in the presence of specific growth factors, NPCs proliferate and differentiate into functional neurons. Figure created with Biorender.com. Human induced pluripotent stem cells (hiPSCs). Neural progenitor cells (NPCs).

1.7.1 Human iPSC-derived neuronal models in previous studies

Two studies have examined ischemia in healthy hiPSC-derived neuronal models. The first study that investigated ischemia in hiPSC-derived neuronal models was performed in 2020 by Juntunen et al. They compared the effect of OGD on hiPSC-derived neurons and differentiated SHSY5Y cells, which is a human-derived neuroblastoma cell line commonly used as a model for studying neuronal function (Agholme et al., 2010) (Juntunen et al., 2020). They showed that OGD conditions caused cytotoxicity and apoptosis in both SH-SY5Y- and hiPSC-derived neuron with SH-SY5Y-derived neurons experiencing more severe damage than hiPSC-derived neurons. The other study was performed in 2021 by Pires Monteiro et al (Pires Monteiro et al., 2021). Using micro-electrode arrays, they measured the electrophysiological activity of hiPSC-derived neuronal networks including excitatory and inhibitory neurons under controlled hypoxic conditions (Pires Monteiro et al., 2021). They also investigated the neuroprotective effect of hypoxic preconditioning. They discovered that under hypoxia conditions, human neuronal networks experience a decline in activity and synchronicity. However, hypoxic preconditioning was shown to enhance resistance to a subsequent hypoxic event. This method allows researchers to replicate disease conditions *in vitro* and pinpoint potential therapeutic targets. However, there is a significant need for additional studies using human-based models in stroke research to advance our understanding and improve treatment strategies.

1.8 Rationale and goal

Previous studies have shown *in vitro* humanized models used in experimental cerebral ischemia studies. Of this research, only 1.4 percent is conducted on hiPSCs cell models (Voogd et al., 2023). This limited source makes it hard to properly understand the cellular responses of hiPSCs to an ischemic insult. The goal of this study was to establish a human neuronal model of the ischemic penumbra using hiPSC-derived neurons and to assess cellular responses following an ischemic insult.

To address this goal, I have the following two aims: Aim #1: Determine the cellular composition of human iPSC-derived neuron cultures. Aim #2: Assess the acute cellular responses to stroke-like condition in hiPSC-derived neuronal cultures at 24h post OGD.

Experimental model: Human iPSCs → NPCs → differentiate to neurons for 14 days.

The 14-day differentiation and neuronal differentiation medium used in this study was based on the work by Yan et al. (2013), in which hiPSC-derived neural stem cells were efficiently differentiated into neurons using a defined serum-free medium.

Oxygen-Glucose Deprivation/reperfusion model (OGD/R): This is an *in vitro* model that simulates the penumbra *in vivo* with mechanisms primarily involving apoptosis. Human iPSC-derived neurons will be transferred to media lacking glucose and placed in a hypoxic chamber with 3% oxygen for 3h, followed by re-oxygenation and media containing glucose for 24h. The control group will be untreated. The choice of 3% oxygen was based on prior studies indicating that oxygen levels in the ischemic penumbra range between approximately 1–3% during ischemia, reflecting moderate hypoxia sufficient to induce neuronal stress responses without

immediate necrosis (Chen et al., 2018; Singh & Chen, 2023). Additionally, previous work in our lab demonstrated no significant differences between 5% oxygen exposure and control conditions in human organoid cultures, reinforcing the need to optimize hypoxic conditions to better mimic the penumbra. A 3-hour OGD exposure was selected to model a substantial but reversible ischemic event, aligning with the clinically recognized therapeutic window for stroke intervention (Hacke et al., 2008). Following OGD, a 24-hour reperfusion period was used to capture the acute-stage neuronal injury responses, consistent with previous findings that neurons in the ischemic penumbra exhibit significant dendritic and axonal degeneration within 24 hours post-insult (Fifield & Vanderluit, 2020). This timeframe enables the assessment of early apoptotic and degenerative processes before widespread irreversible necrosis occurs.

Significance: Human iPSC-derived neurons provide an excellent model for studying how human neurons function at the cellular and molecular levels and offer valuable insights that could lead to new therapeutic strategies for stroke patients. The application of human neurons has the potential to improve drug discovery and translation of preclinical findings into patient treatments, as well as create new opportunities for personalized studies.

Chapter 2 : MATERIALS AND METHODS

2.1 Human induced pluripotent stem cell and neural induction

The human induced pluripotent stem cell (hiPSC) (wildtype female) used in this study was provided by Dr. Jessica Esseltine's laboratory in the Division of Biomedical Science, Memorial University of Newfoundland. All experiments performed with hiPSCs were covered under Dr. Esseltine's Human Research Ethics Board (HREB) application # 2018.210 (Appendix 1).

The hiPSCs were maintained under feeder-free conditions and cultured in essential 8™ medium (A1517001, Thermo Fisher Scientific, Waltham, MA, USA) on Geltrex-coated dishes (A1413301, Thermo Fisher Scientific). Geltrex was diluted at 1:100 in ice cold Dulbecco's Modified Eagle Medium (DMEM; 319-015-CL, Wisent, Saint-Jean-Baptiste, QC, Canada) and dishes were coated for 1 hour at 37°C. Neural differentiation was initiated when hiPSCs reached ~70-80% confluency, at which point they were transferred into neural induction medium containing neurobasal medium (490 mL, 21103-049, Thermo Fisher Scientific) + neural induction supplement (10 mL, A16477-01, Thermo Fisher Scientific). Cells were cultured under standard conditions (37°C, 5% CO_2), with medium changes every two days. By day 6 of neural induction, cells reached near-maximal confluency and exhibited morphological characteristics of neural progenitor cells (NPCs). At this stage, NPCs were harvested using Accutase (500 µL; A1110501, Thermo Fisher Scientific), then cryopreserved or expanded in NPCs expansion medium (Table 2.1), supplemented with Rho-associated coiled-coil containing protein kinase inhibitor (ROCKi; 10005583-5, Cayman chemical, Ann Arbor, Michigan, USA) for initial plating.

2.1.1 NPCs expansion

A 6-well plate was coated with Geltrex. After coating, one vial of 2 x 10^6 NPCs (P0) was thawed and plated in NPC expansion media (Table 2.1). NPCs were cultured for 3-5 days and were passaged when NPCs became confluent.

To passage NPCs, culture media was removed, and cells were washed with 1 x Dulbecco's phosphate-buffered saline (dPBS; 311-425-CL, Wisent). Accutase (500 µL) was added for 5-10 minutes at 37°C to detach the NPCs from the dish. NPCs were collected in a 15 mL falcon tube containing 3-5 mL of DMEM (or 1 x dPBS). Cells were centrifuged at 300 x g for 5 min (Eppendorf 5810r, Fisher Scientific), counted using a hemocytometer and re-seeded at a density of 1 x 10^5 cells/cm^2 onto a 6-well Geltrex coated dish with NPC expansion medium. NPC expansion media was changed 24h after each passage. After the fourth passage, expansion media without ROCKi was changed every second day. To evaluate the purity of NPC culture prior to differentiation, cells were regularly assessed with every passage for the expression of NPC-specific markers (e.g., Nestin, PAX6) using immunocytochemistry. Non-specific differentiation was minimized by maintaining cells in NPC expansion medium and passaging before initiating differentiation. NPCs at passage 4-6 were used for all neuronal differentiation experiments. This neural differentiation and expansion method is adapted from manufacturer's instructions (Life Technologies), which has been shown to generate functional NPCs from hiPSC.

Table 2.1: NPC Expansion media

Reagents	**10 mL**	**Cat#**	**Suppliers**
Neurobasal (NB)	4900 µL	21103-049	Invitrogen - Thermo Fisher Scientific
Advanced DMEM/F12	4900 µL	12634-010	Thermo Fisher Scientific
Neural Induction Supplement	200 µL	A16477-01	Thermo Fisher Scientific
ROCKi (Y-27632)	10 µL	10005583-5	Cayman chemical

2.1.2 Neuronal Differentiation

To differentiate NPCs to neurons, 8-well chamber slides (154534, Thermo Scientific) were coated with laminin (CB-40232, Fisher Scientific). Laminin was diluted in ice cold PBS (311-011-CL, Wisent) to a final concentration of 10 ug/mL and added into the wells of an 8-well chamber slide and incubated at 37°C for 1-2 hours. After slides were coated, NPCs were plated at a density of 1.5 x 10^4 cells/cm^2 in neuronal differentiation medium (Table 2.2). Media was changed every 2-3 days and cells were differentiated for 14 days before experimentation (Yan et al., 2013).

Table 2.2: Neuronal Differentiation Media

Reagent	Stock	Final conc	10mL	Cat#	Suppliers
NB	-	-	9570 µL	21103049	Thermo Fisher Scientific
B-27	50x	1x	200 µL	17504044	Thermo Fisher Scientific
Glutamax	200 mM	2mM	100 µL	35050061	Thermo Fisher Scientific
MEM	100x	1x	100 µL	321-011-EL	WISENT Inc
BDNF	20 µg/ml	20 ng/ml	10 µL	78005	STEMCELL Technologies, Vancouver, BC, Canada
GDNF	20 µg/ml	20 ng/ml	10 µL	78058	STEMCELL Technologies
L(+)-ascorbic acid	200 mM	200 µM	10 µL	97061-072	VWR, Radnor, PA, USA
ROCKi	10 mM	10 µM	10 µL	10005583-5	Cayman chemical

2.2 Oxygen-glucose deprivation/reperfusion (OGD/R)

Previous work in our lab showed no significant differences between 5% oxygen level and control condition in human organoid cultures, reinforcing the need to optimize more physiologically relevant hypoxic conditions. Based on these findings, subsequent experiments in this study were conducted using a standard condition of 3% oxygen.

To establish the oxygen-glucose deprivation/reperfusion (OGD/R) model, glucose and oxygen were removed from the neuronal culture by changing the media to a mix of glucose-free neurobasal-A (990 μL) (A24775-01, Gibco) and glutamax (10 μL) and de-gassing this media in a hypoxic chamber (Cytation 5.0 imaging reader, Gen 5 version 3.05 software) for 30 minutes. Glucose and oxygen-free media was added to the hiPSC-derived culture dishes, and they were placed in the hypoxic chamber with a controlled atmosphere of 92% N_2, 5% CO_2, and 3% O_2. Cells were kept under OGD conditions for 3 hours at 37°C. After the OGD period, the hypoxic solution was removed from the cell culture plates. For reperfusion, neuronal differentiation media was added back to the neurons. The plates were returned to the standard incubator conditions (37°C, 5% CO_2, humidified atmosphere). Neuronal cultures were collected after 24 h for analysis.

2.3 Immunocytochemistry

Twenty-four hours post-OGD, the cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature, followed by rinsing three times with 1x Phosphate-buffered saline (PBS) (pH 7.4) for 5 minutes each. Primary antibodies (Table 2.3) were diluted in 1 x PBS. The diluted primary antibody was applied to the cells and incubated overnight at 4°C to facilitate protein binding. Following primary antibody incubation, the cells were washed three times with 1

x PBS for 5 minutes each to remove excess unbound primary antibody and to reduce background staining. The appropriate secondary antibodies (Table 2.3) were diluted in 1 x PBS. The secondary antibody solution was applied to the cells and incubated for 1 hour at room temperature while protected from light. Secondary antibody solution was removed, and cells were washed with 1 x PBS for 5 minutes. Cell nuclei were stained with Hoechst stain (bis-benzamide H 33258, B1155, Sigma-Millipore, Burlington, MA, USA) for 3 minutes. Following Hoechst staining cells were washed two times with 1 x PBS for 5 minutes each and then coverslipped using [1:3] glycerol:1 x PBS as a mounting media. Nail polish was used to seal the coverslips onto the slides.

2.4 Propidium Iodide (PI) staining (Live cell staining)

Propidium Iodide (PI) solution (P4864-10ML, Sigma-Millipore) was diluted in 1 x PBS at the final concentration of 1 μg/ml. The PI (5 μL per well) was added directly to the media of unfixed cells at 24 hours post-OGD. Cells were incubated for 10 minutes at 37°C to facilitate labeling dead cells. Following incubation, cells were ready for imaging.

Table 2.3: Antibodies and stains

Name	Target	Cat#	Conc	Suppliers
Primary mouse anti-Tau (Tau 46)	Tau - Presence of neurons	sc-32274	1:200	Santa Cruz Biotechnology, Dallas, TX, USA
Primary mouse anti-Hif-1α (H1α 67)	Hif-1α – Presence of hypoxia	sc-53546	1:100	Santa Cruz Biotechnology
Primary rabbit anti-AC3	AC3 - Presence of apoptotic cell death	559565	1:400	BD Biosciences, Franklin Lakes, New Jersey, USA
Primary anti-Hu Nestin	Hu-Nestin – Presence of NPCs	14-9843-80	1:500	Invitrogen- Thermo Fisher Scientific
Primary polyclonal rabbit anti-Pax6	Presence NPCs	Ab5409	1:300	Sigma-Millipore
Primary polyclonal rabbit anti-Pax6	Presence of NPCs	PRB-278P	1:100	Covance, Princeton, NJ, USA
Primary mouse anti-S-100 β	S-100 - Presence of astrocytes	sc-393919	1:100	Santa Cruz Biotechnology

Primary polyclonal rabbit anti-GFAP	GFAP - Presence of astrocytes	Z0334	1:400	Dakocytomation, Glostrup, Denmark
Primary monoclonal mouse anti-GFAP	GFAP - Presence of astrocytes	G6171	1:400	Sigma-Millipore
Primary mouse anti-Ki67	Ki67 - Presence of proliferation	550609	1:100	BD Pharmingen, San Diego, CA, USA
Secondary donkey anti-rabbit Alexa Fluor 488	Primary rabbit anti-AC3/ anti-MAP-2/ anti-Pax6/ anti-GFAP	A21206	1:200	Invitrogen - Thermo Fisher Scientific
Secondary donkey anti-mouse Alexa Fluor 488	Primary mouse anti-Tau/ anti-Hif-1α/ anti-Nestin/ anti-S-100 β/ anti-GFAP/ anti-Ki67	A21202	1:200	Invitrogen - Thermo Fisher Scientific
Secondary donkey anti-rabbit Alexa Fluor 594	Primary rabbit anti- MAP-2/ anti-GFAP	A20207	1:200	Invitrogen - Thermo Fisher Scientific
Hoechst (Bisbenzimide) stain	Nuclei	B1155	1:500	Sigma-Millipore
Propidium iodide (PI)	Dead cells	P4864-10ML	1:200	Sigma-Millipore

2.5 Quantification of axon area

For quantifying neurodegeneration, neuronal cultures were subjected to primary mouse anti-Tau (Tau 46, sc-32274, Santa Cruz Biotechnology) immunostaining. The quantification of axon degeneration was conducted using ImageJ software (version 1.53t, http://imagej.nih.gov/ij). To ensure that analysis of the images were performed blind, a colleague renamed all images. Scale was set to 3.125 pixel/µm. Images were imported into program and converted into an 8-bit format. Brightness and contrast were adjusted to enhance thresholding accuracy. ImageJ provided the total area and percentage of total Tau positive area within the image. Subsequently, the area fraction was selected in the set measurement prior to the final measurement. Percentage of the Tau positive area relative to each individual Tau positive cell was calculated using the formula provided below:

$$\%\ Tau\ area\ per\ tau\ positive\ neuron = \frac{\%\ Tau\ positive\ area}{Total\ number\ of\ Tau\ positive\ cells}$$

We considered a reduction of >10% loss of tau+ area per tau+ neuron as indicative of neurodegeneration which could include loss of neuronal processes and neuronal cell volume.

2.6 Microscopy and cell counting

PI staining of cells was visualized using a Zeiss AxioObserver D1 inverted microscope, utilizing a 100-watt mercury bulb as the light source. The objectives used were 20x Korr M27

LD Plan-Neofluar (Cat# 421350-9970) with a numerical aperture (NA) of 0.4. The HE DsRed filter set (excitation: 545–570 nm, emission: 580–620 nm) was used for PI visualization.

Following immunocytochemistry, cells were visualized using a Zeiss AxioImager Z1 upright microscope, equipped with a Colibri LED light. The objective used was 20x/0.5 ECPlan (Cat# 420350-9900). The following filter sets were used for fluorescence imaging on the Zeiss AxioImager Z1: DAPI filter set (excitation: 358–405 nm, emission: 415–485 nm), DsRed filter set (excitation: 545–570 nm, emission: 580–620 nm), HE mPlum filter set (excitation: 590–650 nm, emission: 660–720 nm), GFP filter set (excitation: 450–490 nm, emission: 500–550 nm).

Images of the neuronal cell culture were collected using a Carl Zeiss AxioCam MRm Rev3 camera and Axiovision Rel. 4.8 software. Five images were captured from each corner and the center of the wells per experiment, assessing an average of 1500 cells. Image size is 444.16 µm x 332.8 µm and experiments repeated 5-7 times. Cells of interests were counted manually using ImageJ software. All images were blinded prior to cell counting by changing the name of the files. Adobe Photoshop 2022 (version 23.3.1) software was used to generate figures and to adjust the brightness and contrast of the images.

2.7 Statistical Analysis

Statistical analysis was performed using paired samples t-test between cultures to assess differences in the percentage of cells of interest across experimental groups. GraphPad Prism V software (GraphPad Software Inc., La Jolla, CA, USA) program was used to make these statistical analyses. $P<0.05$ was considered significant.

Chapter 3 : RESULTS

AIM 1: Determine the cellular composition of human iPSC-derived neuron cultures

The human induced pluripotent stem cell (hiPSC)-derived neuronal culture represents a platform for studying neurodevelopmental processes, disease modeling, and drug screening (Ebert et al., 2012). Our objective was to examine the cellular composition of the neuronal cultures derived from hiPSCs and assess their responses to ischemic injury at 24-hour. This approach allows us to assess the cellular dynamics and provides valuable insights into cellular responses under ischemic conditions *in vitro.*

3.1 hiPSC-derived neuronal culture consists of neurons and neural progenitor cells (NPCs)

To determine the cellular composition of cultures derived from hiPSCs, immunocytochemistry was conducted to identify the main cell types, namely neurons, NPCs, and astrocytes (Figure 3.1A, B). Nestin and Tau are considered markers of distinct developmental stages, labeling NPCs and post-mitotic neurons, respectively. Immunostaining for neurons was performed using Tau antibody (Figure 3.1A). Analysis of this immunostaining results revealed that 29.8% ± 6.1 of cells were Tau positive (Tau+) neurons (Figure 3.1C). The presence of NPCs was examined using Nestin antibody (Figure 3.1B). This analysis revealed the percentage of Nestin+ NPCs was 41.4% ± 2.8 of the cells (Figure 3.1C). Furthermore, the presence of Glial fibrillary acidic protein (GFAP)+ astrocytes within the cultures was examined. However, the analysis revealed no detectable GFAP+ cells (data not shown). Similarly, S100β immunostaining, another astrocyte marker, did not show any S100β+ cells within the cultures (data not shown). These results indicate the absence of astrocytes expressing GFAP and S100β in our culture condition.

Overall cell death can be detected using Propidium Iodide (PI) staining (Lema et al., 2011). Vital dyes such as PI only enter cells with a compromised cell membrane, thus selectively labeling

dead cells (Lema et al., 2011). Measuring membrane integrity is a direct and reliable way to assess cellular viability (Kroemer et al., 2005).

To assess cell viability in the control neuronal culture at 14-days, PI staining was used to label dying cells with compromised membrane integrity (Figure 3.1C). Quantitative analysis revealed a mean percentage of 13.6% ± 7.1 PI+ cells in hiPSC-derived neuronal cultures. This finding shows the level of background cell death in the culture.

Overall, this demonstrates that after 14 days *in vitro*, hiPSCs cell cultures consisted of 41% NPCs, 30% neurons with a background level of 14% cell death and the remaining 15% cells were unlabeled.

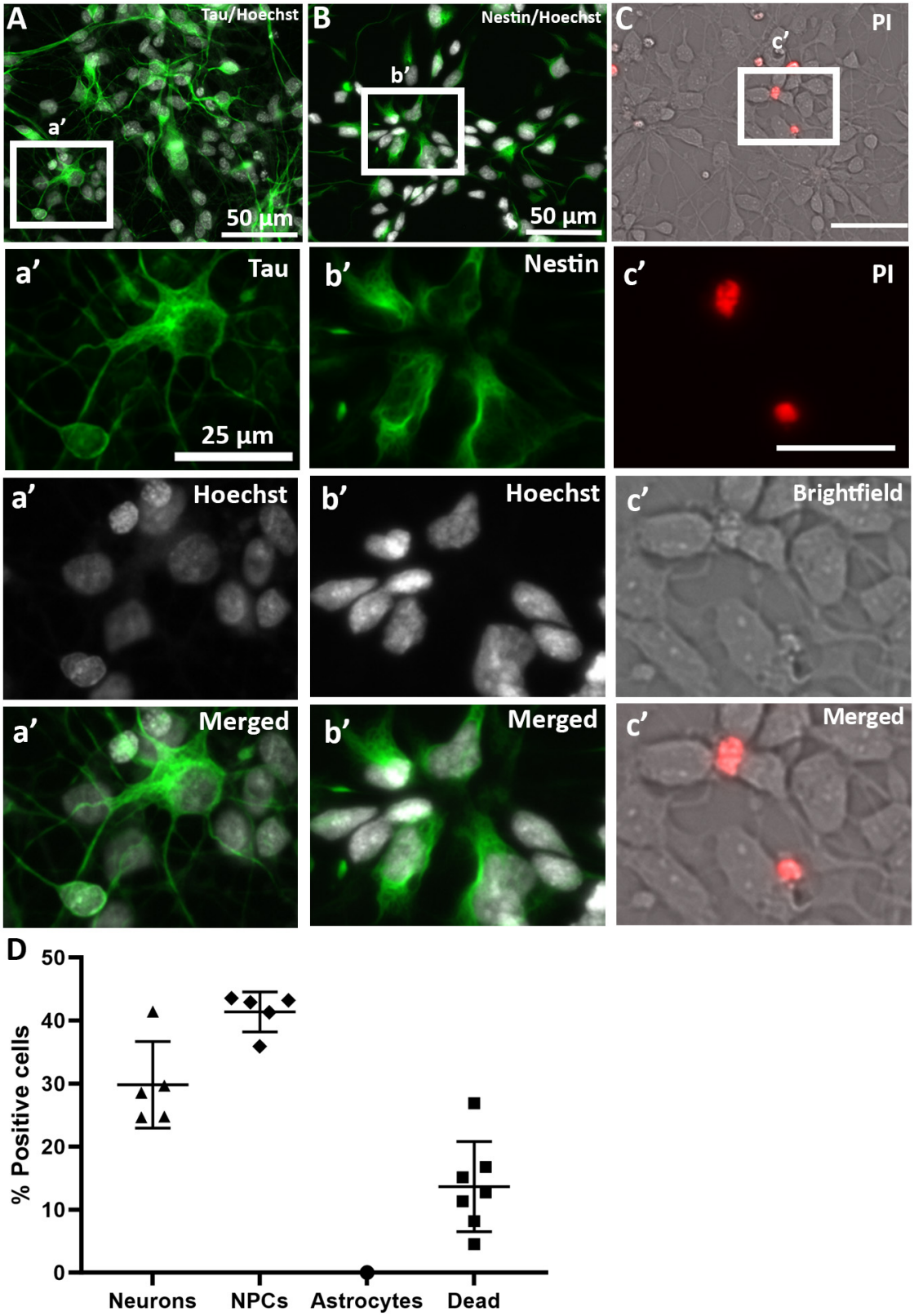
A
Tau/Hoechst
a'
50 µm
B
Nestin/Hoechst
b'
50 µm
C
PI
c'
a'
Tau
25 µm
b'
Nestin
c'
PI
a'
Hoechst
b'
Hoechst
c'
Brightfield
a'
Merged
b'
Merged
c'
Merged
D
% Positive cells
50
40
30
20
10
0
Neurons
NPCs
Astrocytes
Dead

Figure 3.1: Cellular composition of hiPSC-derived neuron cultures in culture

(A) Immunostaining of Tau+ cells (green) indicating neurons and Hoechst staining of cell nuclei (white). (a') Higher magnification of boxed regions in panel A. (B) Immunostaining of Nestin+ cells (green) indicating NPCs and Hoechst staining of cell nuclei (white). (b') Higher magnification of boxed regions in panel B. (C) Staining of PI+ cells (red), indicating compromised cell membrane integrity and cell death. Cells were photographed with a combination of fluorescent microscopy for PI staining and brightfield microscopy for all cells. (c') Magnified views of specific regions from panel C. (D) Quantification of the % Tau+ neurons, % Nestin+ NPCs, %GFAP/S100β+ astrocytes, and % PI+ dead cells after 14 days in culture. Data represent 5 independent experiments (n=5) for neurons, NPCs, and astrocytes and 7 independent experiments (n=7) for cell death. Data expressed as means ± SD.

3.2 Cell proliferation observed in hiPSC-derived neuronal cultures

Ki67 protein expression is observed throughout the active cell cycle phases (G1, S, G2, and mitosis) while it is absent in differentiated cells (G0) (Uxa et al., 2021). This characteristic makes Ki67 an excellent marker for determining cell proliferation (Scholzen & Gerdes, 2000). To examine the extent of cell proliferation in the neuronal culture, cultures were immunostained with Ki67 antibody (Figure 3.2). Quantitative analysis revealed that 35.4% ± 7.5 of the cells were Ki67 positive. This result demonstrates that cultures derived from hiPSCs contain proliferating cells.

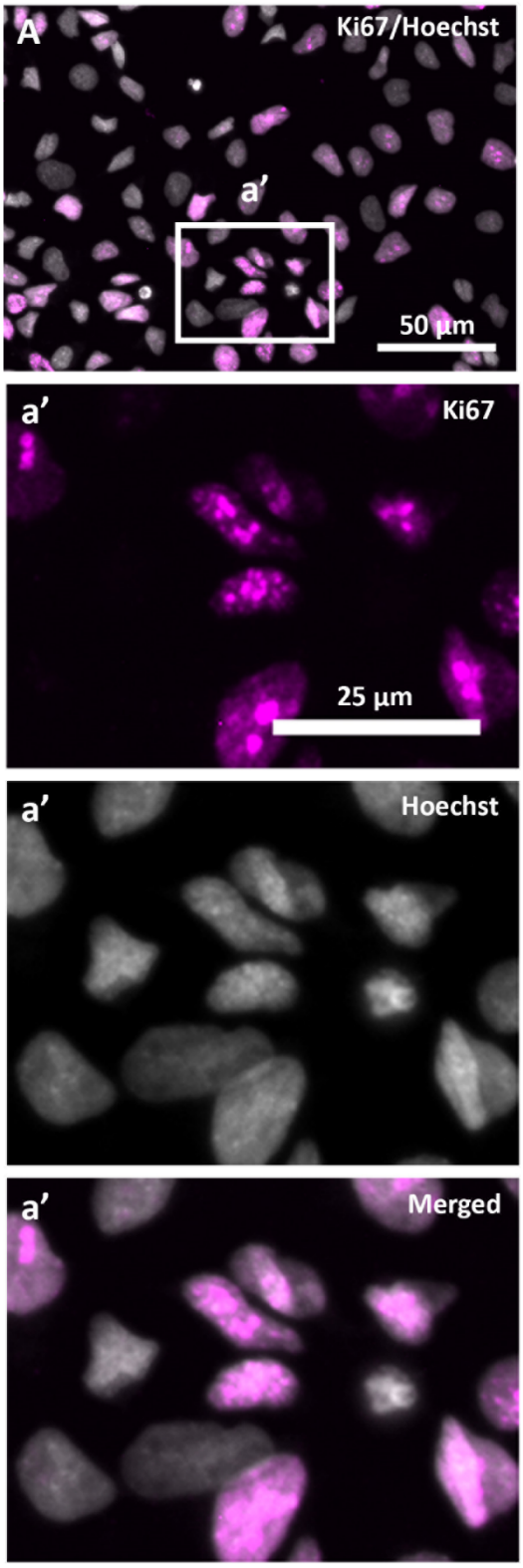

Figure 3.2: Cell proliferation in hiPSC-derived neuron cultures

(A) Immunostaining of Ki67+ cells (magenta), a marker for cell proliferation. Hoechst staining identified cell nuclei (white). (a') Higher magnification of selected region in panel A. Images representative of 6 independent experiments (n=6).

AIM 2: Assess the acute cellular responses to stroke in hiPSC-derived neuronal cultures at 24h post OGD

3.3 The localization of Hypoxia-inducible factor 1-alpha (Hif-1α) changes depending on the levels of oxygen and glucose

Hypoxia-inducible factor 1-alpha (Hif-1α) is a transcription factor that plays a critical role in the cellular response to hypoxia (Ziello et al., 2007). In healthy cells, Hif-1α is localized in the cytoplasm. Following a hypoxic event, Hif-1α translocates from the cytoplasm to the nucleus (Choi et al., 2021).

To determine whether exposing neuronal cultures to OGD for 3 hours was sufficient to produce an ischemic response, immunocytochemistry detection of Hif-1α was performed (Figure 3.3 A, B). Co-staining with Hoechst, a nuclear marker, demonstrated that Hif-1α was predominantly localized within the cytoplasm in control cultures (Figure 3.3 a'). However, following OGD/R, Hif-1α was found to be primarily localized within the nucleus (Figure 3.3 b'). Many of the Hif-1α+ nuclei observed following OGD/R appeared smaller than typical neuronal nuclei. This may reflect nuclear condensation associated with early-stage apoptosis, as nuclear shrinkage is a hallmark of programmed cell death. Quantification of immunopositive cells with Hif-1α localized to the nucleus was significantly increased at 24 h following OGD. The mean percentage of Hif-1α+ nuclei in control groups was 6.9 ± 2.9 cells, however, it increased to 20.7 ± 5.3 cells in the OGD/R group (Figure 3.3 C). Translocation of Hif-1α into the nucleus confirmed cells experienced a hypoxic event.

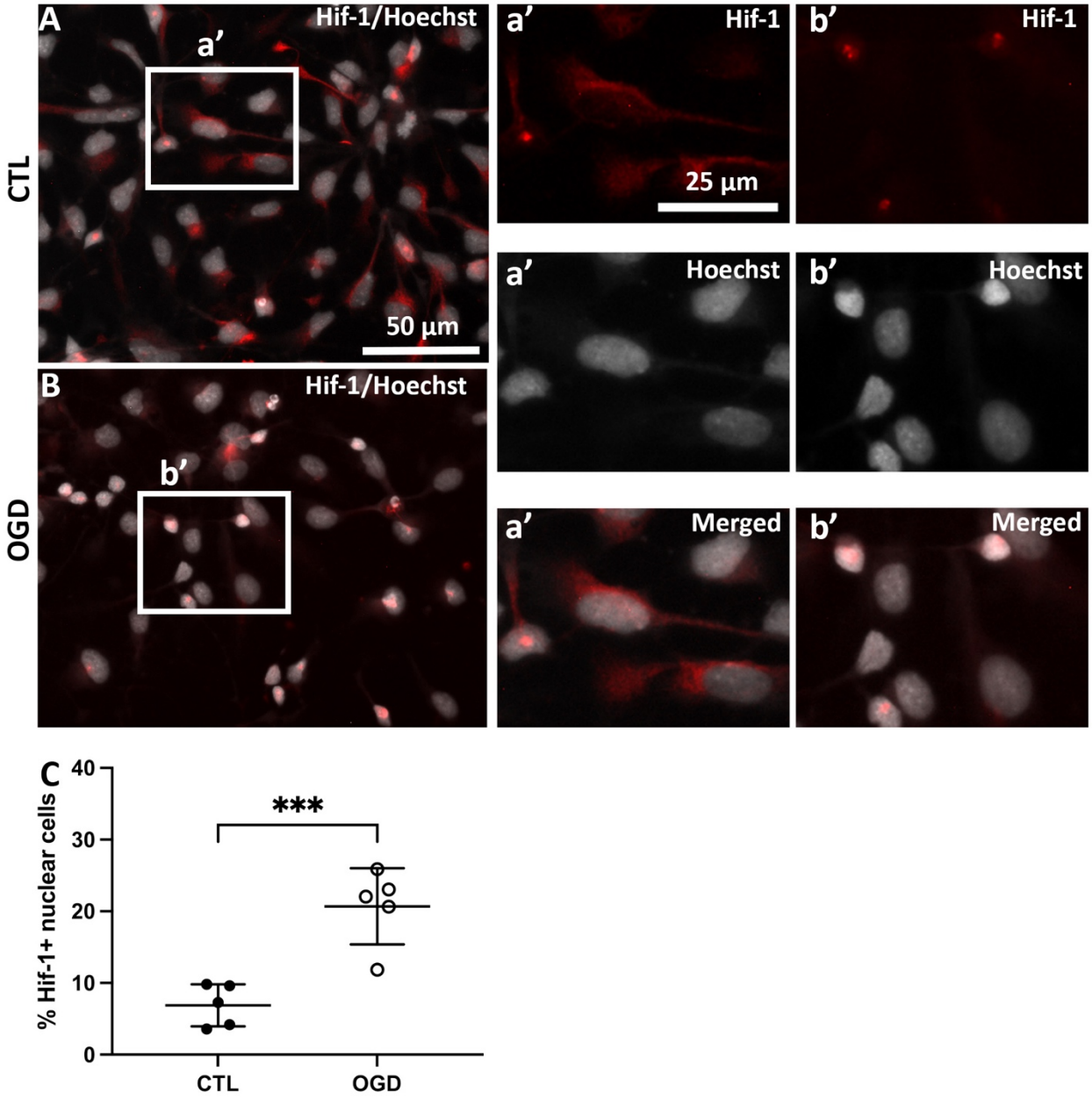

Figure 3.3: Translocation of Hif-1α from cytoplasm to the nucleus at 24h following OGD

(A, B) Immunostaining of Hif-1α+ nuclei (red) and Hoechst staining of cell nuclei (white) within control and OGD/R treated hiPSC-derived neuronal cultures at 24h. (a', b') Higher magnification of boxed regions in panels A and B. Most of the Hif-1α protein translocated from the cytoplasm to the nucleus following OGD/R. (C) Quantification of Hif-1α+ nuclei. Statistical analysis was performed using a two-tailed unpaired t-test. Data represent 5 independent experiments (n=5) and are expressed as means ± SD. ***$p < 0.001$. P value= 0.0009. t=5.094. df=8

3.4 No significant changes were observed in the overall cell death at 24h post OGD.

To determine how cell viability is affected at 24 hours following OGD, PI staining was performed on both control and OGD/R-treated cultures (Figure 3.4 A, B).

The analysis revealed that the percentage of PI-positive cells in control cultures was 13.6% ± 7.1, compared to 21% ± 7.7 in OGD/R-treated cultures (Figure 3.4 C). Although there was an apparent increase in PI-positive cells in OGD/R-treated cultures, statistical analysis indicated that this difference was not significant. These findings suggest that the OGD/R treatment did not have a substantial effect on overall cell death within the initial 24h.

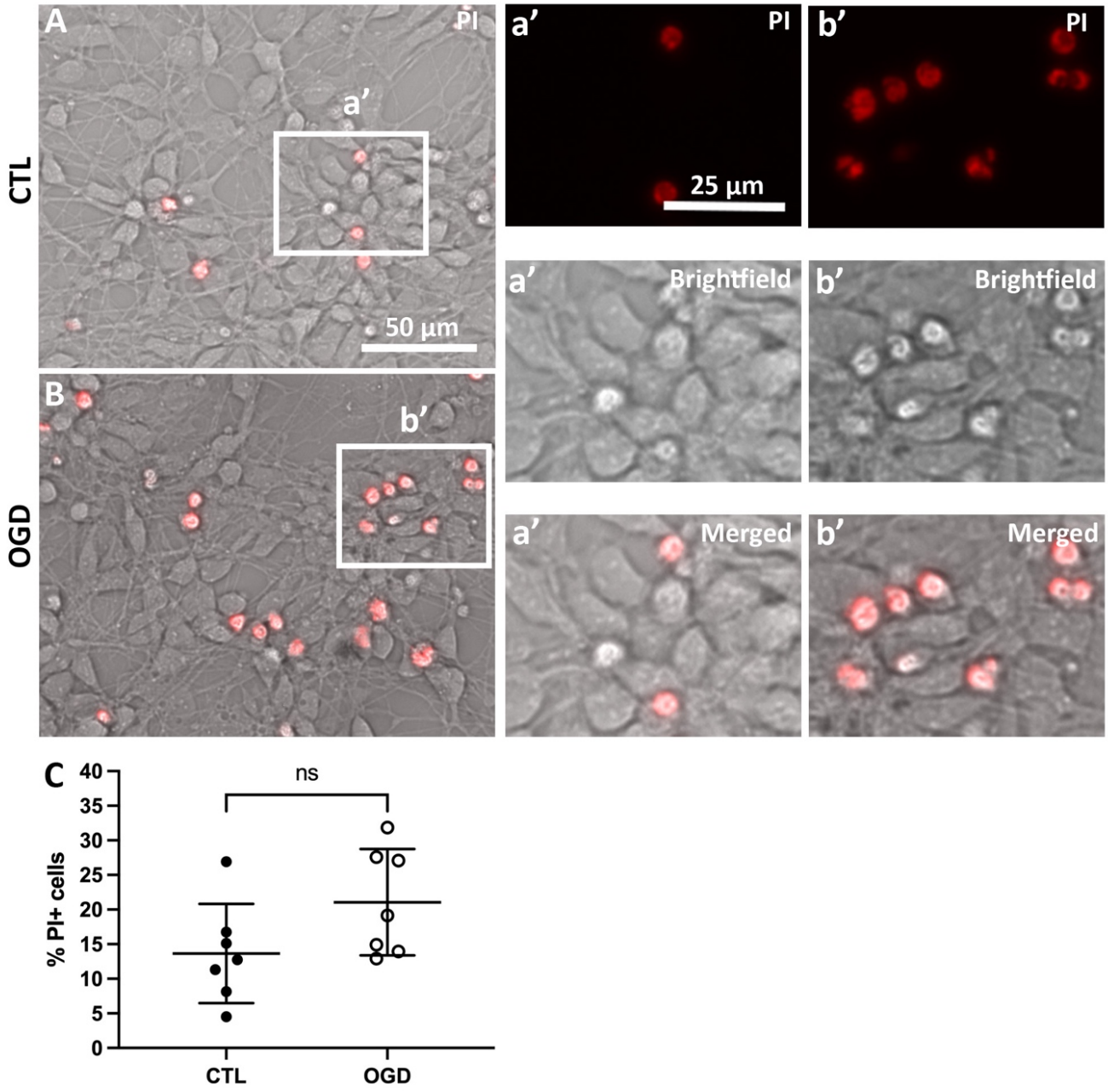

Figure 3.4: Overall cell death in hiPSC-derived neuronal cultures at 24h following OGD

(A, B) PI+ staining (red) of cells in control and OGD/R treated cultures indicating membrane disruption and cell death. Cells were imaged using brightfield microscopy. (a',b') Magnified views of specific regions from panels A and B. (C) Quantification of PI+ cells. Statistical analysis was performed using a two-tailed unpaired t-test. Data represent 7 independent experiments (n=7) and are expressed as means ± SD. n.s. = not significant. P value= 0.0860. t=1.871. df=12

3.5 Apoptosis significantly increases at 24h post OGD

Apoptosis is the primary cause of neuronal death in the penumbra (Ferrer, 2006; Yao et al., 2001). Identifying apoptotic cells typically involves using a combination of biochemical, molecular, and morphological methods including TUNEL assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling) that detects DNA fragmentation, DAPI or Hoechst staining that binds to DNA and visualizes nuclear changes such as chromatin condensation, and immunocytochemistry using antibodies to detect apoptotic markers like antibodies specific to active caspase-3 (AC3) protein. AC3 represents the cleaved form of caspase-3, widely recognized as a marker of apoptosis (Turner et al., 2007).

Here, to assess changes in the level of apoptotic cell death in hiPSC-derived neuronal cultures following OGD/R, immunocytochemistry for AC3 was performed (Figure 3.5 A, B). Immunostaining analysis revealed a significant elevation in the number of AC3+ cells at 24h post OGD (Figure 3.5 C). Quantification shows that the mean percentage of AC3+ cells in control groups was 5% ± 2.8, whereas, in OGD/R groups it increased to 13.7% ± 4.6, accounting for two-thirds of the total cell death observed. This suggests that OGD/R induces apoptotic cell death of hiPSC-derived neurons.

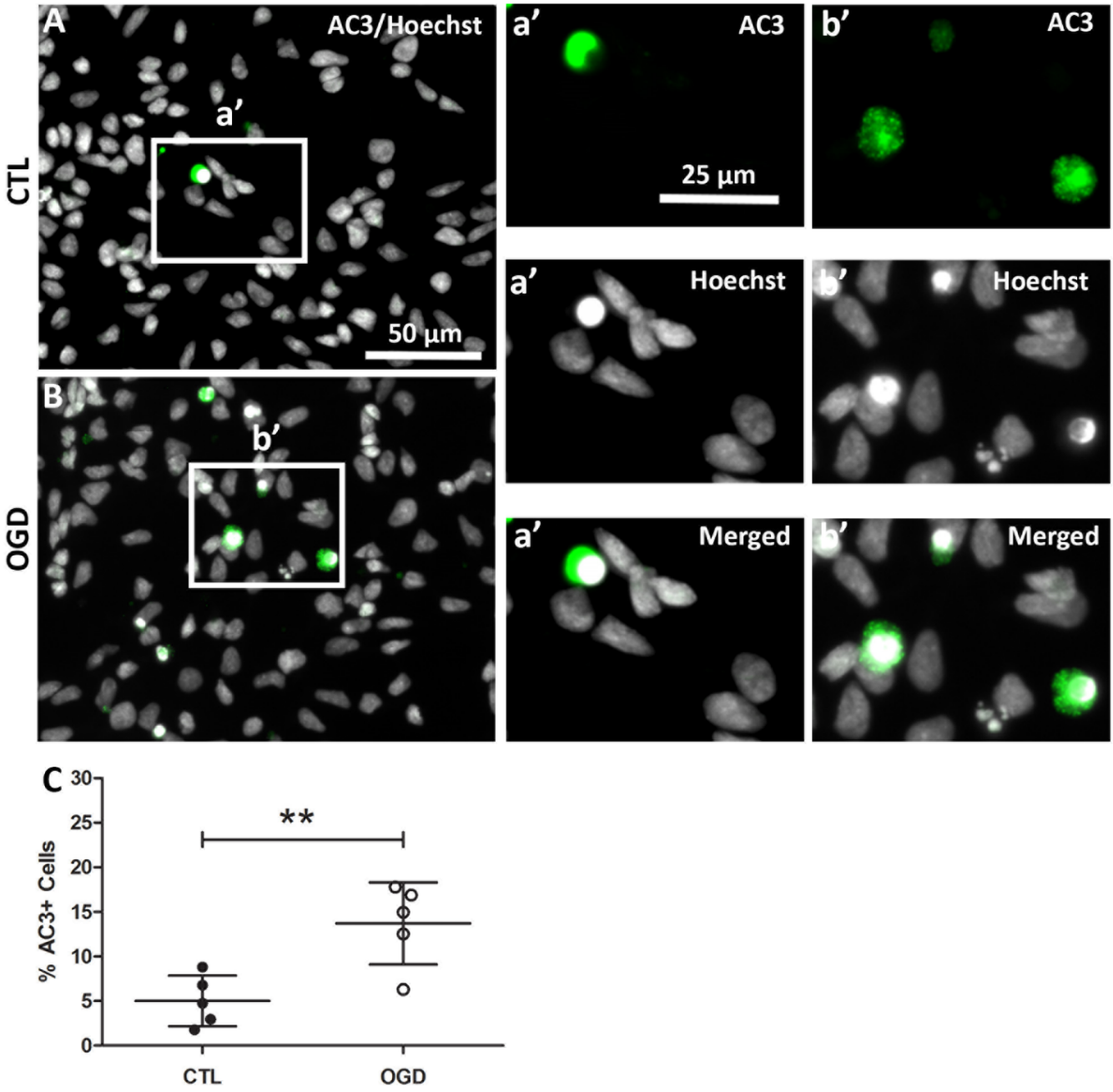

Figure 3.5: Apoptotic cell death in hiPSC-derived neuronal cultures at 24h following OGD.

(A, B) Immunostaining of AC3+ cells (green), indicating apoptotic cell death and Hoechst staining of cell nuclei (white) in control and OGD/R treated cultures at 24h. (a', b') Magnified views of specific regions from panels A and B. (C) Quantification of AC3+ cells show a significant increase following OGD/R. Statistical analysis was performed using two-tailed unpaired t-test. Data represent 5 independent experiments (n=5) and are expressed as means ± SD. $^{**}p < 0.01$. P value= 0.0071. t=3.589. df=8

3.6 The level of cell proliferation did not significantly change at 24h post-OGD

One third of cells in hiPSC-derived neuronal cultures are proliferating cells (Figure 3.2). To investigate whether OGD/R affected the level of cell proliferation in hiPSC-derived neuronal cultures the number of Ki67+ cells was quantified in both control and OGD/R groups (Figure 3.6 A, B). Ki67 immunostaining analysis revealed that at 24h post OGD, there was no significant change in cell proliferation with 35.4% ± 7.5 Ki67+ cells in control cultures and 30.8% ± 13.3 Ki67+ cells in OGD/R cultures (Figure 3.6, C). This suggests that the OGD/R treatment did not significantly impact the level of cell proliferation within the hiPSC-derived neuronal cultures.

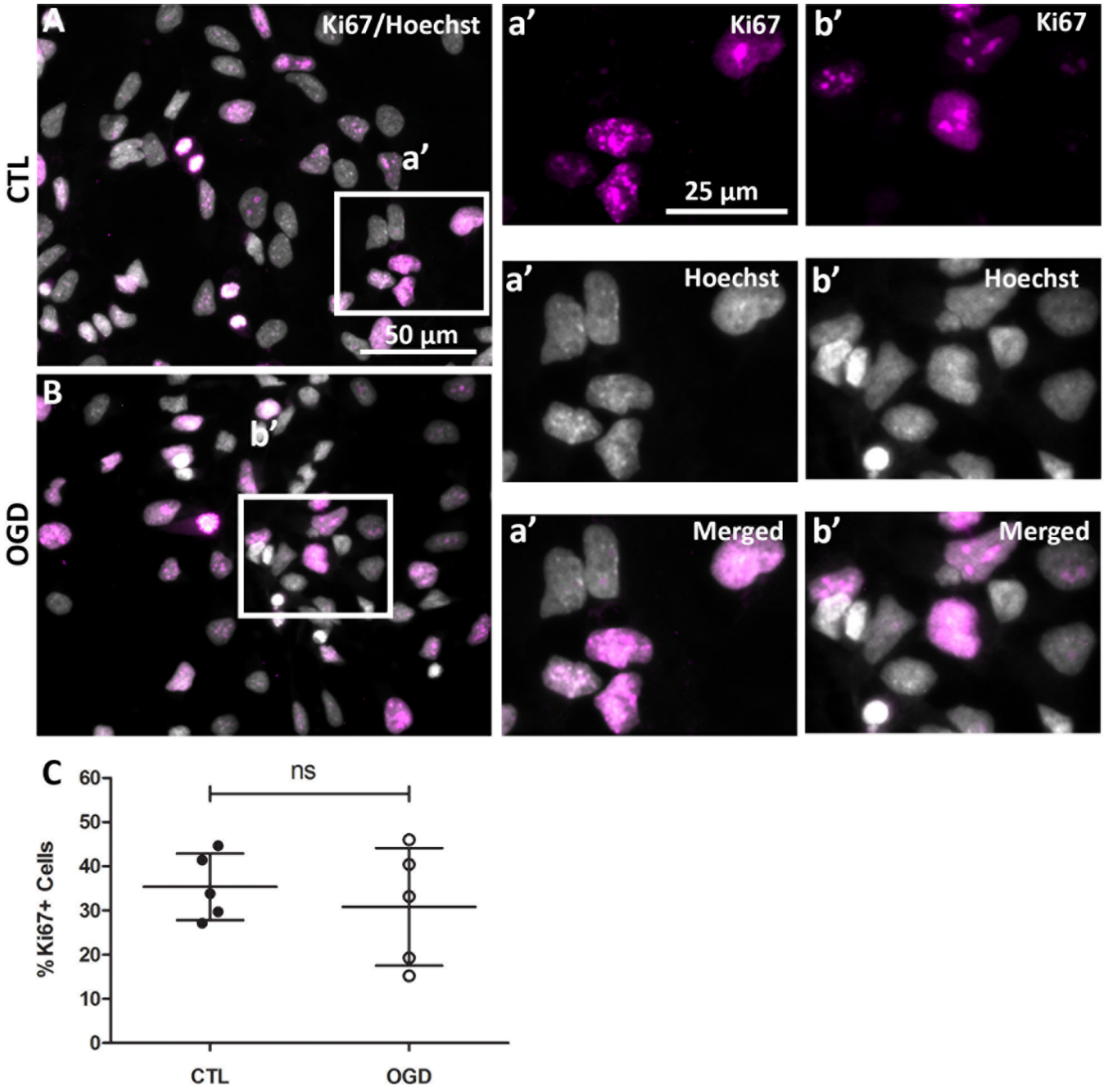

Figure 3.6: Cell proliferation in hiPSC-derived neuronal cultures at 24h following OGD.

(A, B) Immunostaining of Ki67+ cells (magenta) and Hoechst staining of cell nuclei (white) within control and OGD/R treated cultures at 24h. (a', b') Higher magnification of boxed regions in panels A and B. (C) Quantification of Ki67+ cells. Statistical analysis was performed using a two-tailed unpaired t-test. Data represent 5 independent experiments (n=5) and are expressed as means ± SD. n.s. = not significant. P value= 0.5252. t=0.6643. df=8

3.7 No significant changes were observed in the number of Nestin+ NPCs at 24h post OGD

To investigate the influence of OGD/R on NPCs, immunohistochemistry for Nestin was performed on control and OGD/R treated cultures (Figure 3.7 A, B). Immunocytochemistry analysis demonstrated no significant change in the number of Nestin+ NPCs at 24h post OGD (Figure 3.7 C). Quantitative analysis revealed a mean percentage of 44.3% ±6.6 of Nestin+ cells in control cultures and 42.5% ± 8.7 of Nestin+ cells in OGD/R treated cultures. This finding indicates that despite the challenging OGD/R conditions, the NPC population appeared resilient, maintaining its level within the hiPSC-derived neuronal cultures at 24h following OGD.

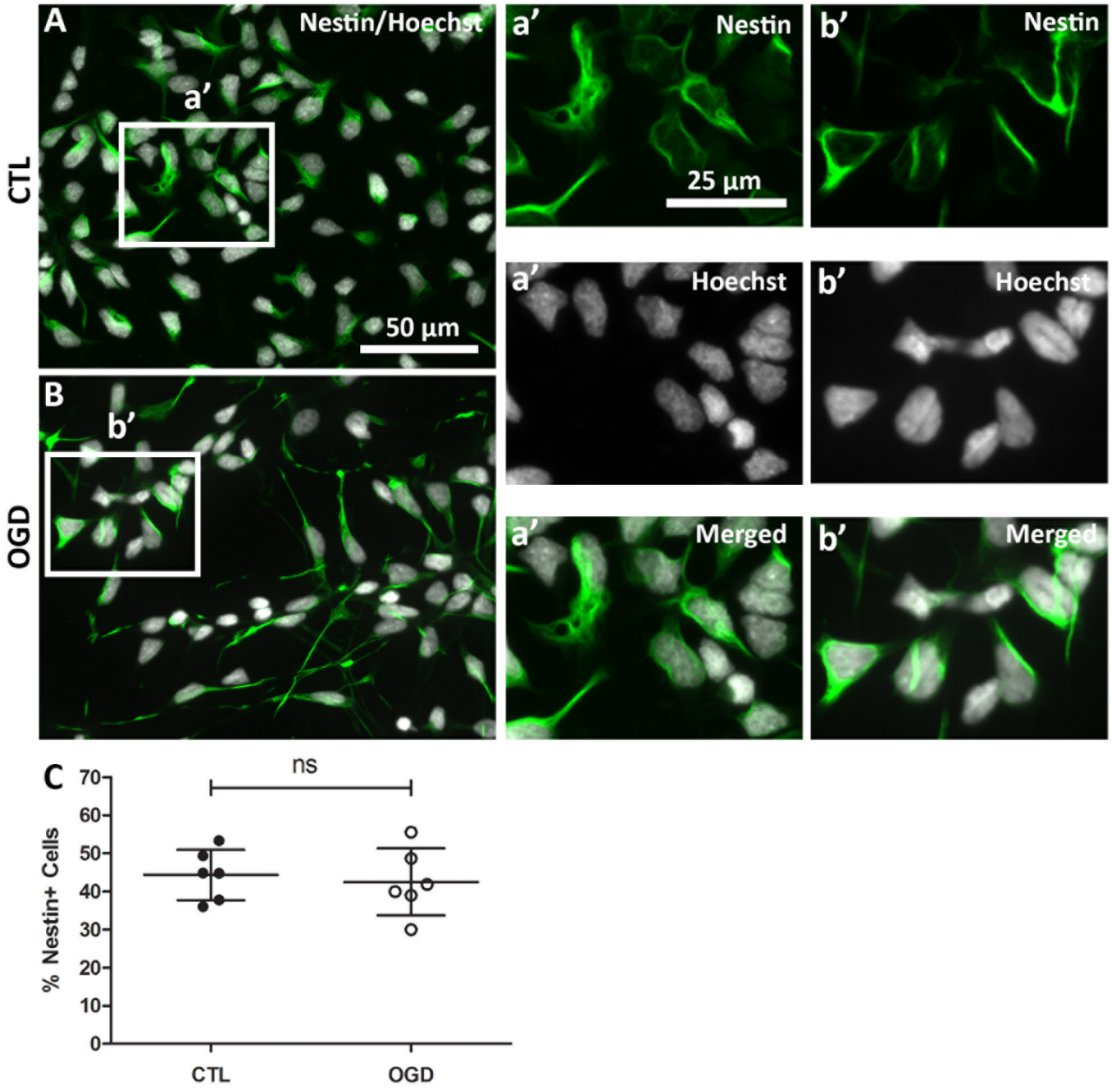

Figure 3.7: Effect of OGD/R on the level of NPCs in hiPSC-derived neuronal cultures at 24h following OGD.

(A, B) Immunostaining of Nestin+ NPCs (green) and Hoechst staining of cell nuclei (white) in control and OGD/R treated cultures at 24h. (a', b') Magnified views of specific regions from panels A and B. (C) Quantification of Nestin+ NPCs showed no significant change in number at 24h following OGD. Statistical analysis was performed using a two-tailed unpaired t-test. Data represent 6 independent experiments (n=6) and are expressed as means ± SD. n.s. = not significant. P value= 0.6920. t=0.4079. df=10

3.8 Number of healthy Tau+ neurons were significantly decreased following OGD/R

To investigate the impact of OGD/R condition on neurons, the percentage of healthy neurons was compared between control vs. OGD/R cultures at 24 hours (Figure 3.8 A, B). Tau immunocytochemistry and Hoechst nuclear staining were performed on cell cultures. Neurons were considered 'healthy' if they were Tau-positive and displayed non-condensed nuclei. Tau immunocytochemistry was performed and significantly fewer healthy Tau+ neurons were observed in OGD/R cultures (Figure 3.8 C). Quantitative analysis showed a mean percentage of 28% ± 4 Tau+ neurons in control cultures and 21.3% ± 1.4 Tau+ neurons in OGD/R cultures. This suggests that the OGD/R significantly impacts the number of healthy neurons within the hiPSC-derived neuronal cultures.

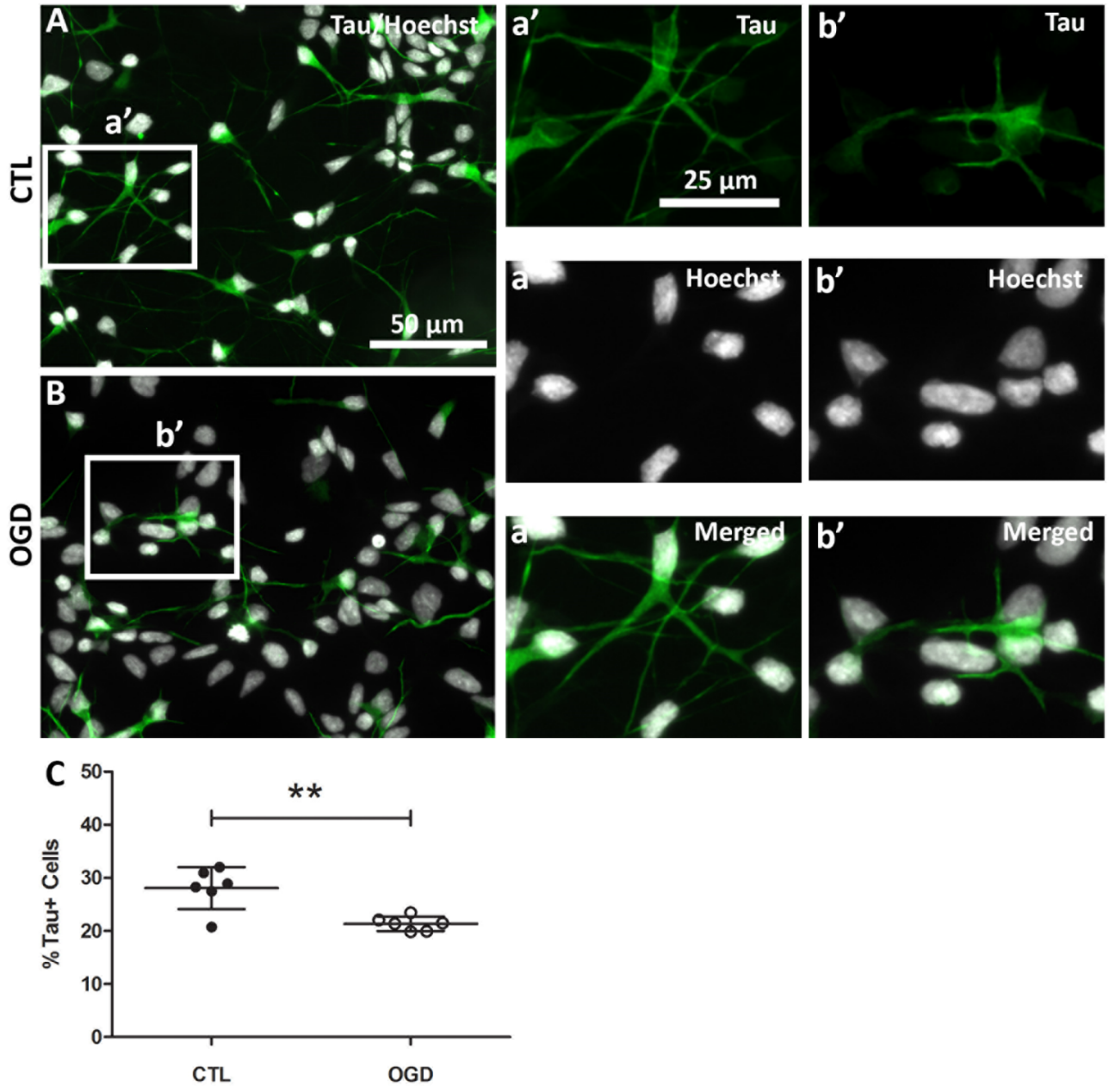

Figure 3.8: The effect of OGD/R on hiPSC-derived neurons at 24h following OGD.

(A, B) Immunostaining of Tau+ neurons (green) and Hoechst staining of cell nuclei (white) in control and OGD/R treated cultures at 24h. (a', b') Magnified views of specific regions from panels A and B.

(C) Quantification of Tau+ neurons showed a significant decrease at 24h following OGD. Statistical analysis was performed using a two-tailed unpaired t-test. Data represent 6 independent experiments (n=6) and are expressed as means ± SD. $^{**}p < 0.01$. P value= 0.0029. t=3.910. df=10

3.9 Neurodegeneration was significantly increased following OGD/R.

To determine the extent of neurodegeneration in hiPSC-derived neuronal cultures at 24h following OGD, the area of Tau+ staining per microscope image (444.16 μm x 332.8 μm) and per Tau+ cell was compared between control vs. OGD/R treated cultures (Figure 3.9 A, B). Quantification revealed a ~50% reduction in Tau+ area per image. The mean percentage of Tau+ area / image was 11.84% ± 4.4 in control cultures and 6.24% ± 3.65 in OGD/R treated cultures (Figure 3.9 C). Similarly, there was a 50% decrease in Tau+ staining per Tau+ neuron. The mean percentage of Tau+ area / Tau + cell was 0.21% ± 0.07 in control cultures and 0.11% ± 0.05 in OGD/R treated cultures (Figure 3.9 D). This likely includes a reduction in neuronal processes and cell size which we are describing it as an indication of neurodegeneration. This data shows that OGD/R treatment significantly decrease the percentage of Tau+ area per image and per Tau+ neuron at 24h post OGD within the hiPSC-derived neuronal cultures.

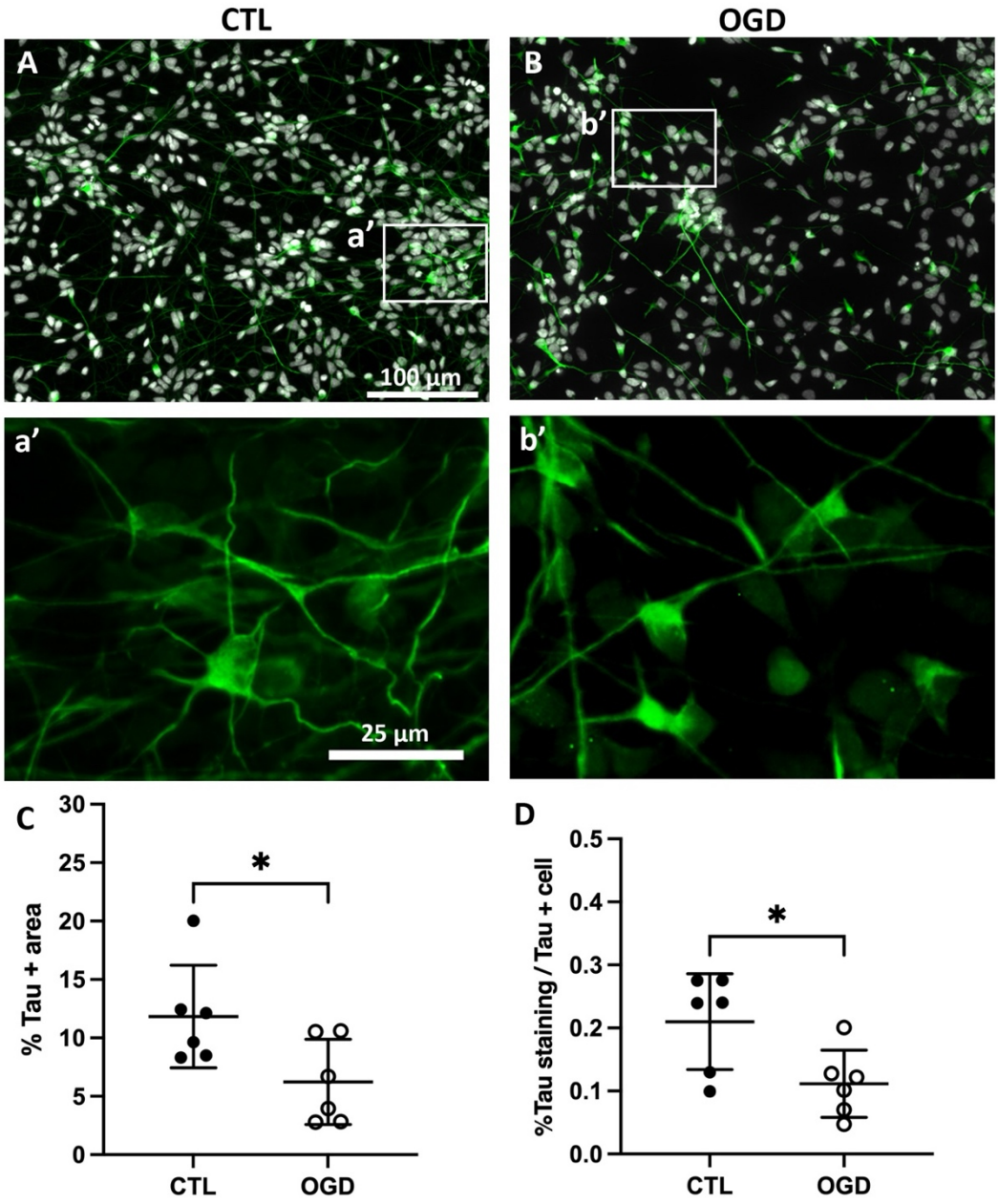

Figure 3.9: Neurodegeneration in hiPSC-derived neuronal cultures at 24h following OGD.

(A, B) Immunostaining of Tau+ neurons and their processes (green) in control and OGD/R treated cultures at 24h. Hoechst staining labeled cell nuclei (white). (a', b') Magnified views of specific regions from panels A and B. (C) Quantification of % Tau + area per image. (D) Quantification of Tau+ area / Tau+ neuron. Statistical analysis was performed using a two-tailed unpaired t-test. Data represent 6 independent experiments (n=6) and are expressed as means ± SD. *$p < 0.05$. % Tau + area P value = 0.0369, t = 2.406, df = 10. % Tau staining/Tau + cell P value = 0.0268. t = 2.592, df = 10

Chapter 4 : DISCUSSION

The goal of this research project was (a) to develop a cell culture model of human cells using hiPSC and (b) to establish an ischemic injury model that mimics the penumbra region to assess cellular responses to ischemic injury. There are three main findings. First, hiPSC-derived neuronal cultures consist of NPCs and neurons. Second, OGD/R caused significantly more cell death and neurodegeneration in neuronal populations versus NPCs demonstrating differential susceptibilities to ischemic injury. Third, no significant changes were observed in cell proliferation or the number of NPCs at 24h post-OGD. By creating a human neural cell-based *in vitro* model, this research project advances our understanding of post-stroke cellular mechanisms and may support the translation of preclinical findings into clinical applications.

4.1 The cellular composition of human iPSC-derived neuron cultures after 14 days in culture.

Human iPSCs can be derived from healthy donors or patients, reflecting their unique genetic profile and can be differentiated into neurons to build neuronal networks (Frega et al., 2017; Mossink et al., 2022). However, utilizing hiPSC-derived neuronal models in ischemic stroke research is a relatively recent advancement. Given the novelty of this approach, further investigation is needed to better understand and optimize these models for stroke studies. The process of deriving functional neurons from iPSCs is facilitated by manipulating the culture environment and applying specific factors that steer pluripotent stem cells toward a neuronal fate. Various protocols have been designed to produce neural cells from iPSCs, with most of them involving an intermediate stage where the cells become NPCs before differentiating into neurons or glial cells (Cheng et al., 2017; Muratore et al., 2014). Variability in genetics and epigenetics between different cell lines, differences in researcher techniques, cell culture environments, and differentiation protocols, often lead to different levels of NPC differentiation.

This inconsistency subsequently affects the purity and overall quality of the resulting neuronal cultures (Hu et al., 2010; Muratore et al., 2014; Paavilainen et al., 2018). Despite these challenges, iPSC-derived neurons remain a valuable tool for modeling human neural network *in vitro* and advancing our understanding of the cellular responses in a controlled manner.

Previous studies have reported differences in cellular composition of hiPSC-derived neuronal cultures depending on how long the cultures were maintained. For instance, D'Aiuto et al. reported that after 4 weeks, over 90% of differentiating cells were TUJ1 positive, a marker for immature neurons (D'Aiuto et al., 2014). In contrast, Gunhanlar et al. found that after initiating NPC differentiation and supplementing with BDNF and GDNF for 8–10 weeks, the culture contained 13% immature neurons, 16% mature neurons, 11% astroglia, and 60% NPCs (Gunhanlar et al., 2018). This thesis study aimed to establish a primarily neural cell culture without glial cells to allow for the investigation of neuron-specific responses to ischemia, which is why the cells were cultured for 2 weeks, following a previously reported protocol (Yan et al., 2013). The presence of glial cells can provide neuroprotection, which can mask the intrinsic vulnerability of neurons to ischemic injury (Goldberg & Choi, 1993). Immunostaining analysis showed the expression of Tau and/or Nestin, along with significant cell proliferation, indicating that our cultures predominantly consist of NPCs and neurons (Figures 3.1-3.2). The remaining 15% of cells were unidentified and may represent undifferentiated hiPSCs or unlabeled precursor cells (Figure 3.1). This approach allows us to specifically examine the impact of an ischemic insult on neurons independent of support cells.

Nestin is a marker of NPCs, however studies have shown its variable expression in developing astrocytes as well (Jurga et al., 2021; Palmer et al., 2000). Our study revealed no detectable GFAP positive or S100β positive astrocytes within the cultures after 2 weeks

(Figure 3.1). However, the absence of detectable GFAP and S100β does not necessarily indicate the absence of astrocytes, as these markers are typically expressed in mature astrocytes (Raponi et al., 2007). In normal human development astrocytes differentiate after neurons have formed (Pal et al., 1999; Wilkinson et al., 1990). Similarly astroglia progenitors or immature astrocytes are derived later than neurons in hiPSCs cultures after 4 to 8 weeks of differentiation (Krencik et al., 2011). From 8-16 weeks of differentiation these cells start expressing other astrocyte-specific markers like GFAP (Krencik et al., 2011). Thus, the findings in this study are consistent with the expected timeline of astrocyte development and maturation in hiPSC-derived cultures. These findings highlight the dynamic nature of hiPSC-derived neuronal cultures and the need for longer differentiation times to achieve maturation.

4.2 Apoptosis increases at 24h post OGD.

The goal of this study was to replicate the ischemic conditions of the penumbra region. While it is well documented that oxygen and glucose deprivation followed by reperfusion creates an ischemia-like insult (or injury) and leads to cell death, the extent of damage depends on the severity and duration of deprivation and reperfusion. Different cells also exhibit varying responses to this insult. Fifteen minutes of OGD with 0% O_2 on a rat culture resulted in 16% apoptotic cells with no necrosis, whereas 75 minutes of OGD led to 26% apoptosis and 23% necrosis after 24 hours (Malagelada et al., 2005). Cell death post-OGD has been also examined in SH-SY5Y- and hiPSC-derived neuronal cultures (Juntunen et al., 2020). After 24 hours of OGD exposure with 1% O_2 followed by 24 and 72 hours of reperfusion, live/dead staining revealed a significant increase in the number of dead cells in SH-SY5Y neurons at all time points. In contrast, hiPSC-derived neuronal cultures showed no significant differences in the

percentage of non-apoptotic dead cells between OGD and control conditions, however, the percentage of live cells significantly decreased (Juntunen et al., 2020). Both cultures showed a higher cleaved caspase-3 positive cells in the OGD group compared to the control group (Juntunen et al., 2020).

Apoptosis is the primary form of cell death in the penumbra, as demonstrated by studies showing elevated caspase-3 activity in the penumbra 24 hours after MCAO *in vivo* or following 24 hours of hypoxia *in vitro* in both human and rodent cultures. (Pires Monteiro et al., 2021; Wang et al., 2014; Wei et al., 2004; Wu et al., 2018). Similarly, in this study, apoptosis was the predominant form of neuronal death in the culture, accounting for two-thirds of all cell death (Figure 3.5). The viability of hiPSC-derived neurons under hypoxic conditions shows a time-dependent increase in apoptosis and cell death. Within the first 24 hours of hypoxia with 2% O_2, the rate of cell death (apoptosis and dead cells) increased to 80% and by 48 hours another 10% of cells died, reflecting the progression and severity of hypoxic damage over time (Pires Monteiro et al., 2021). The importance of apoptosis in ischemic brain is highlighted by studies demonstrating significant protective outcomes when the apoptotic pathway was blocked through different methods. For example, mice with a genetic deletion of caspase-3 had a >50% reduction in infarct volume compared to control mice 24 hours post-stroke (Dojo Soeandy et al., 2019). Transgenic mice with overexpression of Bcl-2 also exhibited decreased cell death and smaller infarct volumes following ischemia (Kitagawa et al., 1998; Martinou et al., 1994).

4.3 The levels of NPCs remained unchanged 24 hours after OGD.

The present study shows that one-third of cells in hiPSC-derived neuronal cultures are proliferating NPCs (Figure 3.1). Immunostaining analysis also revealed that the level of NPCs did not significantly change at 24h after 3 hours of OGD at 3% oxygen. This finding indicates that despite the challenging OGD/R conditions, the cell proliferation and NPC population appeared resilient, maintaining its level within the hiPSC-derived neuronal cultures at 24h post-OGD (Figures 3.6-3.7). In a previous study investigating the effects of hypoxic ischemic injury on the vulnerability of NPCs derived from human embryonic stem cells, researchers found that brief OGD exposure (10, 20, 40, 60, and 90 minutes) at 2% O_2 had no significant impact on the NPC population, their proliferation, or self-renewal capacity (Sowmithra et al., 2020). In contrast, NPCs exposed to 3h of OGD at the same oxygen concentration, followed by 24h of reperfusion reported a 16.59% decline in cell survival compared to the control group (Sowmithra et al., 2022). Additionally, the study found a significant decrease in the Ki67 immunopositive cell population after OGD/R compared to controls, indicating a reduction in the number of proliferating cells (Sowmithra et al., 2022). Thus, the findings of Sowmithra et al. highlight greater vulnerability in NPC cultures. This contrasts with our study that found no significant changes in the number of proliferating cells and resilience in hiPSC-derived NPCs following OGD/R.

These contrasting results can be attributed to several key differences in experimental design. Firstly, our study aimed to replicate the penumbra region by using 3% O_2, whereas Sowmithra et al. utilized 2% O_2. The higher oxygen concentration in our protocol likely created less severe ischemic conditions and neuronal death, which may have contributed to the NPCs survival. This aligns with the goal of our study to model the penumbra, a region characterized by

moderate hypoxia and partial viability, rather than the more extreme hypoxia of the ischemic core. Furthermore, the difference in the origin of cells used to generate NPCs (hiPSC-derived NPCs in our study versus hESC-derived NPCs in theirs) may also play a role. Variations in culture conditions, such as the duration of differentiation, media, and culture composition could also influence the resilience of NPC populations. Sowmithra et al. generated NPCs by plating hESCs and maintaining them in neural induction medium for 7 days prior to OGD. However, in our study, NPCs were generated from hiPSCs in 7 days and underwent a 14-day neuronal differentiation before OGD. Together, our results highlight the importance of selecting OGD/R models and experimental conditions that are appropriate for the biological system and research question at hand. The choice of OGD protocol should be guided by factors such as the cell type used, the maturity of the culture, the severity and duration of ischemia desired, and whether the goal is to model the ischemic core or penumbra. Therefore, no universal protocol applies, and researchers must optimize conditions accordingly in studies investigating cellular responses to OGD/R.

4.4 The number of healthy Tau+ neurons decreased, and neurodegeneration increased at 24h post OGD.

The penumbra region surrounding the infarct core is characterized by delayed neuronal cell death, which depends on the severity of cellular damage. The cell death pathways can be inhibited experimentally, making the neurons in the penumbra rescuable. Murphy et al. showed that rapid tissue reperfusion can rescue neurons and reverse early signs of dendritic blebbing and spine loss (Murphy et al., 2008). This thesis study showed both neuronal cell death and degeneration of neuronal processes at 24h post-stroke (Figures 3.8-3.9). Following our OGD/R

protocol, a significant reduction in the presence of healthy neurons and a significant decrease in the percentage of Tau positive area per Tau positive neuron was observed, pointing to a reduction in neuronal processes. This result suggests that our experimental condition successfully replicated the penumbra condition where most cells survive but are injured.

In vivo studies indicated that 4 hours following an ET-1 stroke, there was a decrease in intact MAP-2+ dendrites and SMI-312+ axons, with extensive loss observed by 24 hours within the penumbra (Fifield & Vanderluit, 2020). *In vitro* studies have also shown that 6 hours OGD on SH-SY5Y cells followed by 24 hours of reoxygenation results in severe cellular damage, however, more than half of the cells remained viable (Wang et al., 2023). These studies demonstrate the reduction of neuronal processes which is consistent with our finding. Since the degeneration of neuronal processes typically occurs within early hours after a stroke, further research is needed to determine if combining reperfusion strategies with inhibitors to prevent axon and dendritic degeneration could improve stroke outcomes.

4.5 Limitations

The work in this thesis establishes a cell culture model of hiPSC-derived neurons and an ischemic injury model to investigate the responses of human neurons to ischemia. While these findings contribute to understanding neuronal responses, several limitations and challenges remain.

Ischemic stroke is a complex neurovascular condition involving the disruption of different interacting cell types, including glial cells, which play a critical role in neuronal support and inflammation. One limitation of this study is the relatively short culture duration of 14 days,

which resulted in the presence of primarily NPCs and early-stage neurons, with no glial cells present. The presence of NPCs may influence the response to ischemia, as NPCs are known to secrete neurotrophic factors such as BDNF that support neuronal survival and repair (Hur et al., 2022; Talaverón et al., 2013). While the present work allowed the assessment of the OGD/R effect on neurons and NPCs in the absence of glial cells, assessing how cells respond to OGD/R when fully mature neurons and glial cells co-exist in the culture would provide a better understanding of neuroinflammation and neuron-glia interactions.

In the current study the decision to use a 14-day culture period was based on prior protocols, however, observations during the 14-day culture period revealed a daily increase in NPC proliferation. One of the challenges in this study was managing the high rate of NPC proliferation in the culture. A recent study shows that when NPCs are cultured in differentiation media, they undergo a brief proliferative phase peaking around DIV7. After this, the cells gradually lose their ability to divide, and by DIV23 to DIV30, neuronal cells enter their final maturation stage, developing bundles of axons (Romito et al., 2024). In our study, the persistent proliferation of NPCs posed a challenge, as we were unable to completely halt this process. The extensive proliferation led to a high cell density in the culture, which interfered with our ability to accurately quantify and track cells over time. This overcrowding made it difficult to perform consistent measurements and would have further complicated data interpretation had the culture period been extended. As a result, we limited the differentiation to 14 days to maintain a manageable cell population for imaging and analysis. While this time frame was sufficient to establish a mixed population of NPCs and neurons, it may not have been long enough for the NPCs to fully transition out of their proliferative phase and into a more differentiated neuronal state.

Cell proliferation also correlates with plating density. Higher culture densities lead to reduced cell proliferation and enhanced propensity for differentiation (Wu et al., 2015). Wu and colleagues showed the fraction of NANOG+ cells and Ki67+ cells dropped with increasing the cell density and similar findings were obtained with hiPSCs (IMR90) culture (Wu et al., 2015). However, overcrowding the culture can form multiple thick layers of cells and hinder experiments if not managed properly. To address this issue, we tried Cytosine Arabinoside (AraC) as a potential solution to stop proliferation. However, this raised concerns about potential damage to neurons in the culture, which we sought to avoid. We also adjusted the plating density to balance proliferation and differentiation. While this approach helped to some extent, further research is needed to control the excessive NPC proliferation fully.

Another limitation of this study is the use of a monolayer culture system, which lacks the three-dimensional (3D) human brain structure. In ischemic stroke research, perfusion is essential, but vascularization cannot be replicated in two-dimensional (2D) cell cultures (Gaston-Breton et al., 2023). Future studies could incorporate 3D culture systems, such as brain organoids. The 3D environment of organoids resembles the organization of cells in the brain (Chiaradia & Lancaster, 2020). Recent advancements in microfluidic “organ-on-a-chip” platforms also provide a promising alternative to static 3D cultures. These models incorporate vascular-like channels, allowing for dynamic perfusion and real-time monitoring of ischemic injury progression (Kim et al., 2024; Lyu et al., 2021). Future research integrating brain organoids with microfluidic systems could enable more physiologically relevant ischemic stroke modeling and drug testing. Despite this limitation, the choice to use a monolayer culture in this study was intentional, as the goal was to examine the direct impact of ischemic insult on neurons. Monolayer culture models offer excellent visualization of individual cells, enabling detailed studies of particular molecular

mechanisms and isolated cellular events. Additionally, these models represent good high-throughput options for studying cellular responses (Cimarosti et al., 2012; Wang et al., 2012). However, the lack of structural complexity limits their ability to accurately model post-stroke tissue remodeling, inflammation, and the role of vascular networks.

Sex is another factor that can impact stroke outcomes, which was not addressed in this thesis. Previous studies have shown that sex-specific differences in hormonal levels, immune responses, and neuroprotective mechanisms can affect stroke severity, neuronal survival, and recovery process in both animal and human models (Ahnstedt et al., 2020; Demeestere et al., 2021). Investigating the role of sex hormones and their influence on neuronal and immune responses during ischemic stroke presents an important research avenue, as it may provide new insights into the underlying mechanisms of ischemic stroke. A deeper understanding of sex differences could inform the development of personalized, sex-specific treatment strategies, ultimately improving therapeutic outcomes.

Future directions

This study established a human cell culture model using hiPSCs and developed an ischemic injury model that mimics the penumbra region to assess cellular responses to ischemia. It provides valuable insights into the effects of OGD/R on hiPSC-derived neuronal culture in the absence of glial cells, offering a simplified yet informative model of ischemic injury. This model can offer a controlled and reproducible *in vitro* system for high-throughput preclinical drug screening, to test for promising neuroprotective compounds before advancing to clinical trials.

This research aims to advance our understanding of the cellular and molecular mechanisms underlying neuronal injury following ischemia, with potential applications in

therapeutic development. Building on these findings, a key direction is to develop more physiologically relevant *in vitro* models that better capture the cellular interactions and complexity of the human nervous system. Extending days *in vitro* will allow for greater neuronal differentiation, synapse formation, and neuron-glia interactions, providing additional insights into ischemic injury progression and repair mechanisms. This model can further expand our understanding of how different cell types respond to ischemic stroke by examining specific neuronal subtypes, including excitatory and inhibitory neurons, and their synaptic interactions both in the absence or presence of glial cells.

Future studies can contribute to therapeutic advancements by investigating the signaling pathways and gene expression changes that occur in neurons and glial cells within the penumbra region, the area surrounding the infarct core that remains partially viable. Identifying key molecular targets could facilitate the development of novel strategies to enhance neuronal survival and promote recovery.

6. Conclusions

In summary, this thesis accomplished two primary objectives: the establishment of an *in vitro* culture model of human neurons derived from hiPSCs and the development of an optimized OGD/R protocol to replicate the conditions of the ischemic penumbra. By developing neuronal cultures consisting of NPCs and neurons, we provided a reliable platform for studying human neuronal responses without glial interference. This OGD/R protocol successfully mimics the level of cell death in the penumbra region, inducing significant apoptosis and neurodegeneration. These advancements are important for future stroke research and therapeutic development. The human neuron culture model offers a physiologically relevant system to investigate neuronal behavior, cellular and molecular mechanisms, and drug responses under ischemic conditions.

This optimized OGD/R protocol allows researchers to model penumbra-like conditions to test neuroprotective strategies and interventions to reduce neuronal death and enhance recovery. Altogether, this thesis provides valuable tools for translational studies and may contribute to developing future therapies and strategies to improve stroke treatment and patient recovery.

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APPENDIX

Ethics approval

From: administrator@hrea.ca
To: Esseltine Jessica(Principal Investigator)
Cc: administrator@hrea.ca
Subject: HREB – Decision on CT event (Approved/Acknowledged) 510169
Date: December 3, 2020 2:12:24 PM

Researcher Portal File #: 20191777

Dear Dr. Jessica Esseltine:

This e-mail is to inform you that your amendment event # 510169 for study HREB # 2018.210 - Connexins and Pannexins in Stem Cell Pluripotency and Cell Fate Decisions - was reviewed by the Full Board on December 1, 2020 and has been approved and/or acknowledged (as indicated in the Researcher Portal). Event details are as follows:

Date event submitted: November 20, 2020

Approval/Acknowledgement of:

	Questions	Answers
2.1	List ALL documents, including version dates, to be approved. Please upload these documents under the 'Attachments' tab.	HREB Protocol version 2 track changes HREB Protocol version 2 clean copy

You may view this decision by logging into the Researcher Portal.

It is your responsibility to seek the necessary organizational approval from the Regional Health Authority (RHA) or other organization as appropriate. You can refer to the HREA website for further guidance on organizational approvals.

Thank you,

Research Ethics Office

(e) info@hrea.ca
(t) 709-777-6974
(f) 709-777-8776
(w) www.hrea.ca
Office Hours: 8:30 a.m. – 4:30 p.m. (NL TIME) Monday-Friday

COVID-19 Impact Statement

The COVID-19 pandemic introduced significant challenges that impacted the progress of this project.

1- Upon my arrival in Newfoundland, Canada, I had to spend 2 weeks in mandatory quarantine due to public health regulations. This delay postponed my lab work since I had no access to any university facilities. Moreover, this quarantine made it difficult to contact mentors in person, adding another layer of challenge to an already challenging period. As much as the restrictions were important for safety during the pandemic, they made my transition more complicated.

2- The COVID-19 pandemic caused supply chain disruptions, which created a big problem for projects that required the on-time delivery of essential materials. Supply chain disruptions led to considerable delays in delivering research supplies. Because of the manufacturing slowdowns and shipping delays, the reagents were often back ordered or arrived weeks or months later than expected. These delays impacted the scheduling of experiments since some critical materials were unavailable when needed.

3. The pandemic made the collaboration between labs complicated due to safety protocols and concerns about cross-contamination. Access to shared resources, equipment, and lab space had to be approved and carefully planned. These extra steps made it difficult to set up experiments or share expertise between teams, which slowed down the progress even more.

4- From January to February 2023, a strike of MUN faculty members limited academic and research activities for about 4 weeks. Since the laboratories were not accessible at this time, the ongoing projects had to be put on hold. This event affected experiments that were time-sensitive,

causing the loss of valuable samples. Several experiments had to be delayed, which postponed data collection. This situation also influenced future experiments, as the timelines for subsequent research phases were changed.

These challenges were beyond my control and impacted my thesis progress by slowing it down at various stages. While I worked hard to overcome each setback, they extended the duration of my project.