Radboud Universiteit UNIVERSITY OF TWENTE.

Eva J. H. F. Voogd, s4552148
Research Master Cognitive Neuroscience, Donders Institute, Radboud University
Department of Clinical Neurophysiology, University of Twente

Pathophysiological cascade of stroke and treatment strategies investigated in hiPSC-derived neuronal cultures modelling the ischemic penumbra

On-site supervisor: Dr. Monica Frega
department of clinical neurophysiology, University of Twente

Educational supervisor: Dr. Dirk Schubert
department of cellular neurophysiology, Donders Institute, Radboud University

January 2021

Acknowledgments

I am grateful for the opportunity that was given to me by the clinical neurophysiology department of the University of Twente and the assistance and advice I received during this research project. Therefore, I would like to acknowledge those who were involved.

First of all I would like to thank my direct supervisor, Monica Frega, for helping me become a better researcher. Her love for neuroscience made my love for neuroscience grow even more. I am grateful for her understanding of my personal situation and for always making sure I was okay. Even during these difficult times of Covid-19, she was always a skype call away. Thank you so much Monica! I would also like to thank Jeanette Hofmeijer for the opportunity she gave to come to the University of Twente and conduct my graduation project here. Furthermore, I would like to show my gratitude to Joost le Feber (who helped me with my MATLAB struggles) and Michel van Putten for their input and interest in my research. I would also like to thank Dirk Schubert who is my educational supervisor from the Radboud University. Dirk is the neuroscientist that enlightened the first spark for neuroscience in me. Dirk's lectures made me curious and started my passion for neuroscience.

This research would not be possible without Marloes Levers and Gerco Hassink, who were always willing to help me in the lab. I learned a lot from Marloes, how to calculate dilutions for example, which was always difficult for me. Thank you for the coffee breaks and the nice talks and for always being there for all of us (students). I would also like to thank Tanja Gerrits for the nice chats and laughs we've had. Furthermore, I would like to thank the rest of the CNPH group for creating such a fun working environment (when we were able to visit the university).

Furthermore, I would like to show my gratitude to my family and partner. They always believed in me and assisted me through the home office period during the spring and during the rest of the internship. Sometimes they were even more excited than I was about the research I was doing. This was very motivating. Lastly, I would like to give my special thanks to a very special person in my life, Nigel Voogd, even though he is not with us anymore, without him I would never have chosen neuroscience as my career path. Thank you so much big brother!

Major thanks to all of you!

Abstract

In ischemic stroke, there is an incredibly complex pathophysiological cascade which augment brain damage. Starting with energy failure caused by low perfusion levels, leading to e.g. synaptic failure, excitotoxicity, mitochondrial dysfunction and ending in different types of cell death like necrosis and apoptosis. The extensive and heterogenous cascade makes stroke a disease that is difficult to treat. In experimental research different treatment strategies are investigated that target one or more molecular events that occur in stroke. Most of these experimental studies show neuroprotection. Translation of these treatment strategies to patients is still difficult. Here we reviewed the pathophysiological cascade and candidate treatment strategies. Then we established a human model of the ischemic penumbra to test the effect of hypoxia on the synaptic puncta, cell viability and the mitochondria and neuronal network functionality. Furthermore, we investigated treatment strategies based on suppression of neuronal activity (NMDAR antagonist and hypothermia) and enhancement of neuronal activity (optogenetic stimulation). Neurons were generated from induced pluripotent stem cells derived from healthy donors and cultured on coverslips and micro-electrode arrays. The effect of different treatment strategies on cell viability and neuronal network functionality was tested. hiPSCs are vulnerable to hypoxia reflected by an increase of cell death and decrease in activity and synchronicity under low oxygen conditions. Results showed that NMDAR antagonist memantine did not rescue the cultures after 48h of hypoxia. Hypothermia and optogenetic stimulation on the other hand did improve resistance to hypoxia after 48h. We showed that hiPSC-derived neurons are vulnerable to hypoxia and that treatment strategies based on preservation of energy and enhancement of neuronal activity can be neuroprotective.

Contents

Chapter 1

Introduction

Ischemic stroke is one of the leading causes of death and disability worldwide [1-3]. Patients are frequently left with moderate to severe neurological deficits [4]. Ischemic stroke is caused by an occlusion of a brain artery by an embolus or a thrombus. The sudden loss of blood perfusion leads to loss of neuronal function, neuronal cell death and loss of neuronal structure, sequentially, which leads to neuronal deficits [5]. To limit brain damage, reperfusion should be established in time, otherwise the brain damage will be permanent [6].

To this day the only treatment that has been proven to be beneficial is acute recanalization by intravenous thrombolysis [7] or mechanical thrombectomy [8]. These treatments show to be beneficial when done within 4.5h [9] or 6h [10] after symptom onset, respectively. Due to the limited time-window and a wide range of contra-indications, only 5-10% of patients is eligible for recanalization treatment and then still most patients experience moderate to severe neurological deficits [11].

The ischemic injury can be divided in the infarct core, which is irreversibly damaged, and the surrounding tissue which is named the penumbra [12]. The penumbra is salvageable, even though there is a significant decrease in remaining perfusion levels [13]. It is assumed that neuronal activity in the penumbra becomes impaired, but neurons initially remain structurally intact and metabolically active [5, 14]. If energy supply is restored in time this dysfunction is in principle reversible, if not the tissue will be irreversibly damaged. Research focuses on the treatment of the penumbra. This area has a large potential to advance recovery. However, current treatments to promote recovery are scarce [15].

The pathophysiological cascade that is initiated by oxygen and glucose deprivation after ischemic stroke is heterogenous. The pathophysiological cascade of ischemic stroke starts with energy failure, which is caused by the depletion of oxygen and glucose and will end in cell death like necrosis and apoptosis. The neuronal network viability and functionality is affected by this and many more molecular events occurring in stroke.

Over the past two decades more than 1200 animals studies showed strong evidence that different treatment strategies are neuroprotective in ischemic stroke models [16]. Many of these treatments included ion channel blockers, neurotransmitter receptor antagonists, or suppressors of inflammation. However, in more than 500 clinical trials, none of these therapies were effective in patients [16].

Previously tested treatment strategies were directed at suppression of neuronal activity, in order to minimize energy consumption to preserve basic cellular function [16]. However, suppression of activity has been associated with progression towards irreversible damage *in vitro* and in patients. In line with these observations, Muzzi et al. showed that mild stimulation improved recovery after hypoxia in rodent neuronal cultures [17]. Secondly, failure of translation of findings from animal studies to human studies may be related to inherent differences between animal and human neurons [18-22]. Furthermore, there exists a large heterogeneity in patients groups, where the pathophysiology of recovery or deterioration may vary, which is not reflected in rodent models [23].

The recent advent of human induced pluripotent stem cell (hiPSC) technology with the possibility to differentiate stem cells into neurons has opened the way to study human neuronal diseases *in vitro* [21]. The use of human neurons may allow identification of human-specific effects and eventually include inter-individual differences of neuronal responses to hypoxia and neuroprotective treatments. To our knowledge, no studies about responses to hypoxia and treatment effects have been performed on human iPSC-derived neurons other than in our lab.

This thesis includes a review about the pathophysiological cascade of stroke and treatment strategies, and an experimental research conducted in hiPSC-derived neuronal cultures. In the experimental research we established a human model system of the ischemic penumbra and we provided an in depth characterization of neuronal responses to hypoxia and different treatments at the network level. The treatments investigated in this study are based on suppression of neuronal activity, with a NMDAR antagonist (memantine), or hypothermia, or on enhancement of neuronal activity, with optogenetic stimulation. We show that viability of the neurons is affected by hypoxia. We show that hypoxia has

deleterious effect on the neuronal network activity. Treatment with memantine shows no positive effect on the cell viability. Furthermore, we show that transient hypoxia combined with different treatments *in vitro* have a positive effect on the network activity. Finally, we observe that treatment based on stimulation of the network shows the best resistance to hypoxic damage. Our findings encourage novel investigations in the ischemic pathological cascade and treatment strategies on human neurons and open new possibilities for personalized medicine.

Chapter 2

Background

A review is written as part of this thesis. In this review a complete overview of the pathophysiological cascade in ischemic stroke is given and candidate treatment strategies to target these molecular events are described (appendix A). In this chapter we summarize the molecular events that are investigated in the experimental research of the thesis.

2.1 Pathophysiological cascade

The infarct core is immediately irreversibly damaged. Within seconds after stroke onset there is a pathophysiological cascade activated that aggravates tissue damage. Toxic signals of dying neurons in the core cause secondary damage to the penumbra. Molecular events that happen in the core will gradually affect the penumbra. The volume of the core expands when the cells in the penumbra go into cell death. These events might occur sequentially or in parallel and might activate different pathways resulting in a complex physiological cascade. A complete overview of the pathophysiological cascade and candidate treatment strategies is given in table 1.

2.1.1 Synaptic dysfunction

Energy failure causes synaptic dysfunction. It has been shown that synaptic failure is an early consequence of energy depletion [12, 24]. This is due to the fact that synaptic transmission is highly susceptible for metabolic perturbations. Hypoxia causes damage to the dendritic structure and causes spine loss. Depression of excitatory synaptic transmission was the very first measured pathophysiological process in experimental study under acute complete anoxia [24]. When the duration of hypoxia is severe enough, damage to the synapses is irreversible [24]. However, it is unclear how the disturbances of synaptic transmission evolve in the outer border of the penumbra under conditions of hours-lasting partial hypoxia in the absence of depolarization. Animal studies suggest that the expression of genes and proteins that are associated with synaptogenesis is increased in the first hours to days after acute ischemic stroke [25]. This indicates that there is a limited period of increased potential for neuroplasticity. This is in line with results of clinical research, which shows improved recovery with early and intensive physical therapy after ischemic stroke [26].

2.1.2 Excitotoxicity

Adenosine triphosphate (ATP) depletion causes failure of the Ca^{2+}/ATP pump. This leads to an influx of Ca^{2+} in the cell [27]. In the core the excessive amounts of Ca^{2+} binds to synaptotagmin in the axon terminal of excitatory neurons. Presynaptic voltage-dependent Ca^{2+} channels become activated and increase the amount of excitatory neurotransmitters (e.g. glutamate) that are released in the synaptic cleft, which is neurotoxic [6]. This leads to hyperexcitation of N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazoleprpionic acid (AMPA) and kainate receptors. Activation of these receptors result in increased energy-consuming electrical activity, especially under conditions of reduced glutamate uptake by astrocytes in ischemia. Neuronal damage is augmented by increase of Ca^{2+} concentration, sodium and water into the neurons, which causes cell swelling [28]. Excitotoxicity increases brain damage and therefore it is an often investigated target for treatment research. Studies try to block the excessive activation of NMDARs by NMDAR antagonists. However, it is important that healthy concentrations of glutamate are released in the synaptic cleft in order to maintain normal neuronal functioning.

2.1.3 Mitochondrial dysfunction

Within minutes after stroke onset, the energy homeostasis is disturbed, which is controlled by mitochondria in the cytoplasm [29]. Mitochondria have an outer membrane that contains protein channel structures which make it permeable for ions, water adenosine diphosphate (ADP) and ATP. The inner membrane is the reactive center of the mitochondrial energy metabolism and contains complexes of electron transport proteins, ATP synthase complex and ATP/ADP transport proteins [30]. Mitochondria are damaged in ischemic stroke due to oxygen and glucose deprivation. The electron transport chain activity is decreased with consequent ATP depletion. Mitochondria are unable to remove Ca^{2+} from the cytoplasm and this promotes loss of mitochondrial membrane potential [31, 32]. This depolarization is in principle reversible. However, it was found that cells undergo a transition from energetic failure to mitochondrial permeability pore opening. This transition leads to a loss of membrane potential that is no longer reversible [33]. It causes release of free radicals, enzymes and proteases that leads to the activation of cell death pathways, increasing tissue damage. Mitochondria

release cytochrome C (cyt C) or apoptosis inducing factor (AIF) and intrinsically activate apoptosis. Neurons try to adapt to ischemic stress by removing dysfunctional mitochondria via mitophagy in an attempt to maintain mitochondrial homeostasis [34, 35].

2.1.4 Apoptosis

Neurons in the penumbra can go into apoptotic cell death [36]. This is initiated by overproduction of free radicals, Ca^{2+} release, excitotoxicity, and mitochondrial dysfunction. Apoptotic stimuli can be classified as intrinsic stimuli or extrinsic stimuli.

Intrinsic stimuli are activated by mitochondrial signaling. The Ca^{2+} influx causes cleavage of Bcl-2 interacting domain (BID) to truncated Bid (tBid). tBid opens the mitochondrial transition pores and Cyt C or AIF is released into the cytosol [33]. Cyt c binds to apoptotic protein-activating factor-1 (Apaf-1) and procaspase-9 to form an apoptosome, which activates the caspase pathway. Caspase is the main driver of the intrinsic apoptotic pathway. Caspase-9 is activated by the apoptosome, followed by activation of caspase-3. This is the last and executive enzyme of the intrinsic apoptotic pathway. Activated caspase-3 fragments nuclear DNA (nDNA) repair enzymes, which leads to nDNA damage and initiates apoptotic cell death [6].

The extrinsic pathway is initiated via ligand-receptor interactions and can activate the caspase pathway independent of Cyt c release. Surface death receptors are activated and induce apoptosis [37]. Caspase-3 is activated and this results in membrane blebbing, mitochondrial membrane permeabilization, chromatin condensation, nDNA fragmentation [38]. Dying cells eventually shrink, condense and are fragmented releasing small membrane bound apoptotic bodies, which are generally engulfed by macrophages. In this way of cell death intracellular content is not released into the extracellular matrix. Inhibition of apoptotic stimuli has the potential to rescue cells from apoptosis.

Table 1. Molecular events and candidate treatment strategies.

Molecular event	**Onset**	**Caused by**	**Tested and effective in animal models**	**Tested in stroke patients?**	**Effective in stroke patients?**
Energy failure	Seconds	Lack of oxygen and glucose	No	No	No
Synaptic dysfunction	Seconds	Energy depletion	No	No	No
Ionic imbalance	Minutes	Failure of Na+/K+ ATPase pumps	Methylene blue injury [39-44]	No	N/A
Excitotoxicity	Minutes	Excessive glutamate excretion	Memantine [45-51]	Yes [52-54]	Yes [52-54]
Spreading depression	Minutes	Waves of depolarization to restore ion gradients	P-188 [55, 56] and citicoline [55, 57-60], Ketamine [61]; [62]	No, in traumatic brain injury (TBI) [57, 63]	Yes, in TBI [57, 63]
Calcium dysregulation	Minutes	Increased calcium influx in neurons	Pseudoginsenoside-F11 [64-67]	No	N/A
Ischemia induced acidosis	Minutes	Increased amount of lactic acid	Ethanol [68-73].	No	N/A
Mitochondrial dysfunction	Minutes	Decrease in electron transport chain activity	Pramipexole [74] or rapamycin [75-78]	No	N/A

Autophagy	Minutes	Dysfunctional proteins	Rapamycin [77, 79-81]	No	N/A
Oxidative and nitrosative stress	Minutes	Imbalance between production of free radicals and neutralization of reactive intermediate products	NR2B9C [82-85]	No	N/A
Inflammation	Hours	Activation of endothelial cells and upregulation of leukocytes	L-3-n butylphthalide [86-89]	Yes [90-92]	Yes[90-92]
Final common pathways to cell death					
Necrosis	Seconds	Cell swelling	No	No	No
Necroptosis	Minutes	Activation of RIPK1/3 and MLKL protein kinases	Ligustroflavone [93]	No	N/A
Apoptosis	Minutes	Activation of caspase pathway	XQ-1H [94-97]	Yes [98]	Yes [98]

2.2 Treatment strategies

Here different treatment strategies that were studied in this research such as the NMDAR receptor antagonist memantine and hypothermia are described. These treatment strategies are based on suppression of neuronal activity. A bigger and more in depth overview of different candidate treatment strategies can be found in the review (appendix A).

Different treatment strategies are investigated in preclinical and clinical trials. Table 1 shows examples of pre- and clinical trials that aimed to target several molecular events. Experimental research showed that it was possible to target cell swelling [39], spreading depression [55, 57-61], calcium overload [64-67], acidosis [72], mitochondrial dysfunction [44, 74-78, 99-102], autophagy [77, 79-81], it was possible to reduce oxidative and nitrosative stress [82-85, 103, 104] and to suppress inflammation [86-89, 105-107]. Experimental studies also aimed to target cell death pathways like necroptosis [93, 108-110] and apoptosis [94, 104, 111-114]. Some of these treatment strategies were investigated in clinical trials and showed some neuroprotection [52-54, 57, 63, 90-92, 98], but failed to improve patient outcome.

2.2.1 Hypothermia

The strategy of cooling is tested by lowering the temperature to 33-35°C and investigating the effect on tissue damage [115]. Hypothermia is a known inhibitor of neuronal activity and has provided strong neuroprotection in experimental stroke studies [116-118]. There are several possible neuroprotective mechanisms that are associated with hypothermia (table 2)[116]. A meta-analysis done in 2007 by Van Der Worp et al. showed an overall reduction of infarct volume of 43.5% in animals models of stroke that received hypothermia as treatment [119]. However, the neuroprotective effect of hypothermia has a limited benefit [120]. In multiple clinical trials there were no significant benefits found, with either surface cooling or endovascular cooling [117, 121, 122].

Table 2. Neuroprotective mechanisms associated with hypothermia adapted from Lyden et al. [116].

Molecular event	**Neuroprotective mechanisms associated with hypothermia**
Energy failure	Lower metabolic rate
Ionic imbalance	Limit edema forming
Excitotoxicity	Reduce glutamate release
Calcium overload	Limit calcium influx after glutamate
Oxidative and nitrosative stress	Suppress formation of reactive oxygen species
Inflammation	Reduce inflammatory response
Cell death	Interrupt necrosis/apoptosis-induced disruption of mitochondrial cyt C

2.2.2 Treating excitotoxicity with memantine.

NMDAR antagonists are investigated to reduce excitotoxicity. Memantine is a moderate affinity, strongly voltage-dependent, uncompetitive NMDAR antagonist and it binds directly within the pore of the channel in its open configuration. Memantine shows a fast blocking/unblocking of NMDAR channels. Memantine can only gain access to the channel in the presence of agonist and remains largely trapped in the channel following removal of the agonist (figure 1) [123]. It blocks the effect of excess glutamate and restores physiological signal transmission. It prevents the Ca^{2+} influx that is caused by excessive glutamate release, therefore it could prevent the disruption of synaptic plasticity [124]. Memantine is an interesting drug to test in hiPSCs, because it was shown that memantine was neuroprotective in rodents [45-51] and in humans [52-54].

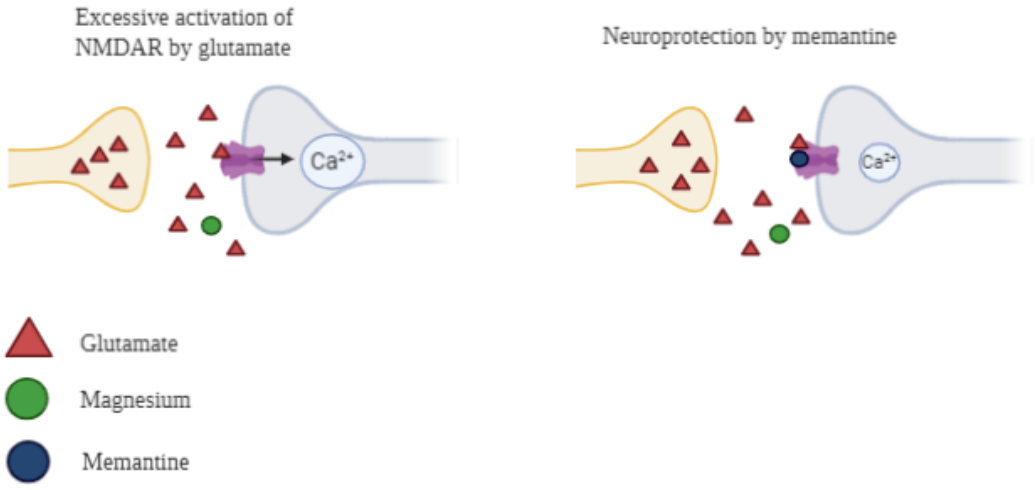


Figure 1. schematic representation of mechanism of action of memantine under ischemic stress. Left: excessive activation of NMDAR by glutamate causing increased influx of Ca^{2+}. Right: Memantine blocks NMDAR in presence of glutamate, decreasing the influx of Ca^{2+} (illustration created using BioRender.com).

2.3 Open issues

Ischemic stroke is a complex and heterogenous disease and so is the penumbra. Despite the effort taken over the years to understand the pathophysiology, we are still not able to salvage this area. Neuronal activation might be the key to recovery in this tissue at risk and stimulation techniques might provide the answers.

The possibility to differentiate hiPSCs into neurons and assess their morphology, viability, and electrophysiological activity on MEAs gives the possibility to study ischemic stroke *in vitro* and might enhance translation of *in vitro* findings to the clinic, when in comparison to rodent models. However, the behaviour of these cells is not fully understood and thus, the effect of hypoxia on cell viability, network connectivity and network activity are not fully understood. In this experimental research we investigate treatment strategies that are tested in animal models and in humans. We aim to answer the question whether activation of neuronal activity is neuroprotective in ischemic stroke. We do this by comparing optogenetic stimulation with previously tested strategies, such as hypothermia and memantine, which are based on suppression. Therefore, in the current study, we aim to create an *in vitro* model of the penumbra and to:

1. Determine the effect of 48 hours of hypoxia on synaptic function, mitochondrial function, cell viability and neuronal network activity.
2. Evaluate the effect of different treatment strategies (memantine, hypothermia and optogenetic stimulation) on the cell viability and on the electrophysiological activity of the networks.

Chapter 3

Material and methods

This chapter addresses the methodology required to achieve all of the previously mentioned aims. In this experimental research we conducted several experiments where we investigated the number of synaptic puncta, cell viability, mitochondrial morphology, and network electrophysiology after hypoxia and after treatment with memantine, hypothermia or optogenetic stimulation.

3.1 Cell cultures

3.1.1 hiPSC generation

HiPSCs required for this study had already been generated from fibroblast obtained from healthy individuals through induced reprogramming by retroviral vectors expressing the Yamanaka factors (Oct4, Sox2, Klf4 and cMyc)[125, 126]. Ngn2 and ASCL1 positive hiPSC lines were generated by transfection with lentiviral vectors (Ngn2 and ASCL1) and rtTa lentiviral vector. To select the cells that were successfully transduced with the viral vectors selection factors (G148 (Sigma Aldrich) and puromycin (Sigma Aldrich)) and E8 medium were supplemented to the cells. These two stable hiPSC lines were the starting point of neuronal differentiation. Excitatory and inhibitory neurons are created by expression of Ngn2 and ASCL1. To force neuronal differentiation doxycycline (Sigma Adlrich) to activate the Tet-on system and forskolin (Sigma Aldrich) were added to the medium as performed by Frega et al. [126]. The two different neuronal populations could grow independently while they are plated in the same well.

3.1.2 Neuronal differentiation

Networks containing only excitatory (glutamatergic) neurons and networks containing both excitatory and inhibitory (GABAergic) neurons were used. The coverslips and MEAs were coated with poly-1-ornithine (Sigma Aldrich) and mouse laminin (Sigma Aldrich) before plating of the Ngn2 and ASCL1 positive hiPSCs according to Frega et al. [126].

To generate excitatory networks, approximately 40.000 Ngn2-positive hiPSCs were plated as single cells on wells with an area of 190 mm^2 containing one coverslip per well. This resulted in a final cell density of around 600 cells/mm^2.

To generate co-cultures of excitatory and inhibitory neurons Ngn2 and ASCL1 positive hiPSCs were plated together in a 60/40 ratio. Approximately 24.000 Ngn2 and 16.000 ASCL1 positive cells were plated as single cells on a sterile 24-well MEA with an area of 32mm^2 or on coverslips in wells with an area of 190 mm^2. This resulted in a final cell density of approximately 2000 cells/mm^2 and 600 cells/mm^2, respectively. The final excitatory/inhibitory ratio was ~80/20 [127, 128].

One day after plating medium containing DMEM/F12 with 1% (v/V) N-2 supplement, 1% (v/v) non-essential amino acids and two human factors (human recombinant neurotrophin-3 (Bioconnect) and human recombinant brain derived neurotrophic factor (Bioconnect)) was added to the cell cultures to help neuronal maturation.

Two days after plating, new-born (P1) Wistar rat cortical astrocytes were added to the hiPSC cultures in a 1:1 ratio to support neuronal maturation. These rat astrocytes were obtained by sacrificing new-born rat pups and isolating the brains. The procedure involving rats was conducted in agreement to Dutch and European laws of animal research and the guidelines of the Dutch Animal Use Committee.

On day three after plating, the neurobasal medium (Thermo Fisher Scientific) which was supplemented with B27 (Thermo Fisher Scientific), glutaMAX (Thermo Fisher Scientific) and cytosine beta-D-arabinofuranoside (Sigma Aldrich) to obstruct the proliferation of the astrocytes and to kill the existent non-differentiated hiPSCs was added to the wells. From this day onwards, each well was filled with ~500 µl of neurobasal medium, plus four other components specific for hiPSCs: BDNF, NT-3, doxycycline. Ten days after plating fetal bovine serum (Sigma Aldrich) was added to the medium. Fresh medium was added every 2-3days. 200ul of old medium was removed from each well and 250ul fresh medium was added to compensate for evaporation loss. Cells were kept in an incubator with a controlled climate (temperature of 37 °C, 90% humidity and 5% $CO_{2)}$ for 3 to 4 weeks for the excitatory cultures and 7 to 9 weeks for the co-cultures.

3.2 Induction of hypoxia

The networks were exposed to a low oxygen condition. The coverslips were placed in an incubator which was controlled for 2% O_2, 5% CO_2, 90% humidity and 37 °C during hypoxia, to create the desired environment.

Flow controllers were used to establish the right mixture of air and N_2 on the MEAs. During normoxia the values were set to 100% air and 0% N_2. During hypoxia this was set to 10% air and 90% N_2, and 5% CO_2 was added constantly to the mixture.

3.3 Experimental protocol

Cultures containing only excitatory neurons (3-4 weeks *in* vitro) and cultures containing excitatory and inhibitory neurons (7-9 weeks *in vitro*), plated on MEAs or coverslips, underwent normoxia of 20 min and were then subjected to hypoxia for 48h. We investigated the number of synaptic puncta, cell viability, number of mitochondria and neuronal network activity.

Different treatment strategies were investigated during 48h of hypoxia.

1. Memantine. 5 µM memantine was added to all wells containing coverslip to assess the effect of memantine on cell viability. Furthermore, memantine was added to 12 wells of a MEA in total in different concentrations (vehicle, 1 µM, 5 µM and 10 µM) to assess the effect of memantine on electrophysiological activity of the networks.

2. Hypothermia. A MEA was placed in the recording setting at 37 °C. Hypoxia was induced and the temperature of the recording setting was lowered to 34 °C to assess the effect of hypothermia. After the experiment, the air flow was set to normoxia and the temperature was set to 37 °C.

3. Optogenetic stimulation. The neurons needed to be manipulated with optogenetics to assess the effect of optogenetic stimulation. The excitatory neurons were infected with channelrhodopsin-2 (ChR2) on seeding day by addition of 0.75 µl of virus per well of a MEA. The neurons acquired blue light (λ=470 nm) sensitive membrane proteins. The cells became excitable when there was exposure to light of this wavelength. Blue light was delivered to the cells, during the entire experimental period, with

the use of a Multiwell-Optogenetic prototype (Multi Channel Systems, Reutlingen, Germany). Light pulses were applied for 3 minutes, every 2hours (200ms, 10mA) by putting the Multiwell-Optogenetic device on top of the MEA. The interpulse interval was set at 5 seconds, so each stimulus would not influence the subsequent one. To avoid light interference, this experiment was conducted in the dark.

3.4 neuronal network activity recordings

The electrophysiological recordings of neuronal networks were performed when the co-cultures were 7 to 9 weeks *in vitro* using the Multiwell Screen software (Multi Channel Systems, Reutlingen, Germany). The 24-well multiwell MEA (mwMEA) was covered by a paper film (Breathe Easier Sealing Membrane; Sigma Z763624) to reduce evaporation of the medium during 48h-lasting recordings, while allowing gases to flow through. The status of the networks was assessed before the start of the network to ensure the networks were healthy and active. Furthermore, this was to assess if wells needed to be eliminated from analyses. The 24-well mwMEA was placed in the recording setup and could acclimate for 20-30 minutes, this allowed for stabilization of the neuronal networks before starting the experiment. First, we recorded 10 minutes normoxia followed by recordings of 10 minutes, every 2h under hypoxia. To promote resistance to hypoxic damage different treatment strategies were applied to the MEAs.

3. 4 Immunohistochemistry

3.4.1 Quantification of synapsin puncta

Coverslips containing hiPSC-derived neurons were fixed by incubating them in 4% paraformaldehyde (PFA, Sigma Aldrich) solution for 15 minutes at room temperature (RT). After washing them thoroughly in phosphate buffered saline (PBS) (home-made), the coverslips were permeabilized for 5 Minutes in 0.2% Triton X-100 (Sigma Aldrich) in PBS, followed by 30 minutes in bovine serum albumin (BSA), a blocking solution. Primary antibody was applied at 4 °C overnight. The coverslips were then washed with PBS and placed on a secondary antibody solution for 1 hour at RT and washed with PBS. Thereafter, DAPI (1:1000) (Sigma Aldrich) was added for 20 minutes at RT. Finally, the coverslips were washed with PBS once more, mounted with Mowiol and dried overnight at RT. The antibodies used

are described in table 3. Epifluorescent pictures were taken at a 60x magnification (0.057µm/pixel) with the use of a Nikon Eclipse 50i epi-fluorescence microscope (Nikon, Japan).

Table 3. Antibodies

Experiment	Antibodies	Source	Identifier	Dilutions
Synapsin puncta	Mouse MAP2	Sigma	M4403-50	1:1000
	Guinea pig synapsin	Synaptic systems	106 004	1:1000
	Goat anti-mouse, Alexa Fluor 488	Invitrogen	A-11029	1:2000
	Goat anti-guinea pig, Alexa Fluor 647	Invitrogen	A-21450	1:2000
Live dead assay	Cell event caspase 3/7	Thermoscientific	C10423	1:500
	Propidium iodide (PI)	Invitrogen/GIBCO	11599296	1:250
Mitotracker protocol	Mouse MAP2	Sigma	M4403-50	1:1000
	Rabbit GFAP	Abcam	AB7260	1:500
	Mito tracker red CMXros	Thermoscientific	M7512	1:500

3.4.2 Live dead assay

At DIV 48 caspase3/7 was added and incubated for 30 minutes. The plates were then placed in the incubator and oxygen was set to 2%. For the treated cultures we added 5µM of the NMDAR blocker memantine to all wells before starting the experiment. PI was added after exposure to hypoxia. The coverslips were washed with PBS, fixated with 3.7% PFA for 15 minutes at RT. DAPI (1:1000) was added and coverslips were then incubated for 20 min at RT. They were washed one more time with PBS and stored at 4°C. This was done for control condition, 24h, 30h, 40h, 48h of hypoxia. Epifluorescent pictures were taken at a 40x magnification (0.085µm/pixel) with the use of a Nikon Eclipse 50i epi-fluorescence microscope (Nikon, Japan).

3.4.3 MITOtracker protocol improvement

Cover slips containing co-cultures at DIV 49 were used to set up the Mito tracker red CMXros protocol from Thermoscientific. First 250 µl was taken out of the medium, so that this could be readded after

washing. 0.5µl (20x prediluted) Mito tracker red CMXros was added and incubated 30 min at 37 °C. Then the medium was taken off and the cover slips were carefully washed with PBS once. The 250 µl old medium and 250 µl fresh medium was added per well. The coverslips were placed in the incubator with 2% O_2 for 6h, 24h and 40h. This was followed by MAP2 staining or GFAP staining as described in quantification of synapsin puncta, without synapsin, to visualize neurons or astrocytes, respectively. Epifluorescent pictures were taken at a 60x magnification (0.057µm/pixel) with the use of a Nikon Eclipse 50i epi-fluorescence microscope (Nikon, Japan).

3.4.4 Data analysis of immunohistochemistry experiments

To analyse the synaptic puncta we merged the pictures that belonged together in FIJI [129]. Images were composed of synaptic puncta (synapsin), neurons (MAP2) and nuclei (DAPI) pictures. Then we measured the length of the dendrites and manually counted the synaptic puncta that were located alongside the dendrites. Further, we calculated the mean number of synaptic puncta per 10 µm per picture. This was done for all pictures of all conditions.

To analyse the live dead assay we used a self-written MATLAB (The MathWorks, Inc., Natik, MA, USA) script where we set different thresholds for the different stainings. Threshold for red (PI) was 20, threshold for green (Cas3/7) was 32 and the threshold for blue (DAPI) was 75. First all nuclei (DAPI positive) were counted to obtain the total cell count. Then the apoptotic cells (Cas3/7 positive) and dead cells (PI positive) were counted. We assumed the cells were death when there was either only red staining or co-localization of red and green staining. Cells with only green staining were assumed to be apoptotic. To calculate the number of non-apoptotic cells we subtracted the dead and apoptotic cell counts of the total cell count.

To analyse the Mito tracker red CMXros we used FIJI [129] to merge the pictures that belonged together to create a red-green-blue image. The images were composed of mitochondria (Mito tracker red CMXros), neurons (MAP2) or astrocytes (GFAP) and nuclei (DAPI). Then we aimed to measure the length of the dendrites of neurons and the limbs of astrocytes and counted the mitochondria (Mito

tracker red CMXros positive) that laid alongside the dendrites and limbs. We aimed to calculate the number of mitochondria per 20 µm per picture.

3.5 Micro electrode array recordings and data analysis

All the electrophysiological recordings were conducted using the 24 mwMEA with 24 independent wells. All wells contain 12 embedded electrodes each (electrode diameter = 30 µm and distance between the electrodes = 200 µm) in a 4 x 4 position excluding corners. Data were acquired at a frequency of 10kHz and the signal was filtered through a high pass filter (2^{nd} order Butterworth filter, 100 Hz) and a low-pass filter (4^{th} order Butterworth filter, 3.5kHz) throughout the recording. A channel was considered active when it is showed a minimum of 10 spikes per minute. Spikes were considered if they exceeded 4.5 times the standard deviation noise threshold. This is based on the activity of hiPSCs. Burst were detected when lasting more than 100 ms containing a minimum of 4 spikes. The minimum interval between burst was set at 100 ms. Network burst were detected when at least 6 channels were participating, of which at least three were simultaneous.

Data analysis was performed with the Multiwell Analyzer software (Multi Channel Systems, Reutlingen, Germany). We extracted spike and burst transcript from the mwMEA system and conducted further analysis in Excel (Microsoft Office) and GraphPad Prism 9 (GraphPad Software, Inc., CA, USA).

Both qualitative and quantitative analyses were performed on the activity of the hiPSCs derived neuronal cultures. Raster plots were used to assess the activity patterns in a visual way. The mean firing rate (MFR) is defined as the number of spikes detected through a specific electrode during the time window of the experiment. Network burst rate (NBR) was calculated by dividing the number of network bursts detected in a well by 10 to obtain the number of network bursts per minute.

3.6 Statistical tools

Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, Inc., CA, USA). We ensured normal distribution using a Kolmogorov-Smirnov normality test. To determine statistical significance for the different experimental conditions p-values <0.05 were considered to be significant. Statistical analysis was performed with two-way ANOVA and post-hoc Bonferroni correction to correct

for multiple comparisons. Data are presented as mean ± standard error of the mean (SEM). The number of independent neuronal networks included for each experiment are reported in the figure legends. All the values of the parameters throughout the different phases of the experiment were normalized with respect to their baseline values to allow comparisons.

Chapter 4

Results

We differentiated hiPSCs into populations of excitatory and inhibitory neurons, by forced expression of the transcription factors Ngn2 and ASCL1. HiPSCs derived neurons, co-cultured with rodent astrocytes to support neuronal maturation, were grown on coverslips and on MEAs. In conditions of normoxia the cultures formed a functionally connected network exhibiting electrical activity composed of spikes and burst appearing in a synchronous matter.

4.1 Effect of hypoxia on neuronal cultures

First, we investigated the effect of hypoxia on neuronal networks that were not treated. We investigated the effect of hypoxia on the number of synaptic puncta, on the cell viability, on mitochondria and on the networks activity.

4.1.1 Effect of hypoxia on synaptic puncta

The synaptic puncta per 10 µm were quantified to analyse the effect of hypoxia on synapses. We found that the number of synaptic puncta was not statistically affected during hypoxia (figure 2 & 3).

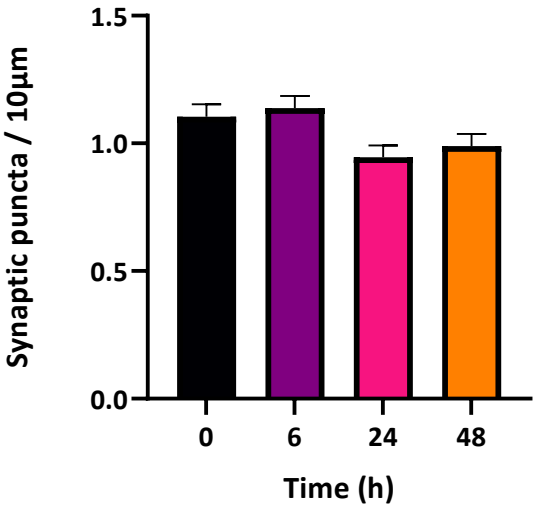


Figure 2. Bar graph showing the effect of hypoxia up to 48h on the mean number of synapsin puncta per 10 µm in co-cultures. Control (n=15), 6h (n=14), 24h (n=14) or 48h (n=14) of hypoxia. Error bars indicate the SEM. Two-way ANOVA test and post-hoc Bonferroni correction was performed between conditions.

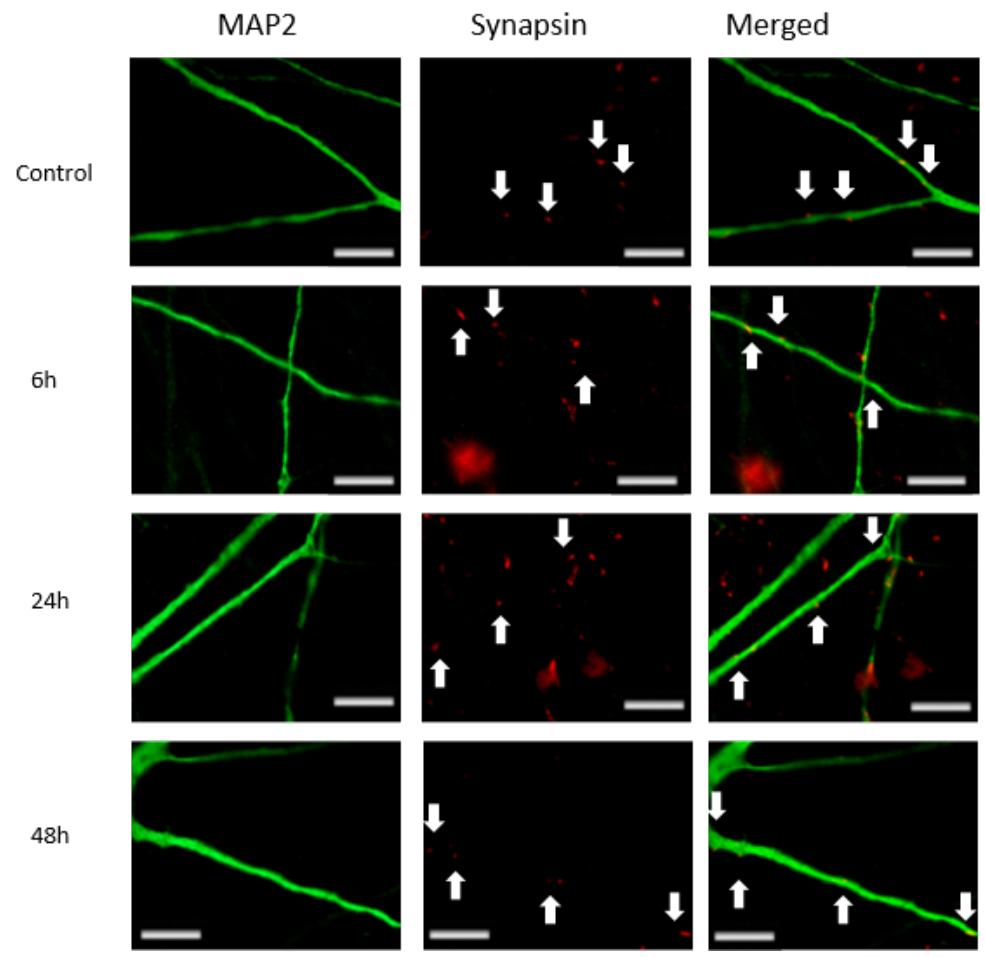


Figure 3. Representative images of neurons stained for MAP2 (green) and synapsin (red) at DIV 49 (scale bar 10 µm) of control (n = 15), 6h (n = 14), 24h (n = 14) and for 48h (n=14) of hypoxia.

This experiment was conducted twice, to increase the *n*. However, in the second experiment we found that the number of synaptic puncta in the control (0 h) was lower than expected (figure 4 & 5). Healthy hiPSC-derived neuronal cultures are expected to have around 1 synaptic puncta per 10 µm [130]. We found a significant increase of synaptic puncta per 10 µm between control and 6h after onset of hypoxia ($p = 0.0043$).

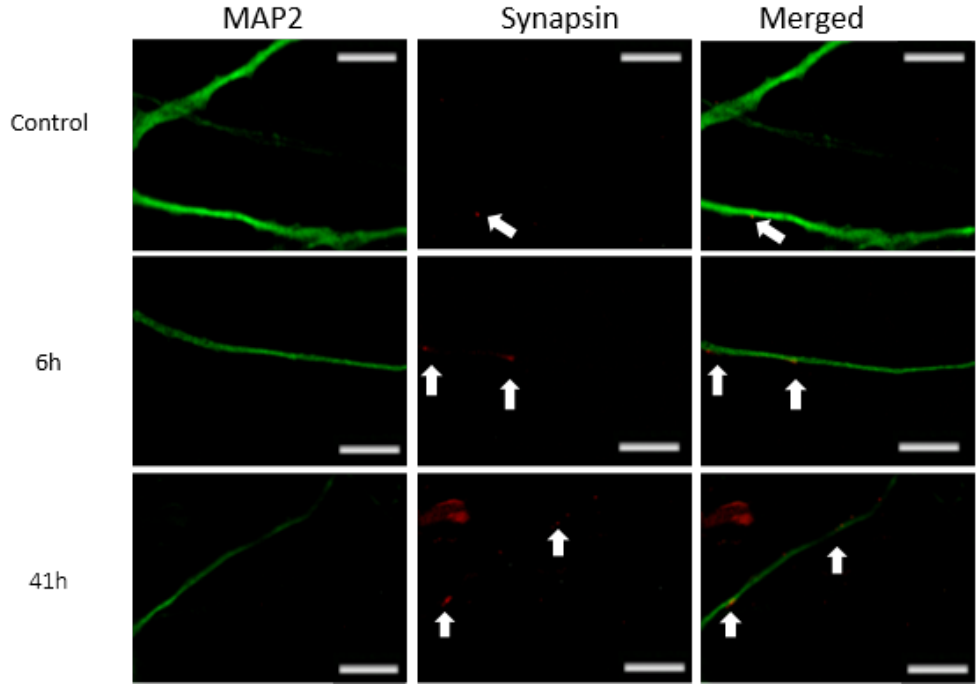


Figure 4. Representative images of neurons stained for MAP2 (green) and synapsin (red) at DIV 49 (scale bar 10 µm) and quantification of synapsin puncta of control (n = 15), 6h (n = 13), and for 41h (n=14) of hypoxia.

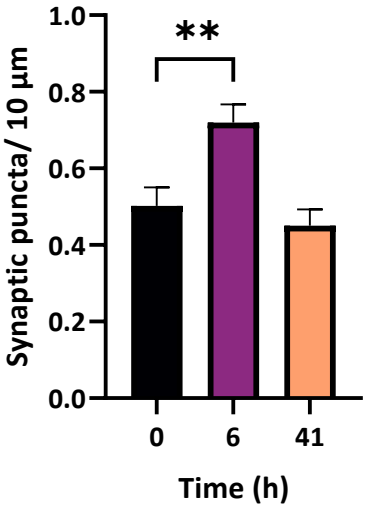


Figure 5. Bar graph showing the effect of hypoxia up to 41h on the number of synaptic puncta per 10 µm. Error bars indicate the SEM. ** p<0.005, two-way ANOVA test and post-hoc Bonferroni correction was performed between conditions.

4.1.2 Effect of hypoxia on cell viability

The effect of 48h of hypoxia on cell viability was investigated. We studied the cell death rate in all cells (neurons and astrocytes) present in the cultures. We assessed the cell count for non-apoptotic, apoptotic, and dead cells in the cultures that were exposed to 48h of hypoxia (figure 7). Total cell count of all conditions can be found in appendix B table S1A.

There was a significant decrease found in non-apoptotic cells over the time course of 48h of hypoxia. After 24h of hypoxia the non-apoptotic cell count significantly decreased (p = 0.0115)(figure 6 A). A significant decrease of non-apoptotic cells is also found between 24h and 30h of hypoxia (p = 0.0475). Furthermore, there was a significant decrease found in non-apoptotic cell count between 24h and 48h of hypoxia (p = 0.0002).

Next, the apoptotic cell count was assessed. Hypoxia of 24h did not significantly affect the apoptotic cell count (figure 6 B). There was a significant increase found in apoptotic cell count after hypoxia with a duration of 30h (p = 0.0038), after a duration of 40h (p = 0.0031) and after a duration of 48h (p = 0.0009) compared to baseline.

Lastly, we assessed the dead cell count. There was a significant increase after 48h of hypoxia in dead cell count (p = 0.0385). Between control and 24h, 30h and 40h of hypoxia we did not observe a significant difference (figure 6 C).

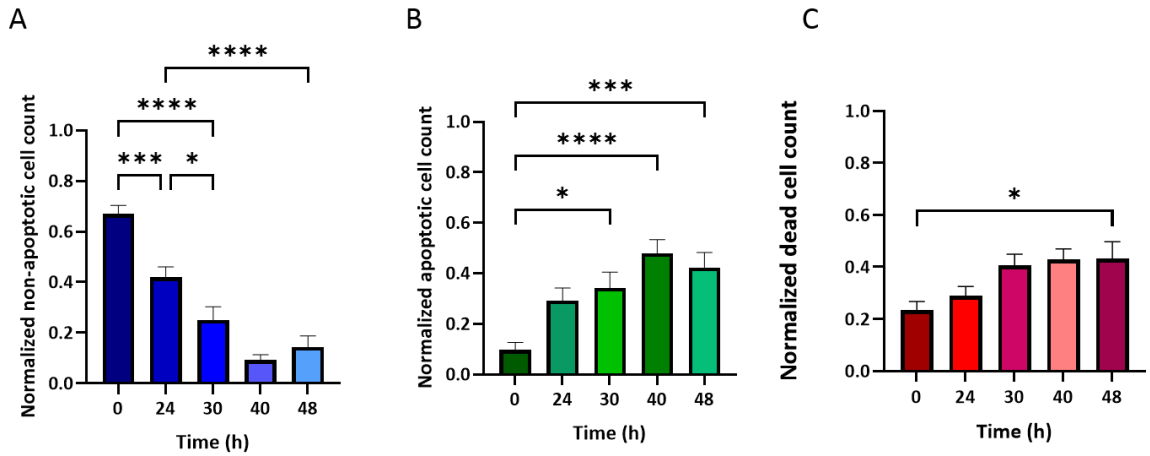


Figure 6. Bar graph showing the effect of hypoxia up to 48 hours on **A.** non-apoptotic cell count, **B.** apoptotic cell count and **C.** dead cell count. Control (n = 20), 30h (n = 14), 40h (n = 28), 48h (n = 21). Error bars indicate the SEM. * P < 0.05, ** P < 0.005, *** P < 0.0005, two-way ANOVA test and post-hoc Bonferroni correction was performed between conditions. Exact p-values are reported in table S1B (appendix B).

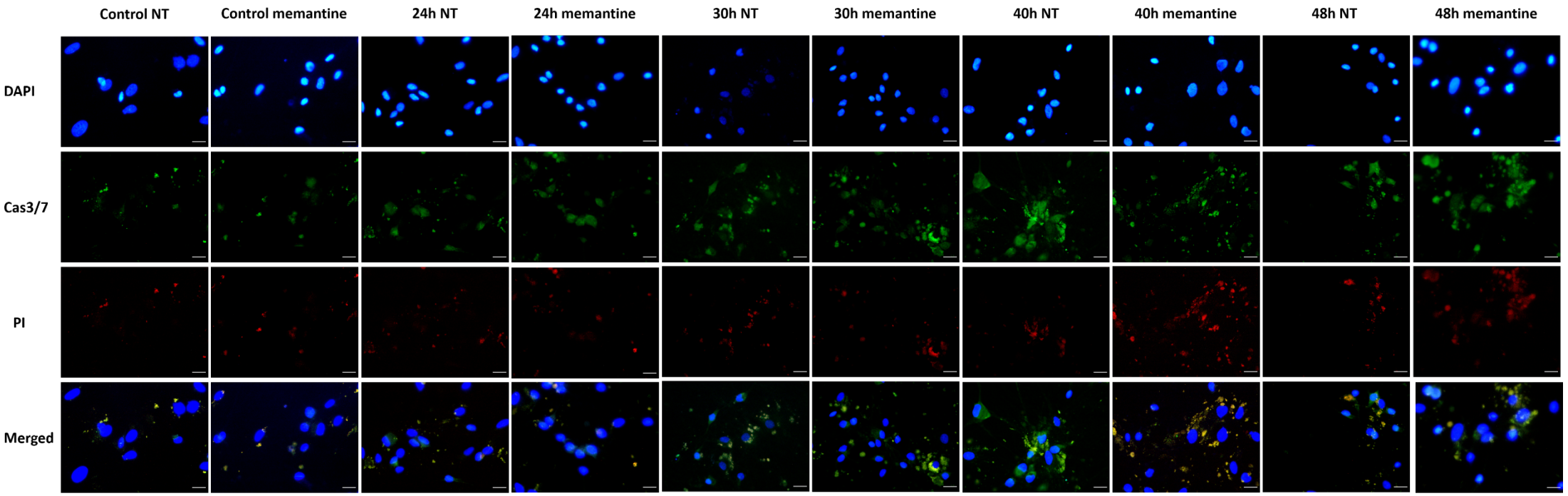


Figure 7. Representative images of non-treated (NT) and cultures treated with memantine stained for Cas3/7 (green), PI (red) and DAPI (blue) at DIV 49 (scale bar 20 µm) and quantification of cell viability in hypoxia, n = 14 for all conditions

4.1.3 Effect of hypoxia on neuronal network activity and synchronicity

To investigate the effect of hypoxia on neuronal network activity and synchronicity, neuronal cultures were grown on MEAs. In conditions of normoxia, the hiPSC-derived neuronal cultures formed a functionally connected network exhibiting electrical activity composed of spikes and burst appearing in a synchronous matter. When the neuronal networks were exposed to hypoxia, the firing rate and network burst rate exhibited by the neuronal networks immediately dropped compared to baseline (figure 10). Significant changes were present one hour after onset of hypoxia ($p < 0.0005$ and $p < 0.0001$, respectively). All neuronal networks completely lost synchronicity within 24h of hypoxia and after 48h the neuronal networks became electrically silent.

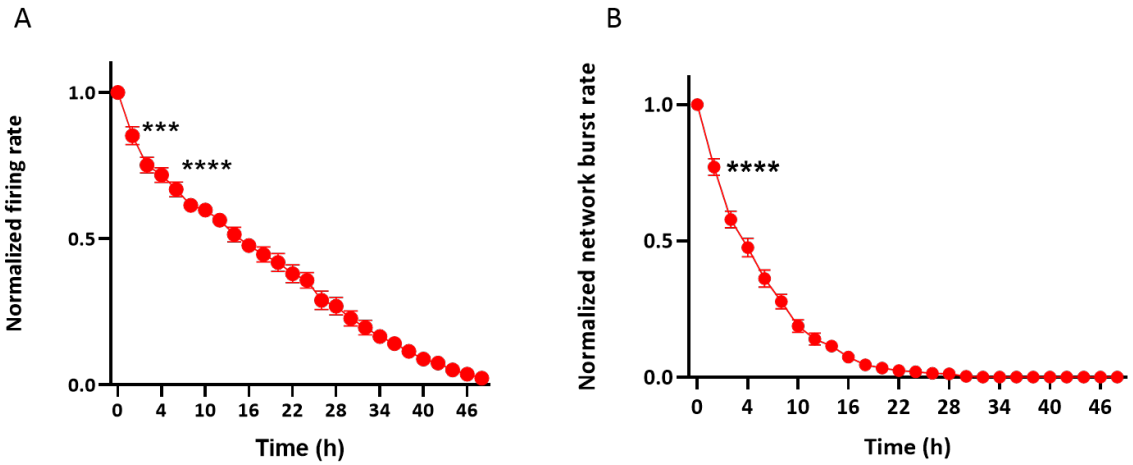


Figure 8 A. Normalized firing rate for non-treated cultures (n=78) over the time course of 48 hours of hypoxia. **B.** Normalized network burst rate in non-treated cultures (n=78) over the time course of 48h. Error bars indicate the SEM. Exact p-values are reported in table S2 (appendix B). *** p <0.0005, **** p <0.0001, two-way ANOVA test and post-hoc Bonferroni correction was performed between conditions.

4.2 Effect of treatment strategies on neuronal networks during hypoxia

Here we investigated the effect of different treatment strategies on cell viability and the network activity. To investigate the effect of treatment strategies that are based on the suppression of activation, we treated neuronal cultures with memantine to assess the effect of this selective NMDAR antagonist on cell viability and neuronal network activity. Furthermore, we investigated the effect of hypothermia on neuronal network activity.

We investigated the effect of optogenetic stimulation, a treatment strategy that is based on activation of the neuronal network on neuronal network activity during hypoxia.

4.2.1 Treatment with memantine

To investigate the effect of memantine on cell viability after 48h of hypoxia we compared non-treated cultures with treated cultures (figure 7). There was no significant difference found in the total cell count between treated and non-treated cultures (appendix B, table S1A & figure S1).

The results showed that there is a significant difference in non-apoptotic cell count between non-treated and treated cultures in the control ($p = 0.0469$). There are less non-apoptotic cells in neuronal cultures treated with memantine compared to non-treated cultures (figure 9 A).

Next, we assessed if memantine had an effect on apoptosis in hypoxia compared to no treatment. The results showed that there was a significant difference in apoptotic cell count in treated cultures and non-treated cultures after 40h of hypoxia ($p = 0.0042$). There are less apoptotic cells in memantine compared to no treatment (figure 9 B).

Lastly, we assessed the number of dead cells in treated and non-treated cultures. The cultures treated with memantine had significant higher dead cell count compared to the non-treated cultures at 40h of hypoxia ($p = 0.0003$) (figure 9 C).

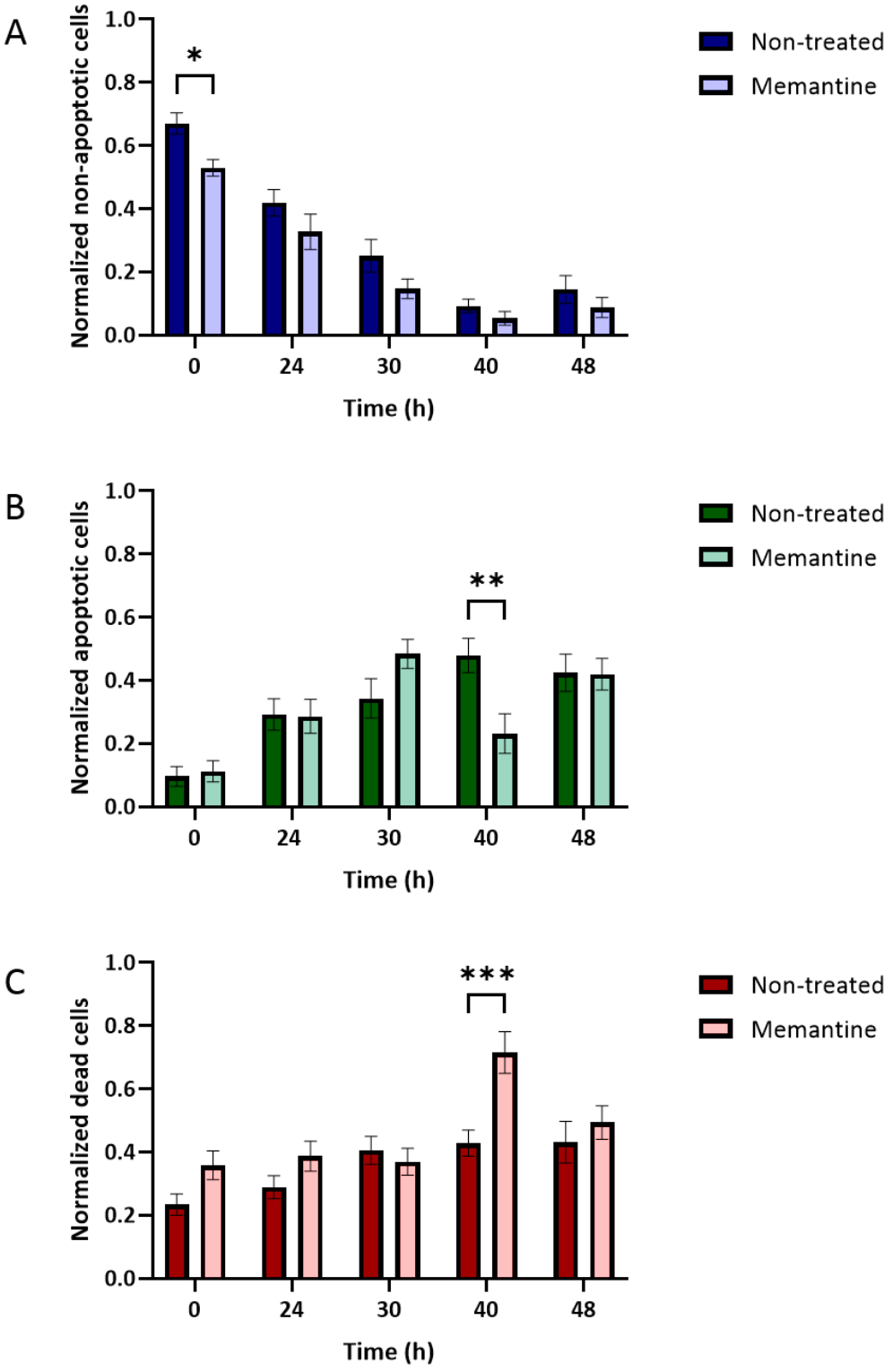


Figure 9. Bar graph showing the effect of 48h of hypoxia on **A**. non-apoptotic cell count, **B.** apoptotic cell count and **C.** dead cell count. Control (n = 14), 30h (n = 14), 40h (n = 14) and 48h (n = 14) for non-treated cultures and cultures treated with memantine. Error bars indicate the SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, two-way ANOVA test and post-hoc Bonferroni correction was performed between conditions.

We then investigated the effect of memantine different doses of memantine (1µM, 5 µM or 10 µM) on neuronal network functionality after 48h of hypoxia. The results showed that the activity decreased over time as observed in non-treated cultures and no activity was observed after 48h of hypoxia (figure 10 & 11).

There was no significant difference between the non-treated, vehicle and different doses up and until 14h after onset of hypoxia in terms of normalized firing rate. The dose of 1µM memantine showed a significantly higher normalized firing rate after 16h of hypoxia when compared to the non-treated cultures ($p = 0.0100$). A significant difference in firing rate was found between non-treated cultures and cultures treated with 1µM memantine up to 34h of hypoxia (table S3) (figure 10A).

Furthermore, after 26h of hypoxia the cultures treated with 10 µM memantine showed a significantly higher normalized firing rate compared to non-treated cultures ($p = 0.0047$). This significant difference between 10 µM memantine and non-treated cultures was found up to 34h of hypoxia (table S3). After 28h of hypoxia, cultures treated with 5 µM memantine also showed a significant higher normalized firing rate when compared to non-treated cultures ($p = 0.0436$) up to 34h of hypoxia (figure 10 A).

The cultures treated with vehicle (PBS) showed a significantly higher normalized firing rate compared to non-treated cultures ($p = 0.0070$) after 34h of hypoxia. There was no significant difference found between the different doses of memantine compared to the vehicle over the time course of 48h (figure 10).

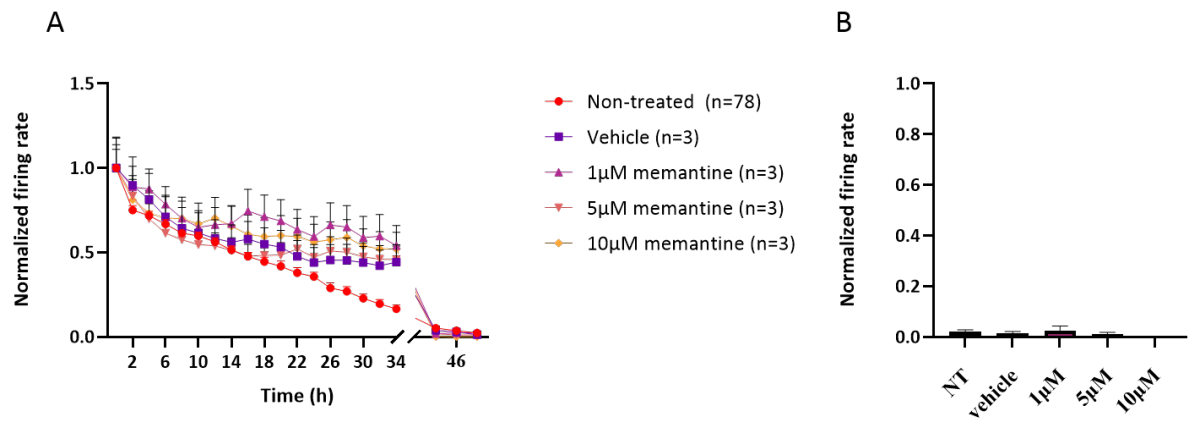


Figure 10. **A.** Normalized firing rate for non-treated (n=78), vehicle (n=3), 1µM memantine (n=3), 5 µM memantine (n=3) and 10 µM memantine (n=3) after 48 hours of hypoxia. **B.** bar graph representing the normalized firing rate after 34h of hypoxia in non-treated (NT), vehicle (PBS), 1 µM, 5 µM and 10 µM. Error bars indicate the SEM. Two-way ANOVA test and post-hoc Bonferroni correction was performed between conditions. Exact p-values are reported in table S3 (appendix B).

The results of the NBR showed already a significant difference 2h after onset of hypoxia when the vehicle, 1 µM, 5 µM and 10 µM memantine were compared to non-treated cultures ($p < 0.0001$). This significantly higher NBR was found for all treated cultures (even for the vehicle) compared to non-treated cultures up and until 34h of hypoxia (figure 11).

Furthermore, after 8h of hypoxia the dose of 1 µM memantine showed a significantly higher NBR compared to the vehicle ($p = 0.0027$). Besides this difference, 1 µM memantine showed to have a significantly higher NBR compared to 5 µM memantine ($p = 0.0418$) at 8h of hypoxia.

After 18h of hypoxia a significant difference between vehicle and 5 µM memantine ($p = 0.0303$) and between vehicle and 10 µM memantine ($p = 0.0010$) was found. Furthermore, 1 µM memantine showed to have a significantly lower NBR compared to 10 µM memantine ($p = 0.0449$).

The significant difference between the vehicle and 5 µM memantine and between the vehicle and 10 µM memantine was found up to 24h of hypoxia and after 28h of hypoxia (table S3).

To conclude the treated cultures had significantly higher NBRs compared to the non-treated cultures from 2h of hypoxia up to 34h of hypoxia. The significant differences between the treated cultures show a lot of variation over time (table S3). Furthermore, 5 µM memantine showed to be the least deviant

to baseline activity (1 network burst / min) and showed to be the most stable in terms of network burst rate.

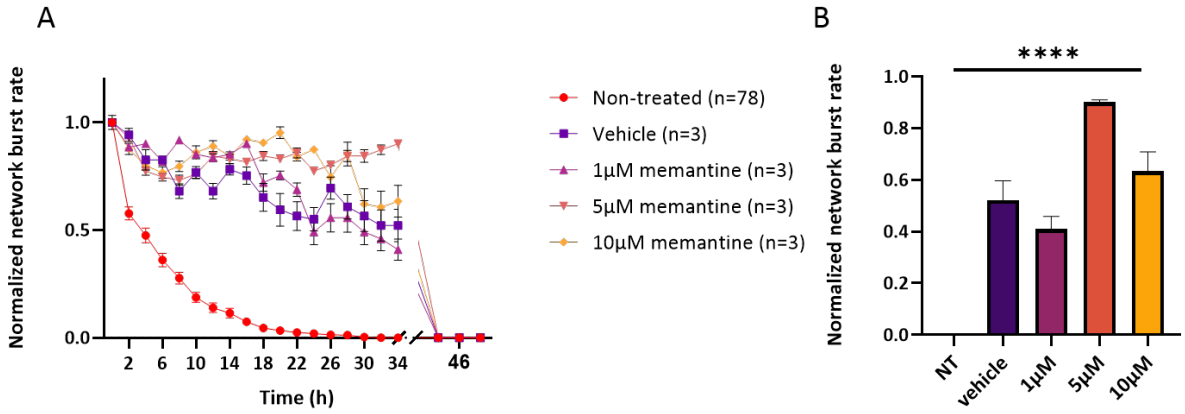


Figure 11. A. Normalized network burst rate for non-treated (n=78), vehicle (n=3), 1µM memantine (n=3), 5 µM memantine (n=3) and 10 µM memantine (n=3) over the time course of 48 hours of hypoxia. **B.** bar graph representing the network burst rate after 34h of hypoxia in non-treated (NT), vehicle (PBS), 1 µM, 5 µM and 10 µM. Error bars indicate the SEM. Two-way ANOVA test and post-hoc Bonferroni correction was performed between conditions. Exact p-values are reported in table S3 (appendix B).

4.2.2 Treatment with hypothermia

Next, we investigated the effect of cooling (34 °C) on the network activity and synchronicity during hypoxia. When the non-treated cultures were exposed to hypoxia, the firing activity and synchronicity exhibited by hiPSC-derived neuronal networks immediately dropped. The cultures subjected to hypoxia combined with hypothermia did not drop as much in firing rate, although this was not found to be significant (figure 12). The non-treated cultures become electrically silent after 48h after hypoxia onset, this was for treated cultures not the case.

The NBR showed significant changes 4h after onset of hypoxia and onwards ($p < 0.0001$) (figure 13). After 26h, the treated cultures showed an increase of NBR that was increased from baseline (1.000 ± 0.018) to 2.259 ± 0.058 at 30h of hypoxia($p < 0.0001$). After 30h of hypoxia there is a decrease in NBR in the treated cultures, however after 48h of hypoxia there is still network synchronicity (0.617 ± 0.034) (figure 13B). The NBR in non-treated cultures reduced and was almost zero, 20h after onset of hypoxia. These cultures completely lose synchronicity within 24h of hypoxia. These results show that hypothermia has a positive effect on the network burst rate, but do not have a significantly positive effect on the firing rate of neuronal cultures during hypoxia in this experiment.

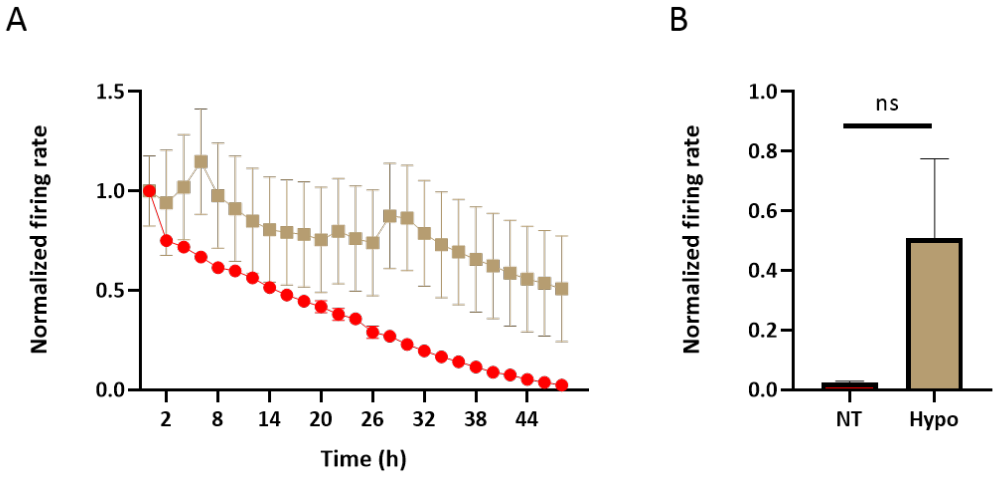


Figure 12. A. Normalized firing rate of non-treated cultures (red, n=78) and cultures treated with 34 °C hypothermia (taupe, n=24). **B.** bar graph representing firing rate after 48h of hypoxia in in non-treated (NT) and hypothermia cultures. Error bars indicate the SEM. Two-way ANOVA test and post-hoc Bonferroni correction was performed between conditions.

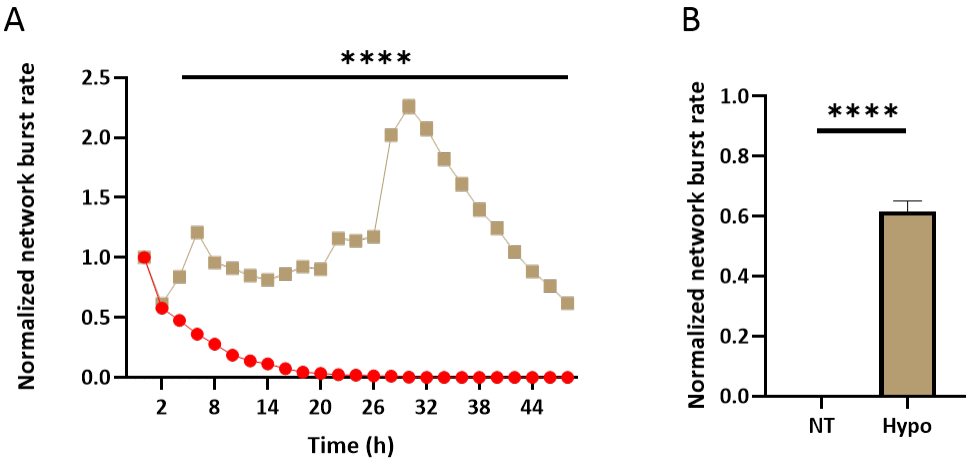


Figure 13. A. Normalized network burst rate (NBR) of non-treated cultures (red, n=78) and cultures treated with 34 °C hypothermia (taupe, n=24)). **B.** bar graph representing network burst rate after 48h of hypoxia non-treated (NT) and hypothermia. Error bars indicate the SEM. **** $P < 0.0001$, two-way ANOVA test and post-hoc Bonferroni correction was performed between conditions.

4.2.3 Treatment with optogenetic stimulation

We showed that during hypoxia memantine and hypothermia had a positive effect on network synchronicity. Both of these strategies are based on the suppression of activity. We suggest that stimulation of the networks might also show a significant increase of network activity and synchronicity compared to non-treated cultures. We tested this hypothesis by optogenetically manipulating excitatory neurons and exciting them with blue light. When neuronal networks were exposed to 40h

of hypoxia and optogenetic stimulation, they maintained baseline levels of firing rate and NBR throughout the entire duration of hypoxia (figure 14 and 15). These results show that the network activity was stable during hypoxia, this indicated that activation of the entire network has a protective effect during hypoxia.

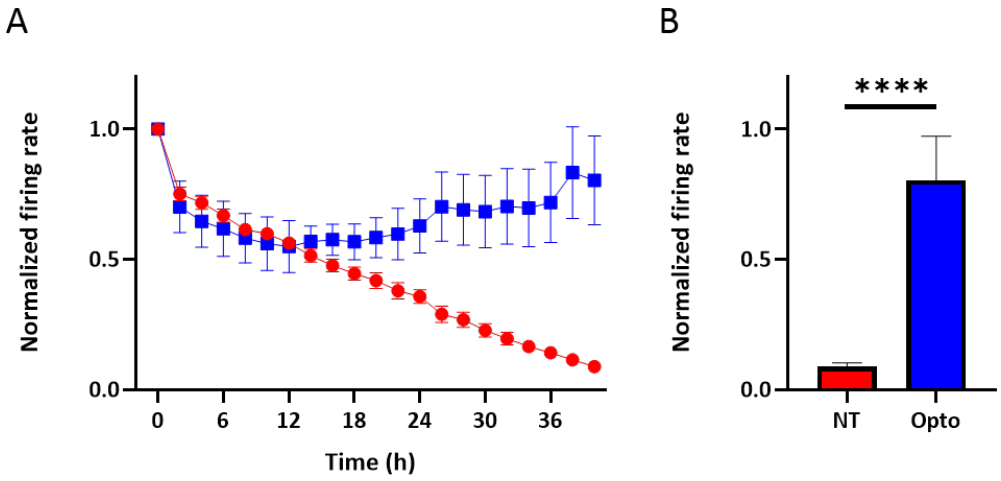


Figure 14. A. Normalized firing rate of non-treated cultures (red, n=78) and cultures treated with optogenetic stimulation (blue, n=8). **B.** Bar graph representing the firing rate after 40h of hypoxia for non-treated (NT) and optogenetic stimulation (Opto). Error bars indicate the SEM. **** P < 0.0001, two-way ANOVA test and post hoc Bonferroni correction was performed between conditions. Exact p-values are reported in table S3 (appendix B).

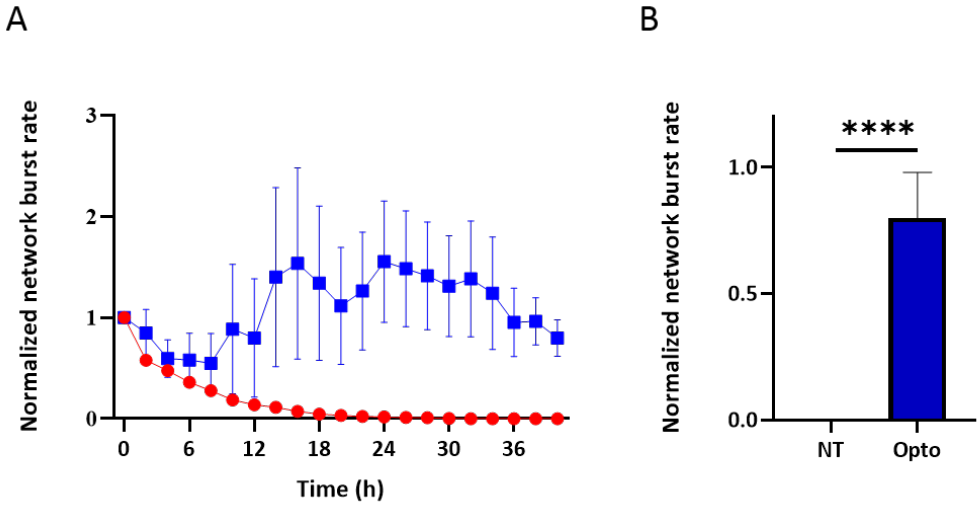


Figure 15. Normalized network burst rate (NBR) of non-treated cultures (red, n=78) and cultures treated with optogenetic stimulation (blue, n=8). Error bars indicate the SEM. **** P < 0.0001, two-way ANOVA test and post hoc Bonferroni correction was performed between conditions. Exact p-values are reported in table S3 (appendix B).

Chapter 5

Discussion

In this thesis, we established a human neuronal model of the ischemic penumbra to (i) study the effect of hypoxia on synaptic puncta, (ii) study the effect of hypoxia on cell viability, (iii) study the effect of hypoxia on network activity and synchronicity, and (iv) test different treatment strategies to support neuronal functionality. In cultures exposed to hypoxia, we showed that hypoxia affects cell viability by increasing apoptosis and cell death. Furthermore, we showed that hiPSCs derived neuronal cultures become electrically silent after 48h of hypoxia.

In cultures exposed to hypoxia and different treatment strategies, we showed that memantine had no significant overall effect on cell death, which is probably caused by the fact that memantine is not an inhibitor of apoptosis. We observed that memantine has some positive effect on firing rate and network burst rate compared to non-treated cultures. However, so did the cultures who were treated with vehicle (PBS). After 48h of hypoxia all cultures became electrically silent.

Furthermore, we showed that hypothermia seemed to have a higher firing rate after 48h of hypoxia. However, this was not found to be significant. The network burst rate of hypothermia was higher compared to non-treated cultures. After 48h of hypoxia the cultures treated with 34°C were still active in terms of firing rate and network burst rate. Lastly, we investigated the effect of optogenetic stimulation. We observed that this had a protective effect, by maintaining the network levels of activity stable during 48h of hypoxia.

5.1 Hypoxia affects neuronal functionality.

5.1.2 Synaptic puncta seem to be not affected by hypoxia.

Hypoxia did not seem to affect the number of synaptic puncta. We hypothesized that the number of synaptic puncta would decrease due to hypoxia. We expected that this decrease would be found already after 6h of hypoxia. Others have shown that synaptic dysfunction is an early consequence of energy depletion [12, 24]. Hypoxia causes damage to the dendritic structure and causes spine loss. Our findings did not show a decrease in synaptic puncta after 48h of hypoxia. In the second experiment we

observed that the number of synapsin puncta seemed to show a decrease from 6h of hypoxia to 41 hours. However, this was not found to be significant.

There is always a chance to have variability in hiPSC-derived neuronal cultures from different batches. Therefore, we propose to establish another batch and create a bigger population to get more insight in the effect of hypoxia on the number of synaptic puncta in hiPSCs derived neuronal model of the ischemic penumbra.

Furthermore, in this thesis we investigated all neurons (inhibitory and excitatory) and did not quantify the effect of hypoxia on excitatory and inhibitory neurons. Research showed that inhibitory neurons are quite resistant to hypoxia [132, 133]. Therefore, we propose to investigate whether there is a difference between the number of synapses of excitatory and inhibitory hiPSC-derived neurons in hypoxia.

5.1.3 Cell viability affected by hypoxia.

We found that the number of non-apoptotic cells decreases rapidly. After 24 hours of hypoxia most of the cells are either apoptotic or dead. After 30h of hypoxia there is an increase of apoptosis. In other studies conducted in non-neuronal cells it was found that apoptosis is reversible when the inducer was taken away. This concept is named 'anastasis', Greek for 'rising to life' [134, 135]. In our experiments that would mean, when normoxia is initiated again a decrease in apoptotic cells could be found. However, our results showed that after 24h of hypoxia with 24h of reoxygenation there was no decrease in the number of apoptotic cells in hiPSC-derived neuronal cultures containing only excitatory neurons (supplementary figure 2). This does not suggest that apoptosis in hypoxia is not reversible, or that neuronal cells cannot undergo anastasis.

These findings suggest that 24h of hypoxia causes to much damage to the cells. Perhaps is anastasis not possible anymore. Furthermore, it is possible that the recovery period was too short. Or that toxins built up in the medium and cause damage to the cells. If this were true, we would expect to see an increase of dead cells from 24h to 30h of hypoxia. However, we did not see this in our results. After

48h of hypoxia the number of dead cells has significantly increased compared to all other time points. If normoxia is initiated earlier, there is a possibility that apoptosis is reversible. The time window to treat ischemic stroke in patients is relatively short. Patients need to be treated within max. 6 hours of symptom onset to have the best chance on recovery. Therefore, it may be possible that the number of apoptotic cells decrease when reoxygenation is established earlier in time.

We propose to assess whether apoptosis is reversible in hypoxia when the duration of hypoxia is no more than 12h. After 12h of hypoxia hiPSC-derived neurons lose their network burst rate as showed in the results of '4.1.3. Effect of hypoxia neuronal network activity and synchronicity.'. By losing their synchronicity, the neurons lose contact with each other. When neurons don't get input from other neurons, they will die ('use it, or lose it') [5, 17, 136-138]. It is possible that before they lose their network, we can save them from apoptosis and with that minimize the loss of neuronal network.

5.1.4 MITOtracker protocol

To investigate the mitochondria in hiPSC-derived neuronal cultures we set up a protocol. The way this protocol was set up made us unable to quantify the mitochondria in the time schedule that was planned for this thesis (appendix C). Furthermore, the quantification of mitochondria with this protocol in general is going to be difficult. A lot of background noise was found, and it was hard to distinguish whether red puncta were mitochondria or noise (supplementary figure 3). Furthermore, it was hard to see the single mitochondria in the soma and the cell bodies. The mitochondria are located in a 3D space and therefore we could not assess whether it were single mitochondria or clusters of mitochondria. This protocol did not give us the correct images to analyse. We were aiming to have results more similar to what Klein Gunnewiek et al. found this year in hiPSC-derived neuronal cultures from mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) patients [139]. They transfected the neurons with a DNA-In Neuro Transfection Reagent in combination with a DSRed2-Mito7 plasmid. With this technique the mitochondria expressed DSRed2-Mito7 during differentiation. Our protocol used mature neurons (DIV 49) where we tried to force Mito tracker red CMXros (Thermo Fisher) into the mitochondria. Future research into the morphology and the number

of mitochondria in ischemic stroke could consider the transfection protocol used in Klein Gunnewiek et al [139].

5.1.5 Neuronal activity and synchronicity affected by hypoxia in hiPSC-derived neuronal cultures.

We found that hiPSCs derived neuronal networks are vulnerable to hypoxia since the activity and synchronicity decreased under low oxygen conditions. After 6h and 12h of hypoxia, neurons remained active and synchronized. If low oxygen persisted for 24h, network burst activity ceased, together with firing activity. This suggest functional neuronal impairments such as synaptic failure [24] and depression of excitatory synaptic transmission [24]. When low oxygen condition lasted longer than 24h the cultures became electrically silent. This could suggest that they are still alive, however there are not enough substrates (i.e. glucose and ATP) to fire action potentials. It is also possible that the firing and synchronous activities are related to distinguishable energy supports, hence the difference between decrease in mean firing rate and NBR. The steeper decrease in the NBR shows that hypoxia primarily affects synchronous activity and indicates that synaptic network transmission has a greater energy consumption than spontaneous spiking activity. Furthermore, given that synaptic transmission requires a set of coordinated pre- and post-synaptic ATP-dependent processes, our results suggest that network bursting requires a minimum amount of energy to occur. Thus it might be a good endpoint measure of energy usage in neuronal networks.

5.2 Effect of treatment strategies on neuronal network during hypoxia in hiPSCs derived neuronal cultures.

We investigated the effect of different treatment strategies in hypoxia. We choose treatment strategies based on suppression of neuronal activation (memantine and hypothermia) and based on activation of the neuronal network (optogenetic stimulation). We assessed the effect of memantine on cell viability and on neuronal network activity. Furthermore, we tested the effect of hypothermia and optogenetic stimulation on neuronal network activity in hypoxia.

5.2.1 Memantine does not improve cell viability or neuronal network activity after 48h of hypoxia in hiPSC-derived neuronal cultures.

We investigated the effect of memantine on cell viability in hypoxia. We hypothesized that excitoxicity decreases due to memantine and prevent apoptosis caused by excitotoxicity. However, we found no difference in reduction in non-apoptotic cell count. Our results showed that after 40h of hypoxia in the memantine treated cultures there are less apoptotic cell count compared to non-treated cultures, but there was an increase in dead cell count after 40h in the cultures treated with memantine. This suggest that memantine has a negative effect after 40h of hypoxia on cell death. It might suggest that memantine blocks healthy glutamate signalling and the cells die due to this blockage.

The pathophysiological cascade of ischemic stroke is incredibly complex. When a molecular event is targeted (e.g. excitotoxicity) it does not mean that no other molecular events will occur. What this suggests is that the cells still can go into apoptosis or other cell death pathways, when only one molecular event is targeted. For example, mitochondrial dysfunction is initiated by ATP depletion and this causes apoptosis. This is just another molecular event in the pathophysiological cascade that can lead to cell death. Therefore, it is interesting to investigate combination treatment strategies. These are treatment strategies that target multiple molecular events of the pathophysiological cascade [73, 103, 140]. First, it is important to investigate how the pathophysiological cascade evolves when one of the molecular events is blocked or prevented. When excitotoxicity is blocked, what mechanism is then initiated to ensure cell death? Future research can provide better insight in which molecular events to target in combination strategies.

Next, we assessed the effect of different doses of memantine (1µM, 5 µM or 10 µM) on neuronal network activity. We chose these doses because they were found to be the least toxic for neurons [51]. Memantine is an uncompetitive NMDAR antagonist, this means that NMDAR activation is required by glutamate before memantine can bind. Memantine preferentially blocks excessive NMDAR activity, possibly without disrupting normal function. Therefore, this could be a promising drug for ischemic stroke because healthy activation of NMDARs can be maintained [141].

Our results show that 1µM of memantine helped the networks maintaining physiological activity levels from 14h up to 34h. Furthermore, 10 µM of memantine showed neuroprotection after 26h of hypoxia and 5 µM of memantine showed neuroprotection after 28h of hypoxia. The synchronicity was the highest over the time course of 34h of hypoxia with 5 µM of memantine. Cultures treated with 5 µM of memantine showed a NBR close to baseline, suggesting that this maintained the network synchronicity overtime. However, all cultures were electrically silent after 48h of hypoxia. This suggest that memantine does not rescue the neurons in the end, but it shows that memantine can be neuroprotective until a certain duration of hypoxia. The effect of memantine can wear off because of the working mechanism of this drug. Memantine binds to NMDARs when glutamate is available to bind to NMDARs. However, after 44h of hypoxia there is possibly so much damage done to the cells (e.g. disruption of mitochondrial function, excessive release of reactive oxygen species etc.) that even though the cells were not overexcited by excessive release of glutamate, they still die.

Furthermore, there was not a big difference found between the three doses and the vehicle (PBS). This could suggest that PBS has a neuroprotective effect. PBS is a water-based salt solution containing disodium hydrogen phosphate, and sodium chloride. The buffer helps to maintain a constant pH. Perhaps it affected the acidosis of the neurons, by maintaining a healthy pH (more information about acidosis in ischemic stroke can be found in the review (appendix A)). Our findings suggest that PBS has an effect on the activity of neuronal cultures during hypoxia. We found that 5 µM of memantine shows more neuroprotection of the networks physiology than the vehicle. From this we can not conclude that memantine affects the neuronal networks, because we found that PBS affects the neuronal networks as well. The experiments had a small n, only 3 experiments were done per dose. Therefore, a next step could be to increase the n by doing more experiments and using a different solution to dilute memantine to test its effect. Perhaps memantine can be diluted with the medium that is used for the neurons (neurobasal). Furthermore, future research could investigate what the effect is of an inhibitor of apoptosis on network physiology and may be combine this with memantine.

5.2.3 Hypothermia improves neuronal network synchronisation during hypoxia in hiPSC-derived neuronal cultures.

The second strategy we employed that was based on suppression, was hypothermia. We found that hypothermia showed to be neuroprotective for the synchronicity of the neuronal networks. We observed the same trend in the firing rate of the cultures treated with hypothermia, however this was not found to be significant. After 8h of hypoxia we observed an increase in firing rate and network burst rate. Furthermore, after 28h of hypoxia we observed an increase of the network burst rate that was twice as high as the baseline. To ensure, this was not due to malfunctioning flow controllers, we assessed the read out of the flow controllers and we did not see any deviant events. This suggest that the increase in synchronicity is caused by hypothermia. A possibility is that energy is preserved and that after 30h of hypoxia the neurons needed to fire action potentials in an attempt to avoid becoming electrically silent. This treatment strategy (33 °C) showed to be neuroprotective in human embryonic stem cell derived neurons by reducing cell death after 4h of hypoxia, and even abolished this effect when normoxia was initiated after 4h of hypoxia [142].

Hypothermia is a multifactorial treatment strategy and targets multiple events of the pathophysiological cascade. The main idea of cooling is to preserve energy by supressing neuronal activity [116]. Hypothermia helped the networks maintaining physiological activity levels throughout the whole duration of 48h of hypoxia. After 48h of hypoxia the neurons were not electrically silent, as we found in non-treated cultures. This suggest that hypothermia is neuroprotective in a hiPSC-derived neuronal model of the ischemic penumbra when initiated immediately at the start of hypoxia. However, in clinical trials system cooling and endovascular cooling of patients did not show neuroprotection in ischemic stroke [117, 121, 122]. Besides that, hypothermia cannot be started immediately after stroke onset in patients. Future research could focus on the effect of hypothermia when started later after onset of hypoxia. Furthermore, future research could investigate new techniques to translate the treatment strategy of hypothermia to the clinic.

5.2.4 optogenetic stimulation shows neuroprotection during hypoxia in hiPSCs derived neuronal cultures.

We investigated a treatment strategy that is based on the activation of excitatory neurons. Optogenetic stimulation of neurons helped the networks maintaining activity the whole duration of hypoxia. Non-treated cultures showed a strong decrease in activity and synchronicity after 40h of hypoxia. These findings suggest that neuronal networks need a certain amount of activity to ensure neuronal viability. Furthermore, it suggest that early stimulatory may be beneficial even during temporary low oxygen conditions such as found in the ischemic penumbra [13]. A primary form of energy production in the brain is the lactate shuttle that exists between neurons and astrocytes [143, 144]. It has been found that lactate administered at early time points after ischemia can be neuroprotective against cell death in vitro, decreases lesion size and improve neurological outcome in vivo [145]. The lactate shuttle is found the be dependent on neuronal activity, the lack of activity that occurred during hypoxia in non-treated cultures could intervene with this shuttle. Therefore, intervention with energy production in the brain is causing tissue damage. Thus, our results suggest that optogenetic stimulation might maintain or trigger the shuttle during hypoxia, by activating signalling molecules (i.e. glutamate, NH4 [146]) and having a neuroprotective effect. Therefore, we propose to investigate whether administration of lactate to hiPSC-derived neuronal cultures subjected to hypoxia shows neuroprotection. Furthermore, future research could try to inhibit the neuroprotective effect of optogenetic stimulation by blocking the lactate shuttle to assess whether the cultures become electrically silent after 40h of hypoxia.

5.3 Conclusions

Combining hiPSCs derived neuronal models with neuronal network dynamics constitutes a promising tool to investigate human neuronal responses to hypoxia. Hypoxia causes an increase in cell death and this is not inhibited by memantine. Hypothermia and optogenetic stimulation show promising neuroprotective effects in neuronal cultures. However, the translation of these treatment strategies is rather difficult. Future research could investigate new translational strategies for these treatments.

The use of hiPSCs opens new perspectives for understanding the effect of hypoxia network activity in ischemic stroke.

Appendix A

Review: Pathophysiological cascade of ischemic stroke and treatment strategies.

Voogd, E.J.H.F

Abstract

Ischemic stroke is a leading cause of death and disability in the world. The only treatments that are proven effective in humans to reduce brain damage is acute recanalization by intravenous thrombolysis or mechanical thrombectomy. The infarct core is irreversibly damaged and the area around the core (penumbra) is salvageable. Research is focused on the treatment to enhance recovery of the penumbra. Preclinical and clinical studies investigated the pathophysiological mechanisms in order to establish neuroprotective treatments. In this review we describe the molecular events that occur upon ischemic attack. These events are placed in a chronological order to provide a clear overview. However, these events might occur sequentially or in parallel and might activate different pathways resulting in a complex pathological cascade. All these molecular events will eventually lead to cell death. For these events we describe candidate treatment strategies that are investigated in preclinical trials. Furthermore, we propose new treatment strategies that target multiple molecular events.

Introduction

Ischemic stroke is a leading cause of death and disability in the world [2, 3, 147]. There are about ten to seventeen million stroke incidences a year worldwide, with at least six million patients dying related to stroke [148]. Epidemiological reports predict an increase due to the aging population. When patients survive, they are frequently left with moderate to severe neurological deficits [4].

Ischemic stroke is characterized by a sudden loss of blood perfusion in part of the brain, resulting from occlusion of a brain artery. The artery can be occluded by an embolus or a thrombus. This leads to loss of neuronal function, neuronal cell death and loss of neuronal structure, sequentially, with consequent neurologic deficits [5]. If perfusion is not reestablished in time, brain damage can be permanent [6]. Therefore, early removal of the occlusion is essential. There is a critical time window where brain damage can be limited, which depends on remaining perfusion levels, which in turn depend on collateral circulation.

The only treatment that is proven to be beneficial to reduce brain damage and neurological impairment is acute recanalization by intravenous thrombolysis [7] or mechanical thrombectomy [8]. The sooner recanalization can be achieved, the better the prognosis will be, reflecting the vulnerability of the brain to hypoperfusion and hypoxia. Beneficial effects of recanalization therapies have been established for intravenous thrombectomy (IVT) within 4.5h [9] and intra-atrial thrombectomy (IAT) within 6h from symptom onset [10]. Treatment windows may be extended based on computed tomography (CT) or Magnetic resonance imaging (MRI) perfusion imaging [149]. Still, due to limited time-windows and a wide range of contra-indications, only 5-10% of patients is eligible for recanalization treatments. Even when treated, most patients experience moderate to severe persistent functional deficits [11]. There is an urgent need to uncover new pathways that could be therapeutically targeted to improve stroke outcome, preferably applicable over a time-window of hours to days.

In order to establish neuroprotective treatment, it is crucial to understand which electrical and biochemical processes are vital for recovery or play a role in progression towards permanent damage.

The core of the infarct is irreversibly damaged, however the tissue surrounding the core ('penumbra') is salvageable, when progression of the damage is limited. Over the past decades, a magnitude of preclinical and clinical research has uncovered relevant pathophysiological mechanisms in order to identify effective treatment targets. Still, there is up to today no effective treatment for ischemic stroke in humans other than intravenous thrombolysis and mechanical thrombectomy. There is no approved agent for neuroprotection. Here we review molecular mechanisms that play a key role in ischemic stroke recovery or deterioration with the aim of providing an overview of potential treatment targets (table 1). Besides this we also show a small overview of different treatment strategies that target the molecular mechanisms in pre- and clinical trials described in this review and propose future directions.

Neuronal damage after ischemic stroke in core and penumbra

The human brain requires relatively much energy: although it only represents 2% of the body weight, it accounts for 20% of oxygen consumption and 25% of glucose utilization [150]. Approximately 25% of the brain's energy expenditure comprises basic cellular activities, such as protein synthesis, intracellular transport, and mitochondrial proton leakage. The remaining 75% is required for signaling processes, including generation of action potentials and synaptic transmission [151]. These functions will be compromised during ischemic stroke and leading to irreversible neuronal damage. This comes in sequential steps, starting with energy failure followed by synaptic dysfunction, ion imbalance, excitotoxicity, and acidification depend on the duration of hypoperfusion and remaining perfusion levels [12, 152]. These are all part of the 'ischemic cascade' that may lead to cell death and inflammation in the first seconds, minutes or hours following stroke. However, it is incompletely understood which electrical and biochemical processes are vital for recovery and which anticipate permanent damage.

The area directly around the infarct is named the infarct core, with perfusion levels below 10 ml/100g/min, in which energy supply is insufficient to preserve ion gradients across the plasma membrane and damage is irreversible [12]. Loss of neuronal function is followed by calcium influx, cell

swelling, and irreversible structural damage on timescales of seconds to hours [152-154]. This starts in the core and gradually spreads towards the penumbra. This is an area with some remaining perfusion from adjacent arteries, however there is a significant decrease in perfusion levels (between 14 and 35 ml/100g/min) [13]. It is assumed that in the penumbra functional neuronal activity becomes impaired, but neurons initially remain structural intact and metabolically active [5, 14]. If energy supply is restored, by reestablishment of the blood flow, this dysfunction is in principle reversible. However, in the absence of reperfusion, ion homeostasis will be affected in parts of the penumbra, leading to cell swelling and cell death. In peripheral regions of the penumbra, ongoing neuronal damage may be unassociated with cell swelling, and continue up to days after the insult [155]. The penumbra is a heterogenous area, this can partly be ascribed to irregularities of perfusion levels [152]. Furthermore, different cell types can have different sensitivity to ischemia, therefore the pathophysiological cascade is heterogenous. Causes for selective ischemic vulnerability are not clear, but possibly include the amount of connectivity [12], differences in ion channel composition [156], or selective synaptic failure, with higher vulnerability of excitatory synapses relative to inhibitory synapses [157]. Moreover, secondary damage of penumbral tissue can lead to additional neurological impairment, which occurs in approximately one third of patients during the first days after the infarct [158]. Improvement of neuronal recovery in the penumbra has a large potential to advance recovery. However, current treatments to promote recovery of penumbral tissue are scarce and only consist of general interventions, such as prevention of pyrexia, hyperglycemia, and systemic hypertension [15].

Molecular events after stroke onset

The tissue of the infarct core dies quickly, within seconds energy failure and synaptic dysfunction occur [24]. The extension of damage is largely determined by the progressive transformation of the penumbra into damaged tissue. The penumbra will be affected over time when there is no intervention, the core will expand, and more tissue is lost [159, 160]. Additionally, the toxic signals of the dying neurons from the infarct core cause damage to the penumbra (e.g. cell death) [161]. Thus, the core increases in volume due to cell death in the penumbra and this does not allow treatment

anymore. In this review we will divide the molecular events happening in seconds, minutes, and hours which are found initially in the core and gradually will affect the penumbra, causing irreversible damage. However, the molecular events involved might occur sequentially or in parallel and might activate different pathways resulting in a complex pathological cascade.

Molecular events after seconds

Energy failure

The depletion of oxygen and glucose slows down or stops the synthesis of adenosine 5'-triphosphate (ATP) through glycolysis and oxidative phosphorylation. The activity of the nerve cells in the brain depend almost exclusively on the oxidative phosphorylation for energy production [162]. Mitochondria in neurons and astrocytes use more than 75% of the oxygen delivered to the brain to generate ATP [154]. When oxidative phosphorylation is disrupted it causes ATP synthase to consume ATP and this accelerates ATP shortage [163]. In mice, a rapid fall of the ATP level occurs within 2-5 seconds of brain ischemia [154, 164]. Ischemic induced arrest of mitochondrial respiration and ATP generation is defined as primary energy failure in ischemic stroke [154].

Synaptic dysfunction

In previous work it has been shown that under complete ischemic or anoxic conditions, synaptic failure is an early consequence of energy depletion [12]. In patients with brain ischemia, early electrophysiological changes include electroencephalographic and evoked potential disturbances, which support the notion of an early alteration of postsynaptic currents [165].

Synaptic transmission is highly vulnerable to metabolic perturbations. Hypoxia damages the dendritic structure and causes spine loss. Bolay et al. [24] conducted in vitro and in vivo experiments with acute complete anoxia and found that depression of excitatory synaptic transmission was the very first measured pathophysiological process. Isolated synaptic disturbances, with intact membrane properties, were irreversible after transient severe anoxia, if the depth or duration of hypoxia was sufficiently severe [24]. Most evidence upholds the assumption that the initial hypoxic synaptic failure is primary a result of presynaptic malfunction caused by a defect in the phosphorylation of synapsin and is associated with impaired neurotransmitter release. This suggestion is based on observations of

depressed postsynaptic potentials evoked by stimulation of afferent fibers, with intact responses to exogenous glutamate or other postsynaptic receptor agonists [24]. Those changes disrupt the neuronal circuit and impairs normal function of the brain [166]. However, it is unclear how functional disturbances of synaptic transmission evolve in the outer border of the penumbra, under conditions of hours-lasting partial hypoxia in the absence of depolarization. Animal studies have indicated that the expression of genes and proteins that are associated with synaptogenesis, which are high during early brain development, undergo repeated increased expression during the first hours to days after acute ischemic stroke [25]. This suggests a limited period of increased potential for neuroplasticity, which is consistent with results of clinical research, showing improved recovery with early and intensive physical therapy [26].

Table 1. Molecular events* and their treatment strategies highlighted in this review.

Molecular event	**Onset**	**Caused by**	**Tested and effective in animal models**	**Tested in stroke patients?**	**Effective in stroke patients?**
Ionic imbalance	Minutes	Failure of Na+/K+ ATPase pumps	Methylene blue [131-136]	No	N/A
Excitotoxicity	Minutes	Excessive glutamate excretion	Memantine [137-143]	Yes [210-212]	Yes [210-212]
Spreading depression	Minutes	Waves of depolarization to restore ion gradients	P-188 [45] and citicoline [148-151], Ketamine [144-145]	Yes [148, 213]	Yes [148, 213]
Calcium dysregulation	Minutes	Increased calcium influx in neurons	Pseudoginsenoside-F11 [152-155]	No	N/A
Ischemia induced acidosis	Minutes	Increased amount of lactic acid	Ethanol [159-164]	No	N/A
Mitochondrial dysfunction	Minutes	Decrease in electron transport chain activity	Pramipexole [167 or rapamycin [168, 169	No	N/A
Autophagy	Minutes	Dysfunctional proteins	Rapamycin [169, 178-180]	No	N/A

Oxidative and nitrosative stress	Minutes	Imbalance between production of free radicals and neutralization of reactive intermediate products	NR2B9C [183-186]	No	N/A
Inflammation	Hours	Activation of endothelial cells and upregulation of leukocytes	L-3-n butylphthalide [103, 189-192]	Yes [214-216]	Yes
Final common pathways to cell death					
Necroptosis	Minutes	Activation of RIPK1/3 and MLKL protein kinases	Ligustroflavone [196]	No	N/A
Apoptosis	Minutes	Activation of caspase pathway	XQ-1H [201-204]	Yes [217]	Yes

* Not all molecular events are targetable in the present day, therefore only targetable molecular events are included in this table.

Molecular events after minutes

Neuronal depolarization

Loss of ATP causes disruption of the ionic pumps resulting in an increase of intracellular Na^+, Ca^{2+} and K^+ concentrations [162]. These ions are redistributed across plasma membranes. ATP depletion causes failure of the Na^+/K^+ ATPase pump, resulting in depolarization [167]. Permanent and anoxic depolarizations develop minutes after stroke onset [6]. Due to the failing Na^+/K^+ ATPase pump, the intracellular concentration of sodium increases, and potassium is released into the extracellular matrix [168]. Membrane potential and osmotic pressure are lost, causing cell swelling [169, 170]. Membrane channels that generate action potentials are inactivated and neurons cannot fire action potentials [168]. Future treatment strategies could try to maintain Na^+/K^+ ATPase pump activity or avoid cell swelling.

Excitotoxicity

ATP depletion causes failure of the plasma membrane Ca2+/ATP pump, causing an increase of Ca2+ concentration inside the cell [27]. In the infarct core excessive amounts of Ca2+ in the excitatory neurons bind to synaptotagmin in the axon terminal. Presynaptic voltage-dependent Ca2+ channels become activated and excitatory neurotransmitters are released into the synaptic cleft, causing a neurotoxic release of excitatory neurotransmitters [6]. The accumulation of glutamate leads to hyperexcitation of the NMDA, AMPA and kainate receptors. Glutamate activation of ionotropic receptors result in increased energy-consuming electrical activity, especially under conditions of reduced glutamate uptake by astrocytes in ischemia. Hyperexcitation causes increase of the influx of calcium, sodium, and water into the neurons, augmenting neuronal damage [28]. Excitotoxicity is deleterious to the brain tissue and is therefore an interesting target to inhibit in stroke treatment. However, it is important that heathy concentrations of glutamate are secreted in order to maintain normal neuronal function.

Spreading depression

Around the infarct core, loss of ion gradients (depolarization) may occur. Waves of depolarization (‘spreading depression’) in the absence of sufficient energy to restore ion gradients, may lead to

calcium influx, cell swelling and cell death [12]. This occurs in human ischemic stroke with high incidence. They arise from the edge of the ischemic core and propagate through the area of the penumbra [171]. Spreading depression (SD) causes transient megachannel opening on the neuronal membrane, this may compromise neuronal survival under ischemic stress [55]. Due to the excess of glutamate and the ionic imbalance waves of neuronal and astroglial mass depolarization occur along with drastic disruption of ionic gradients [6]. These waves propagate across the gray matter with a velocity of 2-5mm/min in animals [55, 172] and 1.7-9.2 mm/min in humans [173]. The number and duration of SDs after ischemic brain injury is correlated to secondary neuronal damage and further infarct expansion [174]. Restoration of the consequently disrupted ion gradients across the plasma membrane is an energy consuming phenomenon and aggravate energy deprivation. Recovery is dependent on remaining energy levels, which in turn depend on perfusion levels. Peri-infarct depolarizations have been demonstrated in animal models and occur with a frequency of several events per hour and can be recorded for at least six to eight hours after stroke onset [6]. In these models it is found that increase in infarct volume is proportional to the number and duration of this phenomenon [6, 175, 176]. Therefore, reducing the number and duration of SDs could be neuroprotective and is an interesting strategy to investigate.

Calcium dysregulation

Membrane depolarization and excitotoxicity lead to the disruption of the calcium homeostasis in the brain. Ca^{2+} is considered to be a mediator of ischemic brain damage. Normally, Ca^{2+} is always leaking in a low amount into the cell and is involved in many different intra- and extracellular processes, such as synaptic activity and cell-cell communication. When ATP production is decreased, Ca^{2+} extruders (e.g. Na^{+}/Ca^{2+} exchanger) stop working, causing the intracellular and mitochondrial Ca^{2+} concentration to increase [177].

Increased Ca^{2+} binds to synaptotagmin in the axon terminal causing glutamate to be released and increasing excitotoxity [178]. The excitatory receptors (NMDAR, AMPAR and Kainite receptor) are well

known for their Ca^{2+} permeability and due to overactivation they are partly responsible for Ca^{2+} influx [179]. Especially the NMDARs are a known route for excessive Ca^{2+} to initiate cell death [32].

Ischemic stress activates different Ca^{2+} routes and induces Ca^{2+} overload in neurons and non-neuronal cells [170]. Mitochondria store Ca^{2+} in the cell and releases their content upon ischemic attack [180]. However, this cannot fully account for the irreversible build-up of Ca^{2+} after excitotoxic stimulation. Other channels and ion pumps are activated up on ischemic attack.

The Na^{+}/Ca^{2+} exchanger (NCX) normally takes up sodium and secrete Ca^{2+}. However, the intracellular accumulation of sodium may reverse the operational direction of the NCX to cause Na^{+}-dependent Ca^{2+} uptake, which is mostly found in glial cells [181]. It is thought that in non-neuronal cells the reverse mode contributes to Ca^{2+} uptake in ischemia [182].

In neuronal cells the situation may be different. In these cells the NCXs may work both ways, some work forward and others may work in the reversed direction, perhaps in different membrane locations. Cytotoxic glutamate exposure enhanced the exchanger forward current (efflux of Ca^{+2}) while the reverse activity was inhibited [183]. Block of NCX damages neurons while increasing the NCX is could be neuroprotective [184]. It was found that knock-down of the exchanger NCKX2 resulted in greater increases of Ca^{2+} in cortical neurons [185]. An impaired function of NCX contributes to necrosis in the infarct core and aggravates apoptosis in the penumbra. Enhancing the NCX is thought to be neuroprotective, whereas the inhibition of NCX further aggravates tissue injury [170]. However, the exact role of NCX in ischemic brain injury and its therapeutic potential remain to be further investigated.

Another type of channel that plays a role in Ca^{2+} overload is the transient receptor potential (TRP) channel. This is a non-selective cation channel. These TRP channels regulate membrane potential and manipulate intracellular Na^{+}, K^{+} and Ca^{2+} concentrations in both excitable and non-excitable cells. Opening of TRP channels depolarizes the cell membrane and thereby activates voltage-dependent Na^{+} and Ca^{2+} channels, leading to intracellular Na^{+} and Ca^{2+} accumulation [186]. Prolonged Ca^{2+} entry and

cytotoxicity mainly result from activation of TRPM7 channels. These channels are activated by ROS from nitric oxide signaling and may be a possible therapeutic target [187].

The increased amount of Ca^{2+} entering the cells will activate phospholipases, disrupt mitochondrial electron transport, and causes release of free radicals [188]. This causes lipid peroxidation, generates more free radicals and releases arachidonic acid [189]. Moreover, Ca^{2+} overload causes excessive stimulation of Ca^{2+}/calmodulin dependent enzymes such as nitric oxide synthase, as well as a host of Ca^{2+}-dependent enzymes as proteases and endonucleases [190]. These are death signaling enzymes. Overactivation of these catalytic enzymes cause protein degradation, phospholipid hydrolysis and DNA damage as well as a disruption of cellular signaling and enzymatic reactions resulting in cell death [191]. Maintenance of normal calcium homeostasis is important to avoid further tissue damage. Therefore, treatment strategies that try to maintain healthy Ca^{2+} concentrations could be promising.

Ischemia induced acidosis

Acidification is a consequence of oxygen deprivation. Ischemia induced acidosis is a complex cascade and is thought to be caused by elevation in blood glucose concentration and can exacerbate cell injury. Excessive lactic acidosis of brain tissue is an important contributor to irreversible damage [192, 193]. The level of lactic acid accumulation is strongly associated with post ischemic recovery. The amount of lactic acid is dependent on the amount of glucose that is available for anaerobic glycolysis. In the infarct core, where the blood flow is interrupted completely, the increase in the concentration of lactic acid is limited by the pre-ischemic tissue stores of glucose and glycogen [154]. However, these stores are rapidly exhausted. The maximal concentration lactic acid is reached within 5 minutes. In the penumbra on the other hand, the production of lactic acid depends on the duration of hypoperfusion, remaining perfusion levels, and on the blood glucose concentration. In this situation there is a continued delivery of substrate for glycolysis and this may exacerbate tissue lactic acidosis [193]. Glycolysis is stimulated during hypoxia when there is increased ATP hydrolysis, along with increased H^+ release. Normal brain tissue has a pH of approximately 7.2 [194]. Under healthy circumstances glucose is transformed into pyruvate. However, insufficient oxygen delivery causes the production of lactic acid from pyruvate.

Excessive amounts of lactic acid accumulate in the tissue and causes the pH to drop to a pH of 6.6 under normal glucose levels ("normoglycemic conditions") and even to drop to under a pH of 6.0 under high glucose levels ("hyperglycemic conditions") [195]. When the pH drops below a pH of 6.7, brain injury will be exacerbated due to denaturation of proteins, activation of acid sensing ion channels (ASICs) and the release of ferrous iron [196, 197]. On the other hand, acidosis with a pH of 6.7 inhibits the production of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and is thought to be neuroprotective, because NADPH oxidase cannot form harmful reactive oxygen species (ROS) under these conditions [198]. ASICs are cation channels that are activated by a pH of 6.7. ASICs are gated by extracellular H^+ and conduct the acid-evoked inward Na^+ currents leading to depolarization and cell swelling. Homomeric ASIC1a channels are also Ca^{2+} permeable and have been implicated in acidosis-mediated neuronal injury under ischemic conditions [199]. Therefore, a future treatment strategy could be the maintenance of a healthy pH or try at least to maintain a pH of 6.7 to inhibit NADPH production.

Mitochondrial dysfunction

Energy homeostasis, which is controlled by the mitochondria, is disturbed within minutes after ischemic stroke[29]. Mitochondria exist of an outer phospholipid bilayer and an inner membrane. The outer membrane contains protein channel structures, which makes it permeable for molecules up to 10kDa like ions, water, adenosine diphosphate (ADP), and ATP. The inner membrane is the reactive center of mitochondrial energy metabolism and contains complexes of the electron transport proteins, ATP synthetase complex and ATP/ADP transport proteins [30].

Energy balance is disrupted because there is a critical decrease in the electron transport chain activity with consequent ATP depletion. Ischemia in neurons is characterized by this depletion and overproduction of ROS. Low perfusion levels cause mitochondrial dysfunction, this affects the working mechanisms of the mitochondria. The mitochondria remove the Ca^{2+} from the cell in healthy metabolism [29]. Excessive concentration of calcium promotes loss of mitochondrial membrane potential [31, 32].

The mitochondrial membrane depolarization is triggered by an energetic failure driven by a combination of increased energy demand and poly(ADP-ribose) polymerase (PARP) activation, this depolarization is in principle reversible. However, Abramov and Duchen found that cells undergo a transition from the energetic failure to mitochondrial permeability transition pore (mtPTP) opening and revealed that this transition leads to a loss of potential that is no longer reversable [33].

This leads to disturbed mitochondrial functions which in turn activate release of free radicals, enzymes and proteases that also leads to activation of cellular death pathways (e.g. release of cytochrome C). Mitochondria are considered as one of the regulators of stress signaling. Structural reorganization occurs when mitochondria are exposed to ischemic stress. Neurons adapt to stress by removing impaired mitochondria via mitophagy, this is to maintain the mitochondrial homeostasis. [34, 35]. It is an attempt to suppress the release of ROS and cell death signaling cascades like the caspase pathway [200].

There is cross talk between healthy and injured cells, making it possible to transfer mitochondria. The recipients can reprogram the mitochondria to activate signals for cell survival [29]. However, these mechanisms are still unclear. Maintaining the mitochondrial function is crucial for neuronal survival in ischemic stroke. Healthy mitochondrial functioning is therefore one of the promising neuroprotective targets for stroke treatment [29].

Autophagy

Autophagy is activated in different cell types of the brain upon nutrient starvation or metabolic stress to maintain tissue homeostasis through clearance of dysfunctional proteins and organelles. It is the phagocytosis of cytoplasmic materials, which are captured by autophagosomes and fuse with lysosomes to form autolysosomes and are then degraded [201]. Autophagy is not only present in the ischemic core, but it is also located in the penumbra [202].

Activation of autophagy is related to signaling pathways. It was found that phosphorylation of protein kinase B (Akt) and cAMP response element binding protein (CREB) are increased in neuronal cells during ischemia [203]. AMP activated protein kinase (AMPK) signaling pathway is a critical regulatory

cascade of autophagy. Several groups found that AMPK is involved in induction of protective autophagy [204, 205].

The effect of autophagy on the tissue may vary according the duration of hypoperfusion, the remaining perfusion levels and animal models used. It is possible that autophagy has a two-way effect on ischemic injury. Moderate activation may be neuroprotective and excessive activation of autophagy may have deleterious effects on neuronal injury and lead to cell death. Neuronal autophagy upon ischemic attack is part of pro-survival signaling [203]. Ischemic injury may be reduced when autophagy is regulated, this could be a promising treatment strategy.

Oxidative and nitrosative stress

In stroke, free radicals may be important mediators of the infarction process. Oxidative stress is an imbalance between the production of ROS and reactive nitrogen species (RNS) and the capability to readily neutralize the reactive intermediate products through antioxidant defense [30]. Both cell calcium overload and acidosis enhance the production of partially reduced oxygen species, resulting in free radical related damage. Free radicals formed due to reperfusion may contribute to a perturbed membrane function, leading to a sustained alteration of cell calcium metabolism with ultimate mitochondrial calcium overload [206]. Rapid increase of the production of ROS overwhelms the antioxidant defenses and causes more tissue damage. ROS can damage macromolecules that lead to autophagy, apoptosis, and necrosis, which determine final infarct size. The brain is especially vulnerable to free radicals because there is low neuron antioxidant enzyme activity, high oxygen consumption, high concentration peroxidizable lipids and high levels of iron. These characteristics are pro-oxidants under pathological conditions [207].

Superoxide is the most important ROS in the central nervous system (CNS). This oxide harms the ROS producing cell, but also neighbouring cells. Superoxide is a by-product of the respiratory chain or a product generated by enzymes as NADPH oxidase *in vitro* and *in vivo* during ischemia [208]. This enzyme is an important treatment target because NADPH oxidase generates ROS that promote mitochondrial uncoupling and triggers a secondary ROS release by mitochondria. Mitochondria are a

well-known ROS source during electron transport and oxidative phosphorylation [209]. Free radicals mediate mitochondrial damage and activate matrix metalloproteases which disrupt the blood-brain-barrier by degrading the basal layer of collagen and laminin [210].

RNS inhibit key mitochondrial enzymes, facilitate mitochondrial transition pore formation, DNA damage, PARP activation and activation of Ca^{2+} permeable TRPM7 channels [211, 212]. Furthermore, RNS causes post translational modification that significantly impacts cell survival by altering the function of caspase, metalloprotease [213], and glycolytic enzyme GAPDH [214]. Lack of ATP and the overproduction of ROS further impairs the activity of Na^+/K^+ ATPase pump, causing more accumulation of sodium in the intracellular matrix [187].

This shows that increased production of ROS/RNS is deleterious for brain tissue, however homeostatic levels of these free radicals are critical signaling molecules that play a role in normal neuronal and vascular function [215, 216]. Therefore, it is important to find novel ways to suppress the deleterious release of free radicals, without interfering with the endogenous signaling.

Molecular events after hours

Inflammation

Cerebral ischemic injury and reperfusion cause an inflammatory cascade. The vascular response to ischemia activates endothelial cells and upregulates leukocytes and adhesion molecules. The leukocytes travel across the endothelial cells via adhesion molecules and secrete pro-inflammatory cytokines. The cellular content that is released by the necrotic cells is recognized by infiltrating immune cells [217]. These endogenous molecules released from damaged cells activate various immunological receptors in myeloid cells to induce immune responses [218]. Microglia are the major immune cells resident in the brain to be activated and thereby mediate neuroinflammation in response to brain injury after ischemic stroke [219, 220].

Due to ischemic stress microglia are overactivated. They act as macrophages in brain tissue [221]. Microglia polarize into two different phenotypes with distinct functions after ischemic stroke, either pro-inflammatory or anti-inflammatory phenotype, M1 and M2 respectively [106]. M1 microglia cause pro-inflammatory effects and produce large amounts of cellular mediators like cytokines, proteases,

and ROS [222]. M2 microglia have anti-inflammatory effects and activate phagocytosis of cell debris or damaged neurons and release neurotrophic factor to promote tissue repair [223, 224]. Not only the damaged neurons are phagocytosed but the viable neurons in de ischemic penumbra are also phagocytosed. Early-on inflammation may amplify the ischemic lesion [28]. Therapeutic interventions could try to control post-ischemic inflammation, by enhancing the M2 polarization of microglia.

Final common pathways towards cell death

It is well-known that the mechanisms described above are responsible for brain injury after stroke. However, no matter what mechanism is involved in ischemic injury, the destiny of most injured brain cells is death. These mechanisms may trigger different types of cell death, dependent on the cell type, cell age and the location in the brain [225]. Cell death occurs in the core and can release toxic signals which affect the penumbra. If reperfusion is not established, the cells in the penumbra will go into cell death and the core will expand. Here we describe types of cell death which occur in ischemic stroke.

Necrosis

Necrosis is mostly found in the core. Failure of the Na^+/K^+ ATPase pump causes sodium accumulation in the cytosol leading to cellular edema [167]. The nucleus and the cytosol swell and the lysosomes rupture and release degrative enzymes in the cytosol which is damaging to different organelles [226, 227]. The cell membrane ruptures, releasing its content in the extracellular matrix and causes an inflammatory response in and around the cell [161, 228].

Ischemic stroke causes overactivation of PARP-1. This leads to depletion of NAD^+ and ATP, and results in necrosis. Activated caspase-3 cleaves PARP-1 and cleaved PARP-1 is a surrogate marker of necrosis [229]. Inhibition of PARP-1 overactivation could be a therapeutic strategy to prevent necrosis, decrease of cellular energy loss and downregulation of various pro-inflammatory signal transduction pathways. However, necrosis is most common in the core and the consequences of ischemic stroke are not targetable in the core.

Necroptosis

Necroptosis is a partially programmed event of cellular explosion and is dependent on specific receptors or sensors, such as death receptors which mediate cell death. This type of cell death is

triggered by a cascade of protein kinases including receptor-interacting protein kinase-1/3 (RIPK1/3) and mixed lineage kinase domain like (MLKL) [93, 230]. Necroptosis is often called RIPK-dependent necrosis. It holds several features found in necrosis and apoptosis. There is lysosomal rupture, rapid swelling and membrane rupture, this is also found in necrosis [226, 227]. Furthermore, cytochrome C (Cyt c) is released and caspase-3 cleavage is activated, which is in line with characteristics of apoptosis [231, 232]. In the ischemic penumbra there is an increased expression of Fas ligand (FasL) and tumor necrosis factor alpha (TNF-α) which activate downstream kinases. When these cells cannot go into apoptosis these ligands activate RIPK1/RIPK3/MLKL pathway and the cell will eventually go into necroptosis [233]. This type of cell death can possibly be avoided by inhibiting the RIPK1/RIPK3/MLKL pathway.

Apoptosis

The neurons in the penumbra can undergo apoptotic cell death, this is a programmed cell death and it is characterized by a distinct set of morphological and biochemical changes [36]. Apoptosis is initiated by overproduction of free radicals, Ca^{2+} overload and excitotoxicity. Apoptotic stimuli can be classified as intrinsic stimuli, coming from within the cell, or extrinsic stimuli, coming from the external milieu.

Apoptotic intrinsic stimuli are activated by mitochondrial signaling. The Ca^{2+} influx causes cleavage of Bcl-2 interacting domain (BID) to truncated Bid (tBid). tBid opens the mitochondrial transition pores [33]. Then Cyt c or apoptosis inducing factor (AIF) is released in the cytosol. Cyt c binds with apoptotic protein-activating factor-1 (Apaf-1) and procaspase-9 to form an apoptosome, which activates the caspase pathway. Caspase is the main driver of intrinsic apoptotic pathway. The apoptosome activates caspase-9 and consequently caspase 3, which is the last and executive enzyme. Nuclear DNA (nDNA) repair enzymes are fragmented by activated caspase-3. This leads to nDNA damage and initiates apoptotic cell death [6]. AIF translocates rapidly to the nucleus, where it mediates large-scale DNA fragmentation and cell death, independent of caspase pathway [234]. Caspase proteins are also activated in the penumbra and inhibition of these proteins could protect against ischemic injury [235].

Extrinsic pathway is initiated via ligand-receptor interactions and can activate caspase independent of Cyt c release. Tumor necrosis factor (TNF) ligands bind to TNF receptors, Fas receptors or TNF related apoptosis inducing ligand (TRAIL) receptors, these are so called cell surface death receptors [37]. In animal studies it was reported that Fas ligand (FasL) upregulates the Fas receptor in ischemic stroke. The Fas/FasL system acts as an apoptosis inducer and triggers pro-inflammatory cytokine production [236]. Caspase-8 and caspase -10 are activated as result of these bindings and will eventually activate caspase-3 [237, 238]. This results in membrane blebbing, mitochondrial membrane permeabilization, chromatin condensation, nDNA fragmentation and eventually cell death [38]. Suppression of these death receptors may improve neuronal survival after ischemic stroke.

Furthermore, P53 is implicated in the ischemic pathophysiological cascade. This is a tumor suppressor and a transcription factor, and it initiates apoptosis through transcription of pro-apoptotic genes such as Bcl-2. P53 mediates mitochondrial dysfunction and caspase activation [239]. In human it is thought that p53 can trigger apoptosis by repression of anti-apoptotic genes [240].

Dying cells shrink and condense and are then fragmented, releasing small membrane-bound apoptotic bodies, which generally are engulfed by other cells. Intracellular content is not released into the extra cellular milieu [241]. Inhibition of apoptotic stimuli could rescue the cells from apoptotic cell death and this may be interesting to further investigate.

Treatment strategies

It is for modern day medicine impossible to target the stroke consequences in the infarct core. The penumbra on the other hand is salvageable. Therefore, treatment strategies aim to minimize the damage to the penumbra by increasing neuroprotection and inhibiting cell death. However, the pathophysiological cascade activated upon ischemia makes it difficult to inhibit molecular events that trigger cell death. There are different treatment strategies investigated in preclinical and clinical trials. Numerous animal studies showed neuroprotection in the penumbra, however these treatments failed to be neuroprotective in clinical trials [16].

Molecular events described above make up possible targets for treatment of the penumbra. Here we describe treatment strategies that are investigated in preclinical studies, which tried to target one or several molecular events or consequences that are found in the pathophysiologic cascade.

Treatment strategies targeting the molecular events.

Ionic imbalance

Due to failure of the Na^+/K^+ ATPase pumps, the neurons depolarize, and the membrane potential is lost. Furthermore, the osmotic pressure is lost, which causes cell swelling. Methylene blue (MB) is found to attenuate neuronal survival in traumatic brain injury [242] and acts anti-inflammatory in subarachnoid hemorrhage [243]. Several studies investigated the effect of MB in ischemic brain injury [39-44]. Shi et al. [39] investigated the effect of MB on cytotoxic brain edema. They used a rat model with transient middle cerebral artery occlusion (tMCAO) with the duration of 1 hour. MB was administered for 30 minutes immediately after reperfusion and for another 15 minutes, 3 hours after ischemia. MRI revealed that MB attenuated cytotoxicity. Moreover, they found that MB attenuated the increase of perivascular space, nuclear pyknosis and necrosis caused by ischemia. In an *in vitro* study Shi et al. [39] investigated cell swelling in cultured rat astrocytes by exposure to an excess of glutamate for 48 hours. It was found that 10μM MB significantly inhibited astrocytic cell swelling [39]. This was in line with earlier findings of the same group [43]. MB was also found to reroute electrons in the mitochondrial electron transfer chain directly from NADH to cytochrome c, promoting mitochondrial activity and mitigating oxidative stress [44].

Excitotoxicity

Excitotoxicity leads to neuronal cell death and inhibition of excitotoxicity is thought to be neuroprotective. This can be achieved with pharmacological NMDAR or AMPAR antagonists.

Memantine is a moderate affinity noncompetitive NMDAR antagonist, binding directly within the pore of the channel in its open configuration. This is a drug that is used to treat Alzheimer's Disease (AD) patients. Several animal studies *in vivo* and *in vitro* which investigated the effect of memantine on ischemic stroke showed decrease in ischemic injury [45-51]. Trotman et al. [51] showed this decrease in an *in vitro* and *in vivo* study. Murine neuronal cell cultures were exposed to 90 minutes of oxygen-

glucose deprivation. Low concentrations of memantine (0.5-2.0 µmol/l) showed neuroprotection in the cultures by successfully blocking excitotoxic injury [51].

The *in vivo* study was conducted with adult male mice, which received a right middle cerebral artery occlusion (MCAO) with the duration of 1 hour. The treatment group would receive different doses of memantine starting 24 hours before MCAO and continuing until 48 hours after MCAO. They found a significant volume reduction in both striatal and striato-cortical lesions with treatment of 0.2 mg/kg memantine. This was associated with improved behavioral scores 24 hours after MCAO [51].

Spreading depression

Evidence support that prevention of SD or suppression of its propagation could result in less brain damage [55, 61, 62, 244]. Treating SD could improve functional outcome by preventing the increase of core volume.

One way to attenuate SD is to modify the megachannel opening that is caused by SD. An *in vivo* study conducted by Yildirim et al. [55] investigated the effect of Poloxamer 188 (P-188) and citicoline on the neuronal megachannel opening induced by cortical SD (CSD) in the mouse brain. P-188 is a membrane sealing agent and has been found to decrease neuronal loss and macrophage infiltration in a rat model of excitotoxicity [56]. Citicoline is a membrane stabilizing agent and reduces ischemic injury by stabilizing cell membranes and suppressing ROS production [57-60]. A single SD was induced in the mouse brain. Propidium iodide (PI) was injected, this is a membrane permeable fluorescent dye and is used as a marker for megachannel opening. The treatment groups either received P-188, administered 60 minutes before CSD induction, or citicoline, administered 90 minutes before induction. The results showed that after administration of P-188 or citicoline the number of PI positive cells significantly decreased compared to the control group. These results suggest that P-188 and citicoline may act by way of modifying megachannel opening and suppress propagation of SD [55].

Another target to inhibit SDs is by blocking NMDARs. Ketamine is a non-competitive NMDAR antagonist, and it was found in adult rats that this drug can interfere with SD initiation and expansion by increasing the threshold for K^+ and neurotransmitters. It can also decrease the duration of SDs by

reducing the influx of Na^+ and Ca^{2+} [61]. SD was induced in rats by cathodal DC-stimulation of the frontal cortex. At a dose of 40 mg/kg, ketamine increased the SD threshold and decreased the propagation velocity. It was found that SD propagation was inhibited completely by a dose of 80 mg/kg ketamine [61]. Amemori and Bures found a similar effect by a dose of 100 mg/kg, however the blockade that was induced weakened and finally disappeared [62].

Calcium dysregulation

Calcium overload is one of the main triggers of neuronal cell death and plays a role in excitotoxicity and is therefore an important target to investigate to try and attenuate ischemic injury. A way to target the calcium overload is by repression of Ca^{2+} influx. Evidence supports that Pseudoginsenoside-F11 (PF11) protects against ischemic injury by repressing Ca^{2+} overload c. An *in vivo* and *in vitro* study conducted by Zhang et al. [65], researched the effect of PF11 on Ca^{2+} overload among other mechanisms in ischemic stroke. Rats were subjected to tMCAO for the duration of 2 hours and treated immediately after reperfusion with PF11 and every 24h for 14 days. The study showed that PF11 treatment *in vivo* reduced infarct volume, brain edema, neurological deficit, cortex neuron loss and it repressed calcium overload at 24 hours after reperfusion. The *in vitro* study in cultured primary cortical neurons, which were exposed to oxygen-glucose deprivation/reoxygenation, showed that PF11 increased cell viability, reduced neurites decline, restored ATP levels and decreased Ca^{2+} concentration [65].

Acidosis

Acidification caused by glucose deprivation is thought to exacerbate cellular injury. Several studies showed that pharmacological inhibition or genetic deletion of ASIC1a resulted in a significant reduction in acidosis-mediated and ischemic brain injury, suggesting that ASIC1a is a potential therapeutic target for brain ischemia [197, 245-247].

Several studies showed that acute low to moderate ethanol administration to treat ischemic injury could reduce neuronal death and be neuroprotective [68-73]. A research group tried to treat ischemic induced acidosis with ethanol [72]. They investigated the effect of ethanol on the activity and expression of ASIC1a channels and acidosis-induced neurotoxicity. Murine cortical neuronal cultures

were exposed to 6 hours of ethanol, then incubated with extracellular solution with a pH of 7.4 or pH of 6.0 for 3 hours followed by incubation in normal medium for 21 hours. The results demonstrated that ethanol inhibited ASIC1a currents, ASIC1a-mediated Ca^{2+} accumulation, reduction of ASIC1a protein expression and neuronal cell death through autophagy dependent ASIC1a protein degradation [72].

Mitochondria

Maintaining mitochondrial function is crucial for neuronal survival in ischemic stroke [29]. Mitochondrial dysfunction plays a central role in the activation of death signaling pathways and the generation of ROS. Interventions targeting mitochondrial dysfunction by pharmacological agents have been demonstrated to be neuroprotective in preclinical studies [44, 74-78, 99-102].

One of the pharmacological agents tested was pramipexole, a dopamine D2 receptor antagonist. This drug showed to be neuroprotective in patients with Parkinson's disease and restless leg syndrome. It is thought that pramipexole mediates its neuroprotection through mitochondria. Therefore, Andrabi et al. [74] investigated the effect of pramipexole on mitochondria and tested its neuroprotection in a rat model with tMCAO. The occlusion was removed after 2 hours. The rats received then injections of pramipexole after reperfusion. The results showed that pramipexole had a positive effect on the neurobehavioral tests 24 hours after reperfusion. They also found that post-stroke treatment reduced levels of mitochondrial ROS and Ca^{2+} after ischemic stroke. Pramipexole elevated the mitochondrial membrane potential and mitochondrial oxidative phosphorylation. Furthermore, it was shown that pramipexole inhibited the transfer of Cyt c from mitochondria to cytosol, thereby inhibiting Cyt c induced apoptotic pathway [74].

Another strategy that has been investigated is the activation of mitophagy in an attempt to sustain homeostasis and protect cells from ischemic stress [75-78]. Li et al. [77] tested the effect of rapamycin rats who underwent tMCAO with a duration of 2 hours followed reperfusion. Rapamycin is a macrolide antibiotic, that can reduce injury in several models of neurodegenerative disorders by activating autophagy. The rats were pretreated with rapamycin 30 minutes before tMCAO. The results showed

that rapamycin significantly enhanced mitophagy, inhibited mitochondrial dysfunction, reduced infarct volume, and improved neurological function [77].

Autophagy

There is some controversy in studies about autophagy in ischemic stroke. Some studies found that inhibition of autophagy is beneficial, meaning that the activation of autophagy has deleterious effects on neural tissue after ischemia [248-250]. Whereas other studies found that moderate activation of autophagy is beneficial for ischemic injury [102, 251].

Several groups investigated the effect of rapamycin on autophagy. It was found that rapamycin was neuroprotective [77, 79-81]. This neuroprotection ranged from the reduction of infarct volume to the improvement of neurological outcomes. A study done by Buckley et al. [80] investigated both activation and inhibition of autophagy and their effects on ischemic injury. They investigated the effect of rapamycin and chloroquine in two different stroke models. The mice were subjected to either permanent middle cerebral artery litigation [120], which does not allow for reperfusion of the distal trunk of the middle cerebral artery, or embolic clot middle cerebral artery occlusion (eMCAO), which allows for slow reperfusion similar to human stroke patients. They used rapamycin to induce autophagy and chloroquine to block completion of autophagy. The mice were treated immediately after stroke onset and after 24 hours. The results showed that chloroquine significantly reduced the infarct size with 30% in the eMCAO model 48 hours post-stroke. In this model chloroquine also improved neurological score but did not increase survival. Rapamycin reduced infarct size with 44% (MCAL model) and 50% (eMCAO model). Rapamycin also improved the neurological score and survival more than was found with chloroquine treatment [80].

Oxidative and nitrosative stress

Increase of ROS and RNS is deleterious for brain tissue. These free radicals need to be suppressed without interfering with the endogenous signaling [215, 216]. Several studies tried to target oxidative stress that is caused by ischemia [82-85, 103, 104]. An example of a pharmacological agent that prevents nitric oxide production and blocks superoxide production is the neuroprotective agent NR2B9C [82-85]. A study conducted by Chen et al. [82], investigated the effect of Tat-NR2B9c on

NMDA-induced superoxide formation in murine neuronal cell cultures. They found that Tat-NR9B9c suppressed NMDA-induced superoxide production by about 75%. It prevents the assembly of the active neuronal NADPH oxidase complex [82]. An *in vitro* study done by Lv et al. [84] showed decrease in infarct volume and

ameliorated the neurological deficit in an MCAO rat model treated with NR2B9c.

Inflammation

Inflammation causes more damage of the neural tissue after stroke. Evidence suggests that microglia play an important role in ischemic injury and they polarize into two different phenotypes, either pro- or anti-inflammatory [106]. A research strategy is to manipulate the polarization of the microglia after stroke and create M2 microglia which are anti-inflammatory [105-107]. Several studies investigated the effect of L-3-n butylphthalide (NBP) on ischemic injury and found that it affects axonal growth, promotes neurogenesis and neuroplasticity, alleviates oxidative stress and reduces inflammation [86-89], and that it attenuates neuroinflammation in cultured astrocytes and microglia [106]. Li et al. [106] investigated whether NBP had an effect on microglial polarization. Mice were subjected to tMCAO for a duration of 45 minutes and were treated with NBP. The results showed that mice treated with NBP had a significant reduction of infarct volume. Behavioral tests showed that treatment with NBP improved performance. Immunofluorescent staining indicated that NBP treatment significantly increased the number of anti-inflammatory M2 microglia and reduced the number of pro-inflammatory M1 microglia. They concluded that NBP mitigated ischemic injury and promotes recovery of neurological function in early phase after ischemic stroke [106].

Treatment strategies targeting cell death.

In the previous part several treatment strategies are discussed. However, when these molecular events are not inhibited, they will lead to cell death. Therefore, inhibition of cell death is an interesting strategy to further investigate. Here we discuss treatment strategies that focus on the inhibition of cell death.

Necroptosis

Necroptosis is induced by ligands that activate RIPK1 [233]. It is possible that cells do not go into necroptosis when expression of necroptosis-associated proteins (RIPK1, RIPK3 and MLKL/p-MLKL) is reduced. Several studies tried to target the RIPK1 pathway and have revealed that this strategy improves neurological function and reduces infarct volume [93, 108-110].

Zhang et al. [93] investigated the effect of ligustroflavone on neurological deficits, infarct volume and levels of necroptosis-associated proteins. Ligustroflavone is a compound that possesses a variety of pharmacological activities including anti inflammation [252]. The rats received ligustroflavone 15 minutes before ischemia. They subjected the rats to MCAO for a duration of 2 hours and reperfused. Neurological deficit scores were assessed and showed that pretreatment with ligustroflavone improved the neurological function in a dose dependent matter. Furthermore, infarct volume was measured and it revealed that treatment with ligustroflavone reduced infarct volume and decreased protein levels of RIPK3 and MLKL/p-MLKL. This suggest that necroptosis was suppressed. However, the treatment had no effect on RIPK1 protein levels. This group also conducted an *in vitro* experiment with PC12 cells which were subjected to 8 hours of hypoxia followed by 24 hours of reoxygenation. Ligustroflavone was added to the culture medium before the cells were exposed to hypoxia and cell viability was tested. Results showed that treatment with ligustroflavone restored the viability of the PC12 cells. Furthermore, ligustroflavone attenuated protein levels of RIPK3 and MLKL/p-MLKL. However, it did not affect RIPK1 protein level. This is in line with the results they found in the *in vivo* study [93].

Apoptosis

Several experimental studies have revealed that inhibition of apoptosis decreases ischemic injury [94, 104, 111-114]. For example, inhibition of apoptosis by 10-O-(N,N-dimethylaminoethyl)-ginkgolide B methanosulfonate (XQ-1H). Several studies showed that XQ-1H has a protective effect on ischemic injury in rodents [94-97]. Xu et al. [94] investigated the effect and molecular mechanisms of XQ-1H on neuronal survival after stroke in mice. The mice were subjected to tMCAO for a duration of 45 minutes. Administration of XQ-1H started after reperfusion. They found that XQ-H1 limited cerebral infarction.

XQ-1H reversed the increased water content and therefore decreasing brain edema. It reduced the apoptosis rate of neurons and upregulated the anti-apoptotic molecule, B-cell lymphoma-extra-large (Bcl-xL) which was opposite to cleaved caspase-3. These results indicate the protective activity of XQ-1H against apoptosis [94].

Discussion

Here we reviewed the pathophysiological cascade of ischemic brain injury and several treatment strategies that target the different molecular events. We showed that there are a lot of complex molecular events that cause and extend brain injury. Furthermore, we discussed several treatment strategies that target one or more of these events. Most promising strategies which were successfully conducted in animal studies, seem to fail in clinical trials [16]. We still do not have a complete understanding of the pathophysiological mechanisms that damage brain tissue in ischemic stroke and how the injury can be limited or how recovery in humans can be improved.

There is a critical balance between healthy signaling of several cascades (depolarization of neurons, glutamate excretion, Ca^{2+} influx, ROS/RNS signaling, etc.) and their deleterious effects caused by ischemic stress. Therefore, it is important to try to inhibit the deleterious effects without the interference of healthy signaling. Besides healthy signaling, there is also a balance in activity. Neurons need to fire action potentials in order to communicate with each other and to stay alive [5, 27]. Current treatment strategies (e.g. blockage of excitotoxicity with NMDAR antagonists) are based on suppression of neuronal activity [16]. Investigation into the effect of treatment strategies that not completely block activity, but rather mildly stimulate the neuronal activity could be neuroprotective. This knowledge is not new, it is known that activity is needed for neuronal survival [5, 136-138]. Muzzi et al. [17] showed in rat cortical neurons that mild stimulation either with electrical stimulation or optical stimulation improves neuronal activity and neuronal survival after hypoxia. Further research could investigate the effect of electrical or optical stimulation in human derived neuronal cultures subjected to hypoxia.

The strategies discussed in this review often only target one of the molecular events. Perhaps an effective treatment consists of a combination therapy targeting multiple molecular events or their interaction with neurotoxic pathways and other regulatory mechanisms. We propose to target several molecular mechanisms to help cell viability and prevent them from going into cell death. Excitotoxicity and mitochondria could be promising targets to investigate further in treatment strategies. When excitotoxicity is inhibited or prevented, the cell could suffer less from spreading depression and Ca^{2+} influx, however glutamate excretion cannot be blocked completely. It is also important to prevent mitochondrial damage, so that overproduction of ROS is prevented and that the apoptotic pathway is not activated. When mitochondria are functional, they will not excrete Cyt c to activate apoptotic cell death. With these two targets we may be able to salvage the neurons in the penumbra. Moreover, it could also be helpful to inhibit apoptosis itself. When the neurons are affected but still viable then there is the possibility that they will not go into apoptosis and get more time to recover from ischemic stress.

An example of a compound that targets multiple events is tetramethyl pyrazine nitrone (TBN) [103, 140]. This is a compound that scavenges free radicals, inhibits calcium overload, maintains mitochondrial function, and prevents neuronal damage in primary cortical cultures. TBN reduced infarct volume and ameliorated impairment of behavioral functions in rats. It was also found that TBN down regulated the expression of pro-apoptotic factors and up regulated anti-apoptotic factors in the penumbra of these rats [103, 140]. Compounds like TBN which have multifunctional mechanisms play a role in the neuroprotective effect and make it a promising treatment strategy. Moreover, treatment strategies that are combinations of multiple agents or techniques could also provide improved recovery. For example, normobaric oxygenation combined with ethanol was found to be neuroprotective in rats. It showed a decrease in infarct volume and neurological deficits. This was associated with an indication of efficient preservation of ATP levels, reduction in ROS and NDAPH oxidase activity, decrease of acidosis, reduction of caspase-3 and AIF expression [73].

It is well known that the translational value of stroke research is relatively low. Many clinical trials that tried to treat ischemic injury showed no significant improvement [16], while preclinical trials showed a lot of promising results. Nevertheless, there are a few drugs that showed a positive effect in patients like memantine [52-54], citicoline [57, 63], L-3-n butylphthalide [90-92] and XQ-1H [98]. Furthermore, human genes and rodent genes are different even though we share about 85% of our protein-encoding genes. The morphology of the neurons and glia cells is not exactly the same between humans and rodents [253]. To overcome the difficulty of translation, it is now possible to work with human models, these are induced pluripotent stem cell derived neuronal cultures. Perhaps they can offer use better insight in the efficacy of different treatment strategies. Therefore, we propose to test the efficacy of treatment strategies in human derived neuronal cultures if possible.

The success of clinical trials can also be increased by use of a more carefully patient selection. Patients can be selected more carefully, with smaller cores or better perfusion levels. This is possible due to imaging techniques like CT or MRI provide us with better insight in the core volume, penumbra volume and perfusion levels. Based on these parameters patients selection can be improved. With these techniques, treatment strategies may deserve a second change. More research into the pathophysiological cascade and treatment strategies is needed to increase recovery in patients with ischemic stroke.

Appendix B

1. Effect of hypoxia on neuronal cultures

1.1 Effect of hypoxia and memantine on cell viability

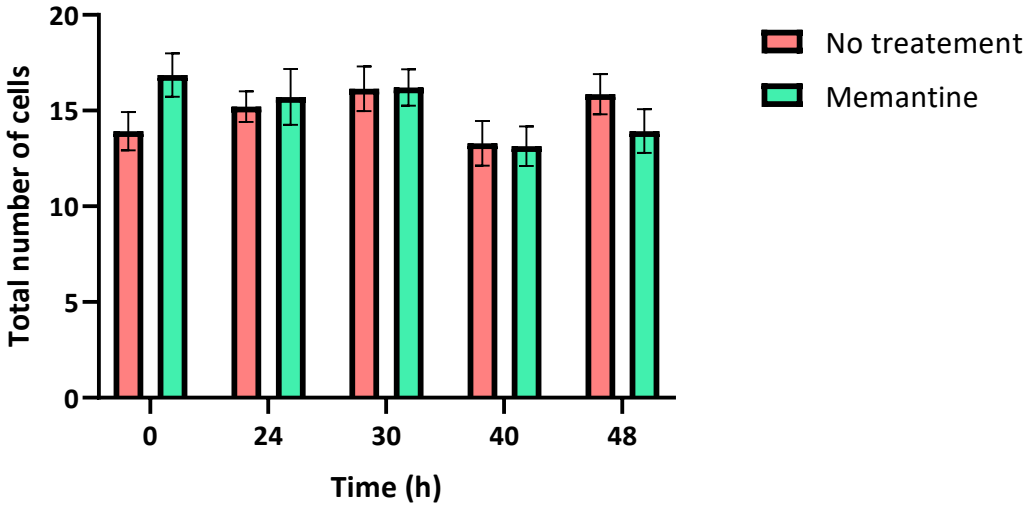
Table S1A. Number of cells relative to live dead assay non-treated cultures and cultures treated with 5µM memantine.

Time (h)	Treatment	Total number of cells (DAPI)	Non-apoptotic cells	Apoptotic cells	Dead cells
0	***Non-treated***	13.93 ± 1.003	9.286 ± 0.7444	1.286 ± 0.45	3.357 ± 0.6167
	Memantine	16.86 ± 1.128	8.929 ± 0.7877	1.875 ± 0.5226	6.071 ± 0.88
24	***Non-treated***	15.21 ± 0.80	6.286± 0.5490	4.357 ± 0.668	4.571 ± 0.6606
	Memantine	15.71 ± 1.458	5.071 ± 0.7947	5.071 ± 1.151	5.571 ± 0.5418
30	***Non-treated***	16.14 ± 1.171	3.714 ± 0.6747	6.143 ± 1.257	6.286 ± 0.624
	Memantine	16.21 ± 0.95	2.429 ± 0.5519	7.929 ± 0.9167	5.875 ± 0.6364
40	***Non-treated***	13.29 ± 1.169	1.429 ± 0.4021	6.214 ± 0.7426	5.643 ± 0.7001
	Memantine	13.14 ± 1.032	0.5714±0.2020	3.143 ± 0.9309	9.429 ± 1.235
48	***Non-treated***	15.86 ± 1.053	2.143 ± 0.645	6.714 ± 1.183	7.000 ± 1.367
	Memantine	13.93 ± 1.146	1 ± 0.3145	5.571 ± 0.6523	7.357 ± 1.175

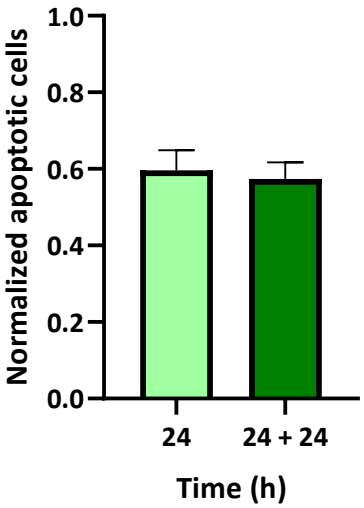
Table S1B. Statistical analysis relative to cell viability in non-treated cultures.

Figure	Parameter	Comparison		p-value
6A	***Non-apoptotic cell count***	24h vs. baseline	***	0,0003
		30h vs. baseline	****	<0,0001
		40h vs. baseline	****	<0,0001
		48h vs. baseline	****	<0,0001
		30h vs. 24h	*	0,0336
		40h vs. 24h	****	<0,0001
		48h vs. 24h	****	<0,0001
6B	***Apoptotic cell count***	30h vs. baseline	*	0,0132
		40h vs. baseline	****	<0,0001
		48h vs. baseline	***	0,0004
6C	***Dead cell count***	48h vs. baseline	*	0.0484

Statistical analyses were performed with two-way ANOVA and post hoc Bonferroni correction.



Supplementary figure 1. Total number of cells (DAPI) counted in non-treated cultures (red, n=14) compared to total number of cells in cultures treated with 5 µM memantine (green, n=14) after 48h of hypoxia. Two-way ANOVA with post hoc Bonferroni correction was performed.



Supplementary figure 2. Bar graph representing the number of apoptotic cells in excitatory cultures (DIV 21) after 24h of hypoxia and after 24h of hypoxia + 24h normoxia. No significant difference was found between recovery and no-recovery in apoptotic cells.

2. Neuronal network activity and synchronicity in treated and non-treated cultures

Table S2. Statistical analysis relative to neuronal activity and synchronicity in non-treated cultures.

Figure	Parameter	Comparison		p-value
8A	***Firing rate***	1h vs. baseline	***	<0.0005
		2h vs. baseline	****	<0.0001
		6h vs. BASELINE	****	*<0,0001*
		12h vs. BASELINE	****	<0,0001
		24h vs. BASELINE	****	<0,0001
		30h vs. BASELINE	****	<0,0001
		48h vs. BASELINE	****	<0,0001
8B	***Network burst rate***	1h vs. BASELINE	****	<0,0001
		6h vs. BASELINE	****	<0,0001
		12h vs. BASELINE	****	<0,0001
		24h vs. BASELINE	****	<0,0001
		30h vs. BASELINE	****	<0,0001
		48h vs. BASELINE	****	<0,0001

Statistical analyses were performed with two-way ANOVA and post hoc Bonferroni correction.

Table S3. Statistical analyses relative to MEA recordings of cultures treated with memantine.

Figure	Parameter	Comparison		p-value
10	***Firing rate***			
	16h	Non-treated vs. 1um	**	0.0100
	18h	Non-treated vs. 1um	*	0.0114
	20h	Non-treated vs. 1um	*	0.0104
	22h	Non-treated vs. 1um	*	0.0172
	24h	Non-treated vs. 1um	*	0.0374
	26h	Non-treated vs. 1um	****	<0.0001
		Non-treated vs. 10um	**	0.0047
	28h	Non-treated vs. 1um	****	<0.0001
		Non-treated vs. 5um	*	0.0436
		Non-treated vs. 10um	***	0.0010
	30h	Non-treated vs. 1um	***	0.0001
		Non-treated vs. 5um	*	0.0256
		Non-treated vs. 10µM	**	0.0014
	32h	Non-treated vs. 1µM	****	<0.0001
		Non-treated vs. 5µM	*	0.0124
		Non-treated vs. 10 µM	***	0.0007
	34h	Non-treated vs. vehicle	**	0.0070
		Non-treated vs. 1µM	****	<0.0001
		Non-treated vs. 5µM	**	0.0035
		Non-treated vs. 10µM	***	0.0002

11	***Network burst rate***			
	4h – 34h	Non-treated vs. vehicle	****	<0.0001
		Non-treated vs. 1µM	****	<0.0001
		Non-treated vs. 5µM	****	<0.0001
		Non-treated vs. 10µM	****	<0.0001
	8h	Vehicle vs. 1µM	**	0.0027
		1µM vs. 5µM	*	0.00418
	12h	Vehicle vs. 10µM	*	0.0130
	18h	Vehicle vs. 5µM	*	0.0303
		Vehicle vs. 10µM	***	0.0010
		1µM vs. 10 µM	*	0.0449
	20h	Vehicle vs. 5µM	**	0.0028
		Vehicle vs. 10µM	****	<0.0001
		1µM vs 5µM	*	0.0228
	22h	Vehicle vs. 5µM	****	<0.0001
		Vehicle vs. 10µM	***	0.0002
	24h	vehicle vs. 5µM	**	0.0059
		vehicle vs. 10µM	****	<0.0001
		1µM vs. 5µM	***	0.0001
		1µM vs. 10µM	****	<0.0001
	26h	1µM vs. 5µM	**	0.0017
		1µM vs. 10µM	*	0.0360
	28h	vehicle vs. 5µM	**	0.0029
		vehicle vs. 10µM	**	0.0017
		1µM vs. 5µM	***	0.0001
		1µM vs. 10µM	****	<0.0001
	30h	vehicle vs. 5µM	***	0.0002
		1µM vs. 5µM	****	<0.0001
		5µM vs. 10µM	**	0.0059
	32h	vehicle vs. 5µM	****	<0.0001
		1µM vs. 5µM	****	<0.0001
		5µM vs. 10µM	***	0.0004
	34h	vehicle vs. 5µM	****	<0.0001
		1µM vs. 5µM	****	<0.0001
		1µM vs. 10µM	**	0.0056
		5µM vs. 10µM	***	0.0004

Statistical analyses were performed with two-way ANOVA and post hoc Bonferroni correction.

Table S3. Statistical analyses relative to MEA recordings of cultures treated with optogenetic stimulation (opto) compared to non-treated cultures (NT).

Figure	Parameter	Comparison		p-value
12	***Firing rate***	Opto vs. NT	****	<0.0001
13	***Network burst rate***	Opto vs. NT	****	<0.0001

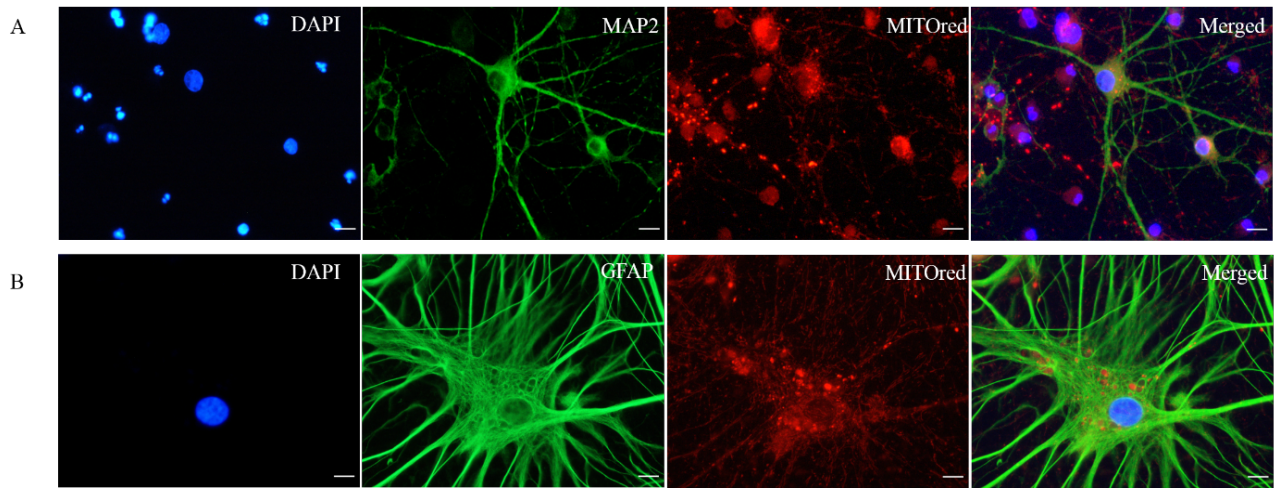
Statistical analyses were performed with two-way ANOVA and post hoc Bonferroni correction

Appendix C

1. Set up of MITOtracker protocol in neuronal cultures.

To assess the number of mitochondria we set up a protocol using Mito tracker red CMXros from Thermofisher. We analysed the number of mitochondria in neurons (MAP2 staining) and in astrocytes (GFAP staining). When we assessed the pictures, we concluded that the mitochondria are quite small, and they are located in a 3D space in the soma and cell body of astrocytes. We were unable to quantify the mitochondria in the soma and cell bodies (supplementary figure 3).

Then we wanted to try to quantify the number of mitochondria that were located on the dendrites. However, the staining was not consistent and showed a lot of noise throughout the image (figure 9). We were unable to quantify the mitochondria on the dendrites.



Supplementary figure 3. Fluorescent images of **A**. a neuron stained for MAP2 (green), mitochondria (red) and DAPI (blue) at DIV 21. **B.** An astrocyte stained for GFAP (green), mitochondria (red) and DAPI (blue) (scale bar 20µm).

References

1. W. Johnson, O.O., M. Owolabi, S. Sachdev. *Stroke: a global response is needed*. [World Health Organization] 2016 [cited 2020 19-02-2020].
2. Bush, C.K., et al., *Endovascular Treatment with Stent-Retriever Devices for Acute Ischemic Stroke: A Meta-Analysis of Randomized Controlled Trials.* PLoS One, 2016. **11**(1): p. e0147287.
3. Alawieh, A., E.F. Langley, and S. Tomlinson, *Targeted complement inhibition salvages stressed neurons and inhibits neuroinflammation after stroke in mice.* Sci Transl Med, 2018. **10**(441).
4. Guan, R., et al., *Mitophagy, a potential therapeutic target for stroke.* J Biomed Sci, 2018. **25**(1): p. 87.
5. Puig, B., S. Brenna, and T. Magnus, *Molecular Communication of a Dying Neuron in Stroke.* Int J Mol Sci, 2018. **19**(9).
6. Dirnagl, U., C. Iadecola, and M.A. Moskowitz, *Pathobiology of ischaemic stroke: an integrated view.* Trends Neurosci, 1999. **22**(9): p. 391-7.
7. Hacke, W., et al., *Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke.* N Engl J Med, 2008. **359**(13): p. 1317-29.
8. Berkhemer, O.A., et al., *A randomized trial of intraarterial treatment for acute ischemic stroke.* N Engl J Med, 2015. **372**(1): p. 11-20.
9. Farbu, E., K.D. Kurz, and M.W. Kurz, *Ischemic stroke--novel therapeutic strategies.* Acta Neurol Scand Suppl, 2011(191): p. 28-37.
10. Fransen, P.S., et al., *MR CLEAN, a multicenter randomized clinical trial of endovascular treatment for acute ischemic stroke in the Netherlands: study protocol for a randomized controlled trial.* Trials, 2014. **15**: p. 343.
11. Liu, X., et al., *The global burden of cerebral small vessel disease related to neurological deficit severity and clinical outcomes of acute ischemic stroke after IV rt-PA treatment.* Neurol Sci, 2019. **40**(6): p. 1157-1166.
12. Hofmeijer, J. and M.J. van Putten, *Ischemic cerebral damage: an appraisal of synaptic failure.* Stroke, 2012. **43**(2): p. 607-15.
13. Astrup, J., B.K. Siesjo, and L. Symon, *Thresholds in cerebral ischemia - the ischemic penumbra.* Stroke, 1981. **12**(6): p. 723-5.
14. Long, D.M., *The ischemic penumbra.* Neurosurgery Quarterly, 2008. **18**(2): p. 151-152.
15. Vitt, J.R., M. Trillanes, and J.C. Hemphill, 3rd, *Management of Blood Pressure During and After Recanalization Therapy for Acute Ischemic Stroke.* Front Neurol, 2019. **10**: p. 138.
16. Ginsberg, M.D., *Neuroprotection for ischemic stroke: past, present and future.* Neuropharmacology, 2008. **55**(3): p. 363-89.
17. Muzzi, L., et al., *Mild stimulation improves neuronal survival in an in vitro model of the ischemic penumbra.* J Neural Eng, 2019. **17**(1): p. 016001.
18. Hawrylycz, M., et al., *Canonical genetic signatures of the adult human brain.* Nat Neurosci, 2015. **18**(12): p. 1832-44.
19. Khaitovich, P., et al., *Regional patterns of gene expression in human and chimpanzee brains.* Genome Res, 2004. **14**(8): p. 1462-73.
20. Hyvarinen, T., et al., *Functional characterization of human pluripotent stem cell-derived cortical networks differentiated on laminin-521 substrate: comparison to rat cortical cultures.* Sci Rep, 2019. **9**(1): p. 17125.
21. Keller, J.M. and M. Frega, *Past, Present, and Future of Neuronal Models In Vitro.* Adv Neurobiol, 2019. **22**: p. 3-17.
22. Tukker, A.M., et al., *Applicability of hiPSC-Derived Neuronal Cocultures and Rodent Primary Cortical Cultures for In Vitro Seizure Liability Assessment.* Toxicol Sci, 2020. **178**(1): p. 71-87.
23. Collaborators, G.B.D.N., *Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016.* Lancet Neurol, 2019. **18**(5): p. 459-480.

24. Bolay, H., et al., *Persistent defect in transmitter release and synapsin phosphorylation in cerebral cortex after transient moderate ischemic injury.* Stroke, 2002. **33**(5): p. 1369-75.
25. Jung, S.H., et al., *Cell proliferation and synaptogenesis in the cerebellum after focal cerebral ischemia.* Brain Res, 2009. **1284**: p. 180-90.
26. Veerbeek, J.M., et al., *What is the evidence for physical therapy poststroke? A systematic review and meta-analysis.* PLoS One, 2014. **9**(2): p. e87987.
27. Sekerdag, E., I. Solaroglu, and Y. Gursoy-Ozdemir, *Cell Death Mechanisms in Stroke and Novel Molecular and Cellular Treatment Options.* Curr Neuropharmacol, 2018. **16**(9): p. 1396-1415.
28. Moskowitz, M.A., E.H. Lo, and C. Iadecola, *The science of stroke: mechanisms in search of treatments.* Neuron, 2010. **67**(2): p. 181-98.
29. Lui, F., et al., *Mitochondria in ischemic stroke: new insight and implications.* Aging and Disease, 2017. **9**(5): p. 924-937.
30. Yang, J.L., S. Mukda, and S.D. Chen, *Diverse roles of mitochondria in ischemic stroke.* Redox Biol, 2018. **16**: p. 263-275.
31. Castilho, R.F., et al., *Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells.* J Neurosci, 1998. **18**(24): p. 10277-86.
32. Stanika, R.I., et al., *Coupling diverse routes of calcium entry to mitochondrial dysfunction and glutamate excitotoxicity.* Proc Natl Acad Sci U S A, 2009. **106**(24): p. 9854-9.
33. Abramov, A.Y. and M.R. Duchen, *Mechanisms underlying the loss of mitochondrial membrane potential in glutamate excitotoxicity.* Biochim Biophys Acta, 2008. **1777**(7-8): p. 953-64.
34. Twig, G., et al., *Fission and selective fusion govern mitochondrial segregation and elimination by autophagy.* EMBO J, 2008. **27**(2): p. 433-46.
35. Kim, I., S. Rodriguez-Enriquez, and J.J. Lemasters, *Selective degradation of mitochondria by mitophagy.* Arch Biochem Biophys, 2007. **462**(2): p. 245-53.
36. Nikoletopoulou, V., et al., *Crosstalk between apoptosis, necrosis and autophagy.* Biochim Biophys Acta, 2013. **1833**(12): p. 3448-3459.
37. Igney, F.H. and P.H. Krammer, *Death and anti-death: tumour resistance to apoptosis.* Nat Rev Cancer, 2002. **2**(4): p. 277-88.
38. Green, D.R., *Apoptotic pathways: ten minutes to dead.* Cell, 2005. **121**(5): p. 671-4.
39. Shi, Z.F., et al., *Methylene blue ameliorates brain edema in rats with experimental ischemic stroke via inhibiting aquaporin 4 expression.* Acta Pharmacol Sin, 2020.
40. Shen, Q., et al., *Neuroprotective efficacy of methylene blue in ischemic stroke: an MRI study.* PLoS One, 2013. **8**(11): p. e79833.
41. Jiang, Z., et al., *The Effects of Methylene Blue on Autophagy and Apoptosis in MRI-Defined Normal Tissue, Ischemic Penumbra and Ischemic Core.* PLoS One, 2015. **10**(6): p. e0131929.
42. Huang, L., et al., *Chronic oral methylene blue treatment in a rat model of focal cerebral ischemia/reperfusion.* Brain Res, 2018. **1678**: p. 322-329.
43. Fang, Q., et al., *Methylene Blue Ameliorates Ischemia/Reperfusion-Induced Cerebral Edema: An MRI and Transmission Electron Microscope Study.* Acta Neurochir Suppl, 2016. **121**: p. 227-36.
44. Tucker, D., Y. Lu, and Q. Zhang, *From Mitochondrial Function to Neuroprotection-an Emerging Role for Methylene Blue.* Mol Neurobiol, 2018. **55**(6): p. 5137-5153.
45. Chen, H.S., et al., *Neuroprotective concentrations of the N-methyl-D-aspartate open-channel blocker memantine are effective without cytoplasmic vacuolation following post-ischemic administration and do not block maze learning or long-term potentiation.* Neuroscience, 1998. **86**(4): p. 1121-1132.
46. Gorgulu, A., et al., *Reduction of edema and infarction by Memantine and MK-801 after focal cerebral ischaemia and reperfusion in rat.* Acta Neurochir (Wien), 2000. **142**(11): p. 1287-92.
47. Babu, C.S. and M. Ramanathan, *Pre-ischemic treatment with memantine reversed the neurochemical and behavioural parameters but not energy metabolites in middle cerebral artery occluded rats.* Pharmacol Biochem Behav, 2009. **92**(3): p. 424-32.

48. Montagne, A., et al., *Memantine improves safety of thrombolysis for stroke.* Stroke, 2012. **43**(10): p. 2774-81.
49. Shih, A.Y., et al., *The smallest stroke: occlusion of one penetrating vessel leads to infarction and a cognitive deficit.* Nat Neurosci, 2013. **16**(1): p. 55-63.
50. Lopez-Valdes, H.E., et al., *Memantine enhances recovery from stroke.* Stroke, 2014. **45**(7): p. 2093-2100.
51. Trotman, M., et al., *The dichotomy of memantine treatment for ischemic stroke: dose-dependent protective and detrimental effects.* J Cereb Blood Flow Metab, 2015. **35**(2): p. 230-9.
52. Bakhshayesh-Eghbali, B., et al., *Comparing the effect of memantine and placebo on clinical outcome of intracranial hemorrage: a randomized double blind clinical trial.* Caspian Journal of Neurological Sciences, 2015. **1**(3): p. 11-18.
53. Berthier, M.L., et al., *Memantine and constraint-induced aphasia therapy in chronic poststroke aphasia.* Ann Neurol, 2009. **65**(5): p. 577-85.
54. Kafi, H., et al., *Study of the neuroprotective effects of memantine in patients with mild to moderate ischemic stroke.* Iran J Pharm Res, 2014. **13**(2): p. 591-8.
55. Yildirim, T., et al., *Poloxamer-188 and citicoline provide neuronal membrane integrity and protect membrane stability in cortical spreading depression.* Int J Neurosci, 2015. **125**(12): p. 941-6.
56. Curry, D.J., et al., *Surfactant poloxamer 188-related decreases in inflammation and tissue damage after experimental brain injury in rats.* J Neurosurg, 2004. **101**(1 Suppl): p. 91-6.
57. Clark, W.M., et al., *A phase III randomized efficacy trial of 2000 mg citicoline in acute ischemic stroke patients.* Neurology, 2001. **57**(9): p. 1595-602.
58. D'Orlando, K.J. and B.W. Sandage, Jr., *Citicoline (CDP-choline): mechanisms of action and effects in ischemic brain injury.* Neurol Res, 1995. **17**(4): p. 281-4.
59. Rao, A.M., J.F. Hatcher, and R.J. Dempsey, *Lipid alterations in transient forebrain ischemia: possible new mechanisms of CDP-choline neuroprotection.* J Neurochem, 2000. **75**(6): p. 2528-35.
60. Adibhatla, R.M. and J.F. Hatcher, *Citicoline mechanisms and clinical efficacy in cerebral ischemia.* J Neurosci Res, 2002. **70**(2): p. 133-9.
61. Marrannes, R., et al., *Evidence for a role of the N-methyl-D-aspartate (NMDA) receptor in cortical spreading depression in the rat.* Brain Res, 1988. **457**(2): p. 226-40.
62. Amemori, T. and J. Bures, *Ketamine blockade of spreading depression: rapid development of tolerance.* Brain Res, 1990. **519**(1-2): p. 351-4.
63. Mitta, M., et al., *Edaravone - citicoline comparative study in acute ischemic stroke (ECCS-AIS).* J Assoc Physicians India, 2012. **60**: p. 36-8.
64. Zheng, M., et al., *Ginsenosides: A Potential Neuroprotective Agent.* Biomed Res Int, 2018. **2018**: p. 8174345.
65. Zhang, T., et al., *Pseudoginsenoside-F11 Protects against Transient Cerebral Ischemia Injury in Rats Involving Repressing Calcium Overload.* Neuroscience, 2019. **411**: p. 86-104.
66. Yuan, L., et al., *Pseudoginsenoside-F11 improves long-term neurological function and promotes neurogenesis after transient cerebral ischemia in mice.* Neurochem Int, 2020. **133**: p. 104586.
67. Hou, Y., et al., *Pseudoginsenoside-F11 ameliorates ischemic neuron injury by regulating the polarization of neutrophils and macrophages in vitro.* Int Immunopharmacol, 2020. **85**: p. 106564.
68. Crews, F.T., et al., *Ethanol, stroke, brain damage, and excitotoxicity.* Pharmacol Biochem Behav, 1998. **59**(4): p. 981-91.
69. Wang, Q., et al., *Ethanol preconditioning protects against ischemia/reperfusion-induced brain damage: role of NADPH oxidase-derived ROS.* Free Radic Biol Med, 2007. **43**(7): p. 1048-60.
70. Fu, P., et al., *Acute administration of ethanol reduces apoptosis following ischemic stroke in rats.* Neurosci Res, 2013. **76**(1-2): p. 93-7.

71. Hafeez, A., et al., *Reduced Apoptosis by Ethanol and Its Association with PKC-delta and Akt Signaling in Ischemic Stroke.* Aging Dis, 2014. **5**(6): p. 366-72.
72. Zhou, R.P., et al., *Acute Ethanol Exposure Promotes Autophagy-Lysosome Pathway-Dependent ASIC1a Protein Degradation and Protects Against Acidosis-Induced Neurotoxicity.* Mol Neurobiol, 2019. **56**(5): p. 3326-3340.
73. Geng, X., et al., *Synergetic neuroprotection of normobaric oxygenation and ethanol in ischemic stroke through improved oxidative mechanism.* Stroke, 2013. **44**(5): p. 1418-25.
74. Andrabi, S.S., et al., *Pramipexole prevents ischemic cell death via mitochondrial pathways in ischemic stroke.* Dis Model Mech, 2019. **12**(8).
75. Sheng, R., et al., *Autophagy activation is associated with neuroprotection in a rat model of focal cerebral ischemic preconditioning.* Autophagy, 2010. **6**(4): p. 482-94.
76. Baek, S.H., et al., *Modulation of mitochondrial function and autophagy mediates carnosine neuroprotection against ischemic brain damage.* Stroke, 2014. **45**(8): p. 2438-2443.
77. Li, Q., et al., *Rapamycin attenuates mitochondrial dysfunction via activation of mitophagy in experimental ischemic stroke.* Biochem Biophys Res Commun, 2014. **444**(2): p. 182-8.
78. Mondal, N.K., et al., *Tetrahydrocurcumin epigenetically mitigates mitochondrial dysfunction in brain vasculature during ischemic stroke.* Neurochem Int, 2019. **122**: p. 120-138.
79. Chauhan, A., et al., *Rapamycin protects against middle cerebral artery occlusion induced focal cerebral ischemia in rats.* Behav Brain Res, 2011. **225**(2): p. 603-9.
80. Buckley, K.M., et al., *Rapamycin up-regulation of autophagy reduces infarct size and improves outcomes in both permanent MCAL, and embolic MCAO, murine models of stroke.* Exp Transl Stroke Med, 2014. **6**: p. 8.
81. Liu, X., et al., *Astrocyte Autophagy Flux Protects Neurons Against Oxygen-Glucose Deprivation and Ischemic/Reperfusion Injury.* Rejuvenation Res, 2018. **21**(5): p. 405-415.
82. Chen, Y., et al., *Tat-NR2B9c prevents excitotoxic neuronal superoxide production.* J Cereb Blood Flow Metab, 2015. **35**(5): p. 739-42.
83. Milani, D., et al., *Neuroprotective efficacy of poly-arginine R18 and NA-1 (TAT-NR2B9c) peptides following transient middle cerebral artery occlusion in the rat.* Neurosci Res, 2017. **114**: p. 9-15.
84. Lv, W., et al., *Bioengineered Boronic Ester Modified Dextran Polymer Nanoparticles as Reactive Oxygen Species Responsive Nanocarrier for Ischemic Stroke Treatment.* ACS Nano, 2018. **12**(6): p. 5417-5426.
85. Li, R., et al., *Targeted delivery of intranasally administered nanoparticles-mediated neuroprotective peptide NR2B9c to brain and neuron for treatment of ischemic stroke.* Nanomedicine, 2019. **18**: p. 380-390.
86. Yang, L.C., et al., *L-3-n-butylphthalide Promotes Neurogenesis and Neuroplasticity in Cerebral Ischemic Rats.* CNS Neurosci Ther, 2015. **21**(9): p. 733-41.
87. Sun, Y., et al., *dl-3-n-butylphthalide promotes neuroplasticity and motor recovery in stroke rats.* Behav Brain Res, 2017. **329**: p. 67-74.
88. Zhao, W., et al., *3-N-butylphthalide improves neuronal morphology after chronic cerebral ischemia.* Neural Regen Res, 2014. **9**(7): p. 719-26.
89. Zhang, P., et al., *N-Butylphthalide (NBP) ameliorated cerebral ischemia reperfusion-induced brain injury via HGF-regulated TLR4/NF-kappaB signaling pathway.* Biomed Pharmacother, 2016. **83**: p. 658-666.
90. Cui, L.Y., et al., *Ninety-day administration of dl-3-n-butylphthalide for acute ischemic stroke: a randomized, double-blind trial.* Chin Med J (Engl), 2013. **126**(18): p. 3405-10.
91. Wang, S., et al., *Dl-3-n-Butylphthalide (NBP): A Promising Therapeutic Agent for Ischemic Stroke.* CNS Neurol Disord Drug Targets, 2018. **17**(5): p. 338-347.
92. Xue, L.X., et al., *Efficacy and safety comparison of DL-3-n-butylphthalide and Cerebrolysin: Effects on neurological and behavioral outcomes in acute ischemic stroke.* Exp Ther Med, 2016. **11**(5): p. 2015-2020.

93. Zhang, Y.Y., et al., *Ligustroflavone reduces necroptosis in rat brain after ischemic stroke through targeting RIPK1/RIPK3/MLKL pathway.* Naunyn Schmiedebergs Arch Pharmacol, 2019. **392**(9): p. 1085-1095.
94. Xu, D., et al., *XQ-1H alleviates cerebral ischemia in mice through inhibition of apoptosis and promotion of neurogenesis in a Wnt/beta-catenin signaling dependent way.* Life Sci, 2019. **235**: p. 116844.
95. Deng, Y., et al., *Blood-brain barrier breakdown by PAF and protection by XQ-1H due to antagonism of PAF effects.* Eur J Pharmacol, 2009. **616**(1-3): p. 43-7.
96. Wei, J., et al., *XQ-1H Suppresses Neutrophils Infiltration and Oxidative Stress Induced by Cerebral Ischemia Injury Both In Vivo and In Vitro.* Neurochem Res, 2013.
97. Fang, W., et al., *Attenuated Blood-Brain Barrier Dysfunction by XQ-1H Following Ischemic Stroke in Hyperlipidemic Rats.* Mol Neurobiol, 2015. **52**(1): p. 162-75.
98. Dong, Y., H. Li, and Q. Dong, *The effect of intravenous ginkgolide on clinical improvement of patients with acute ischemic stroke.* Neurol Res, 2020. **42**(3): p. 260-266.
99. Narayanan, S.V., et al., *Resveratrol Preconditioning Protects Against Cerebral Ischemic Injury via Nuclear Erythroid 2-Related Factor 2.* Stroke, 2015. **46**(6): p. 1626-32.
100. Peng, C., et al., *Mitofusin 2 ameliorates hypoxia-induced apoptosis via mitochondrial function and signaling pathways.* Int J Biochem Cell Biol, 2015. **69**: p. 29-40.
101. Sekiguchi, A., et al., *Rapamycin promotes autophagy and reduces neural tissue damage and locomotor impairment after spinal cord injury in mice.* J Neurotrauma, 2012. **29**(5): p. 946-56.
102. Li, X., et al., *Ginaton improves neurological function in ischemic stroke rats via inducing autophagy and maintaining mitochondrial homeostasis.* Neuropsychiatr Dis Treat, 2019. **15**: p. 1813-1822.
103. Zhang, G., et al., *Neuroprotective Effect and Mechanism of Action of Tetramethylpyrazine Nitrone for Ischemic Stroke Therapy.* Neuromolecular Med, 2018. **20**(1): p. 97-111.
104. Dai, Y., et al., *Isoquercetin attenuates oxidative stress and neuronal apoptosis after ischemia/reperfusion injury via Nrf2-mediated inhibition of the NOX4/ROS/NF-kappaB pathway.* Chem Biol Interact, 2018. **284**: p. 32-40.
105. Cheon, S.Y., et al., *Regulation of Microglia and Macrophage Polarization via Apoptosis Signal-Regulating Kinase 1 Silencing after Ischemic/Hypoxic Injury.* Front Mol Neurosci, 2017. **10**: p. 261.
106. Li, F., et al., *L-3-n-Butylphthalide reduces ischemic stroke injury and increases M2 microglial polarization.* Metab Brain Dis, 2018. **33**(6): p. 1995-2003.
107. Li, F., et al., *Xuesaitong May Protect Against Ischemic Stroke by Modulating Microglial Phenotypes and Inhibiting Neuronal Cell Apoptosis via the STAT3 Signaling Pathway.* CNS Neurol Disord Drug Targets, 2019. **18**(2): p. 115-123.
108. Deng, X.X., S.S. Li, and F.Y. Sun, *Necrostatin-1 Prevents Necroptosis in Brains after Ischemic Stroke via Inhibition of RIPK1-Mediated RIPK3/MLKL Signaling.* Aging Dis, 2019. **10**(4): p. 807-817.
109. Li, X., et al., *Progranulin protects against cerebral ischemia-reperfusion (I/R) injury by inhibiting necroptosis and oxidative stress.* Biochem Biophys Res Commun, 2020. **521**(3): p. 569-576.
110. Yang, M., et al., *Neuroprotective Effect of beta-Caryophyllene on Cerebral Ischemia-Reperfusion Injury via Regulation of Necroptotic Neuronal Death and Inflammation: In Vivo and in Vitro.* Front Neurosci, 2017. **11**: p. 583.
111. Ge, X.L., et al., *Inhibition of miR-19a protects neurons against ischemic stroke through modulating glucose metabolism and neuronal apoptosis.* Cell Mol Biol Lett, 2019. **24**: p. 37.
112. Qiu, C.W., et al., *Post-stroke gastrodin treatment ameliorates ischemic injury and increases neurogenesis and restores the Wnt/beta-Catenin signaling in focal cerebral ischemia in mice.* Brain Res, 2019. **1712**: p. 7-15.
113. Wei, N., et al., *MicroRNA-9 Mediates the Cell Apoptosis by Targeting Bcl2l11 in Ischemic Stroke.* Mol Neurobiol, 2016. **53**(10): p. 6809-6817.

114. Yan, L. and T. Zhu, *Effects of rosuvastatin on neuronal apoptosis in cerebral ischemic stroke rats via Sirt1/NF-kappa B signaling pathway.* Eur Rev Med Pharmacol Sci, 2019. **23**(12): p. 5449-5455.
115. van der Worp, H.B., et al., *Therapeutic hypothermia for acute ischemic stroke: ready to start large randomized trials?* J Cereb Blood Flow Metab, 2010. **30**(6): p. 1079-93.
116. Lyden, P.D., et al., *Therapeutic hypothermia for acute stroke.* Int J Stroke, 2006. **1**(1): p. 9-19.
117. Wu, T.C. and J.C. Grotta, *Hypothermia for acute ischaemic stroke.* Lancet Neurol, 2013. **12**(3): p. 275-84.
118. Baron, J.C., *Protecting the ischaemic penumbra as an adjunct to thrombectomy for acute stroke.* Nat Rev Neurol, 2018. **14**(6): p. 325-337.
119. van der Worp, H.B., et al., *Hypothermia in animal models of acute ischaemic stroke: a systematic review and meta-analysis.* Brain, 2007. **130**(Pt 12): p. 3063-74.
120. Ridenour, T.R., et al., *Mild hypothermia reduces infarct size resulting from temporary but not permanent focal ischemia in rats.* Stroke, 1992. **23**(5): p. 733-8.
121. Piironen, K., et al., *Mild hypothermia after intravenous thrombolysis in patients with acute stroke: a randomized controlled trial.* Stroke, 2014. **45**(2): p. 486-91.
122. Lyden, P., et al., *Results of the ICTuS 2 Trial (Intravascular Cooling in the Treatment of Stroke 2).* Stroke, 2016. **47**(12): p. 2888-2895.
123. Parsons, C.G., et al., *Patch clamp studies on the kinetics and selectivity of N-methyl-D-aspartate receptor antagonism by memantine (1-amino-3,5-dimethyladamantan).* Neuropharmacology, 1993. **32**(12): p. 1337-50.
124. Chilukuri, S. and N. Burela, *Memantine for Prevention of Brain Irradiation-Induced Cognitive Toxicity: A Tale of an Underappreciated and Underused Intervention.* JCO Glob Oncol, 2020. **6**: p. 1384-1388.
125. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. **126**(4): p. 663-76.
126. Frega, M., et al., *Rapid Neuronal Differentiation of Induced Pluripotent Stem Cells for Measuring Network Activity on Micro-electrode Arrays.* J Vis Exp, 2017(119).
127. Ferguson, B.R. and W.J. Gao, *PV Interneurons: Critical Regulators of E/I Balance for Prefrontal Cortex-Dependent Behavior and Psychiatric Disorders.* Front Neural Circuits, 2018. **12**: p. 37.
128. Sultan, K.T. and S.H. Shi, *Generation of diverse cortical inhibitory interneurons.* Wiley Interdiscip Rev Dev Biol, 2018. **7**(2).
129. Schindelin, J., et al., *Fiji: an open-source platform for biological-image analysis.* Nat Methods, 2012. **9**(7): p. 676-82.
130. Frega, M., et al., *Neuronal network dysfunction in a model for Kleefstra syndrome mediated by enhanced NMDAR signaling.* Nat Commun, 2019. **10**(1): p. 4928.
131. Andreadis, C., et al., *Long-term event-free survivors after high-dose therapy and autologous stem-cell transplantation for low-grade follicular lymphoma.* Bone Marrow Transplant, 2005. **36**(11): p. 955-61.
132. Zhu, P.J. and K. Krnjevic, *Anoxia selectively depresses excitatory synaptic transmission in hippocampal slices.* Neurosci Lett, 1994. **166**(1): p. 27-30.
133. Khazipov, R., P. Congar, and Y. Ben-Ari, *Hippocampal CA1 lacunosum-moleculare interneurons: comparison of effects of anoxia on excitatory and inhibitory postsynaptic currents.* J Neurophysiol, 1995. **74**(5): p. 2138-49.
134. Tang, H.L., et al., *Cell survival, DNA damage, and oncogenic transformation after a transient and reversible apoptotic response.* Mol Biol Cell, 2012. **23**(12): p. 2240-52.
135. Tang, H.L., et al., *Reversibility of apoptosis in cancer cells.* Br J Cancer, 2009. **100**(1): p. 118-22.
136. Mao, Z., et al., *Neuronal activity-dependent cell survival mediated by transcription factor MEF2.* Science, 1999. **286**(5440): p. 785-90.
137. Heck, N., et al., *Activity-dependent regulation of neuronal apoptosis in neonatal mouse cerebral cortex.* Cereb Cortex, 2008. **18**(6): p. 1335-49.

138. Dribben, W.H., L.N. Eisenman, and S. Mennerick, *Magnesium induces neuronal apoptosis by suppressing excitability.* Cell Death Dis, 2010. **1**: p. e63.
139. Klein Gunnewiek, T.M., et al., *m.3243A > G-Induced Mitochondrial Dysfunction Impairs Human Neuronal Development and Reduces Neuronal Network Activity and Synchronicity.* Cell Rep, 2020. **31**(3): p. 107538.
140. Wang, Z., et al., *Therapeutic Potential of Novel Twin Compounds Containing Tetramethylpyrazine and Carnitine Substructures in Experimental Ischemic Stroke.* Oxid Med Cell Longev, 2017. **2017**: p. 7191856.
141. Lipton, S.A., *Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults.* NeuroRx, 2004. **1**(1): p. 101-10.
142. Antonic, A., et al., *Hypothermia protects human neurons.* Int J Stroke, 2014. **9**(5): p. 544-52.
143. Barros, L.F. and B. Weber, *CrossTalk proposal: an important astrocyte-to-neuron lactate shuttle couples neuronal activity to glucose utilisation in the brain.* J Physiol, 2018. **596**(3): p. 347-350.
144. Pellerin, L. and P.J. Magistretti, *Food for thought: challenging the dogmas.* J Cereb Blood Flow Metab, 2003. **23**(11): p. 1282-6.
145. Berthet, C., et al., *Neuroprotective role of lactate after cerebral ischemia.* J Cereb Blood Flow Metab, 2009. **29**(11): p. 1780-9.
146. Lerchundi, R., et al., *NH4(+) triggers the release of astrocytic lactate via mitochondrial pyruvate shunting.* Proc Natl Acad Sci U S A, 2015. **112**(35): p. 11090-5.
147. Johnson, W., et al. *Stroke: a global response is needed*. [World Health Organization] 2016 [cited 2020 19-02-2020].
148. Lapchak, P.A. and J.H. Zhang, *The High Cost of Stroke and Stroke Cytoprotection Research.* Transl Stroke Res, 2017. **8**(4): p. 307-317.
149. Yoo, A.J., B. Pulli, and R.G. Gonzalez, *Imaging-based treatment selection for intravenous and intra-arterial stroke therapies: a comprehensive review.* Expert Rev Cardiovasc Ther, 2011. **9**(7): p. 857-76.
150. Raichle, M.E. and D.A. Gusnard, *Appraising the brain's energy budget.* Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10237-9.
151. Magistretti, P.J. and I. Allaman, *A cellular perspective on brain energy metabolism and functional imaging.* Neuron, 2015. **86**(4): p. 883-901.
152. del Zoppo, G.J., et al., *Heterogeneity in the penumbra.* J Cereb Blood Flow Metab, 2011. **31**(9): p. 1836-51.
153. Lansberg, M.G., E. Bluhmki, and V.N. Thijs, *Efficacy and safety of tissue plasminogen activator 3 to 4.5 hours after acute ischemic stroke: a meta-analysis.* Stroke, 2009. **40**: p. 2438-2441.
154. Galkin, A., *Brain Ischemia/Reperfusion Injury and Mitochondrial Complex I Damage.* Biochemistry (Mosc), 2019. **84**(11): p. 1411-1423.
155. le Feber, J., et al., *Progression of Neuronal Damage in an In Vitro Model of the Ischemic Penumbra.* PLoS One, 2016. **11**(2): p. e0147231.
156. Hofmeijer, J., et al., *Why are sensory axons more vulnerable for ischemia than motor axons?* PLoS One, 2013. **8**(6): p. e67113.
157. Tjepkema-Cloostermans, M.C., et al., *Generalized periodic discharges after acute cerebral ischemia: reflection of selective synaptic failure?* Clin Neurophysiol, 2014. **125**(2): p. 255-62.
158. Roger, V.L., et al., *Heart disease and stroke statistics--2011 update: a report from the American Heart Association.* Circulation, 2011. **123**(4): p. e18-e209.
159. Song, D., et al., *Value of utilizing both ASPECTS and CT angiography collateral score for outcome prediction in acute ischemic stroke.* Int J Stroke, 2015. **10**(7): p. 1018-23.
160. Saver, J.L., et al., *Time to Treatment With Endovascular Thrombectomy and Outcomes From Ischemic Stroke: A Meta-analysis.* JAMA, 2016. **316**(12): p. 1279-88.
161. Kalogeris, T., et al., *Ischemia/Reperfusion.* Compr Physiol, 2016. **7**(1): p. 113-170.

162. Taoufik, E. and L. Probert, *Ischemic neuronal damage.* Curr Pharm Des, 2008. **14**(33): p. 3565-73.
163. Abramov, A.Y., A. Scorziello, and M.R. Duchen, *Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation.* J Neurosci, 2007. **27**(5): p. 1129-38.
164. Lowry, O.H., et al., *Effect of Ischemia on Known Substrates and Cofactors of the Glycolytic Pathway in Brain.* J Biol Chem, 1964. **239**: p. 18-30.
165. Hofmeijer, J., et al., *Detecting Cortical Spreading Depolarization with Full Band Scalp Electroencephalography: An Illusion?* Front Neurol, 2018. **9**: p. 17.
166. Shu, L., et al., *Brain ischemic insult induces cofilin rod formation leading to synaptic dysfunction in neurons.* J Cereb Blood Flow Metab, 2019. **39**(11): p. 2181-2195.
167. Caplan, L., *Caplan's Stroke: A Clinical Aproach*. 4 ed. 2009: Saunders. 688.
168. Kager, H., W.J. Wadman, and G.G. Somjen, *Conditions for the triggering of spreading depression studied with computer simulations.* J Neurophysiol, 2002. **88**(5): p. 2700-12.
169. Katsura, K., T. Kristian, and B.K. Siesjo, *Energy metabolism, ion homeostasis, and cell damage in the brain.* Biochem Soc Trans, 1994. **22**(4): p. 991-6.
170. Hu, H.J. and M. Song, *Disrupted Ionic Homeostasis in Ischemic Stroke and New Therapeutic Targets.* J Stroke Cerebrovasc Dis, 2017. **26**(12): p. 2706-2719.
171. Dohmen, C., et al., *Spreading depolarizations occur in human ischemic stroke with high incidence.* Ann Neurol, 2008. **63**(6): p. 720-8.
172. Leao, A.A., *Further observations on the spreading depression of activity in the cerebral cortex.* J Neurophysiol, 1947. **10**(6): p. 409-14.
173. Woitzik, J., et al., *Propagation of cortical spreading depolarization in the human cortex after malignant stroke.* Neurology, 2013. **80**(12): p. 1095-102.
174. Nakamura, H., et al., *Spreading depolarizations cycle around and enlarge focal ischaemic brain lesions.* Brain, 2010. **133**(Pt 7): p. 1994-2006.
175. Back, T., et al., *Induction of spreading depression in the ischemic hemisphere following experimental middle cerebral artery occlusion: effect on infarct morphology.* J Cereb Blood Flow Metab, 1996. **16**(2): p. 202-13.
176. Takano, K., et al., *The role of spreading depression in focal ischemia evaluated by diffusion mapping.* Ann Neurol, 1996. **39**(3): p. 308-18.
177. Annunziato, L., G. Pignataro, and G.F. Di Renzo, *Pharmacology of brain Na+/Ca2+ exchanger: from molecular biology to therapeutic perspectives.* Pharmacol Rev, 2004. **56**(4): p. 633-54.
178. Mehta, S.L., N. Manhas, and R. Raghubir, *Molecular targets in cerebral ischemia for developing novel therapeutics.* Brain Res Rev, 2007. **54**(1): p. 34-66.
179. Kwak, S. and J.H. Weiss, *Calcium-permeable AMPA channels in neurodegenerative disease and ischemia.* Curr Opin Neurobiol, 2006. **16**(3): p. 281-7.
180. Nicholls, D.G., *Mitochondrial calcium function and dysfunction in the central nervous system.* Biochim Biophys Acta, 2009. **1787**(11): p. 1416-24.
181. Garcia, J.H., et al., *Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat.* Am J Pathol, 1993. **142**(2): p. 623-35.
182. Song, M. and S.P. Yu, *Ionic regulation of cell volume changes and cell death after ischemic stroke.* Transl Stroke Res, 2014. **5**(1): p. 17-27.
183. Yu, S.P. and D.W. Choi, *Na(+)-Ca2+ exchange currents in cortical neurons: concomitant forward and reverse operation and effect of glutamate.* Eur J Neurosci, 1997. **9**(6): p. 1273-81.
184. Jeffs, G.J., et al., *NCX3 knockout mice exhibit increased hippocampal CA1 and CA2 neuronal damage compared to wild-type mice following global cerebral ischemia.* Exp Neurol, 2008. **210**(1): p. 268-73.
185. Cuomo, O., et al., *A critical role for the potassium-dependent sodium-calcium exchanger NCKX2 in protection against focal ischemic brain damage.* J Neurosci, 2008. **28**(9): p. 2053-63.

186. Abramowitz, J. and L. Birnbaumer, *Physiology and pathophysiology of canonical transient receptor potential channels.* FASEB J, 2009. **23**(2): p. 297-328.
187. Aarts, M., et al., *A key role for TRPM7 channels in anoxic neuronal death.* Cell, 2003. **115**(7): p. 863-77.
188. Young, W., *Role of calcium in central nervous system injuries.* J Neurotrauma, 1992. **9 Suppl 1**: p. S9-25.
189. Banik, N.L., E.L. Hogan, and C.Y. Hsu, *Molecular and anatomical correlates of spinal cord injury.* Cent Nerv Syst Trauma, 1985. **2**(2): p. 99-107.
190. Thompson, B.J. and P.T. Ronaldson, *Drug delivery to the ischemic brain.* Adv Pharmacol, 2014. **71**: p. 165-202.
191. Fellman, V. and K.O. Raivio, *Reperfusion injury as the mechanism of brain damage after perinatal asphyxia.* Pediatr Res, 1997. **41**(5): p. 599-606.
192. Rehncrona, S., *Brain acidosis.* Ann Emerg Med, 1985. **14**(8): p. 770-6.
193. Rehncrona, S. and E. Kagström, *Tissue lactic acidosis and ischemic brain damage.* The American Journal of Emergency Medicine, 1983. **1**(2): p. 168-174.
194. Robbins, N.M. and R.A. Swanson, *Opposing effects of glucose on stroke and reperfusion injury: acidosis, oxidative stress, and energy metabolism.* Stroke, 2014. **45**(6): p. 1881-6.
195. Siemkowicz, E. and A.J. Hansen, *Brain extracellular ion composition and EEG activity following 10 minutes ischemia in normo- and hyperglycemic rats.* Stroke, 1981. **12**(2): p. 236-40.
196. Ying, W., et al., *Acidosis potentiates oxidative neuronal death by multiple mechanisms.* J Neurochem, 1999. **73**(4): p. 1549-56.
197. Xiong, Z.G., et al., *Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels.* Cell, 2004. **118**(6): p. 687-98.
198. Lam, T.I., et al., *Intracellular pH reduction prevents excitotoxic and ischemic neuronal death by inhibiting NADPH oxidase.* Proc Natl Acad Sci U S A, 2013. **110**(46): p. E4362-8.
199. Grunder, S. and M. Pusch, *Biophysical properties of acid-sensing ion channels (ASICs).* Neuropharmacology, 2015. **94**: p. 9-18.
200. Marchi, S., et al., *Mitochondria-ros crosstalk in the control of cell death and aging.* J Signal Transduct, 2012. **2012**: p. 329635.
201. Wang, J.F., et al., *Puerarin protects rat brain against ischemia/reperfusion injury by suppressing autophagy via the AMPK-mTOR-ULK1 signaling pathway.* Neural Regen Res, 2018. **13**(6): p. 989-998.
202. Pamenter, M.E., et al., *Autophagy and apoptosis are differentially induced in neurons and astrocytes treated with an in vitro mimic of the ischemic penumbra.* PLoS One, 2012. **7**(12): p. e51469.
203. Carloni, S., et al., *Activation of autophagy and Akt/CREB signaling play an equivalent role in the neuroprotective effect of rapamycin in neonatal hypoxia-ischemia.* Autophagy, 2010. **6**(3): p. 366-77.
204. Gabryel, B., et al., *AMP-activated protein kinase is involved in induction of protective autophagy in astrocytes exposed to oxygen-glucose deprivation.* Cell Biol Int, 2017. **41**(8): p. 928-931.
205. Jiang, T., et al., *Acute metformin preconditioning confers neuroprotection against focal cerebral ischaemia by pre-activation of AMPK-dependent autophagy.* Br J Pharmacol, 2014. **171**(13): p. 3146-57.
206. Siesjo, B.K., et al., *Glutamate, calcium, and free radicals as mediators of ischemic brain damage.* Ann Thorac Surg, 1995. **59**(5): p. 1316-20.
207. Gadoth, N. and H.H. Göbel, *Oxidative stress in ischemic and hemorrhagic stroke*, in *Oxidative stress and free radical damage in neurology*, N. Gadoth and H.H. Göbel, Editors. 2011, Humana Press: New York. p. 139-141.
208. Drose, S. and U. Brandt, *Molecular mechanisms of superoxide production by the mitochondrial respiratory chain.* Adv Exp Med Biol, 2012. **748**: p. 145-69.

209. Mookerjee, S.A., et al., *Mitochondrial uncoupling and lifespan.* Mech Ageing Dev, 2010. **131**(7-8): p. 463-72.
210. del Zoppo, G.J. and J.M. Hallenbeck, *Advances in the vascular pathophysiology of ischemic stroke.* Thromb Res, 2000. **98**(3): p. 73-81.
211. Pacher, P., J.S. Beckman, and L. Liaudet, *Nitric oxide and peroxynitrite in health and disease.* Physiol Rev, 2007. **87**(1): p. 315-424.
212. Aarts, M.M. and M. Tymianski, *TRPMs and neuronal cell death.* Pflugers Arch, 2005. **451**(1): p. 243-9.
213. Gu, Z., et al., *S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death.* Science, 2002. **297**(5584): p. 1186-90.
214. Nakamura, T. and S.A. Lipton, *According to GOSPEL: filling in the GAP(DH) of NO-mediated neurotoxicity.* Neuron, 2009. **63**(1): p. 3-6.
215. Faraci, F.M., *Reactive oxygen species: influence on cerebral vascular tone.* J Appl Physiol (1985), 2006. **100**(2): p. 739-43.
216. Zorov, D.B., M. Juhaszova, and S.J. Sollott, *Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release.* Physiol Rev, 2014. **94**(3): p. 909-50.
217. Nakamura, K. and T. Shichita, *Cellular and molecular mechanisms of sterile inflammation in ischaemic stroke.* J Biochem, 2019. **165**(6): p. 459-464.
218. Mildner, A., et al., *Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions.* Nat Neurosci, 2007. **10**(12): p. 1544-53.
219. Hu, X., et al., *Neurobiology of microglial action in CNS injuries: receptor-mediated signaling mechanisms and functional roles.* Prog Neurobiol, 2014. **119-120**: p. 60-84.
220. Prinz, M. and J. Priller, *Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease.* Nat Rev Neurosci, 2014. **15**(5): p. 300-12.
221. Kono, H. and K.L. Rock, *How dying cells alert the immune system to danger.* Nat Rev Immunol, 2008. **8**(4): p. 279-89.
222. Moehle, M.S. and A.B. West, *M1 and M2 immune activation in Parkinson's Disease: Foe and ally?* Neuroscience, 2015. **302**: p. 59-73.
223. Cherry, J.D., J.A. Olschowka, and M.K. O'Banion, *Neuroinflammation and M2 microglia: the good, the bad, and the inflamed.* J Neuroinflammation, 2014. **11**: p. 98.
224. Mo, Y., Y.Y. Sun, and K.Y. Liu, *Autophagy and inflammation in ischemic stroke.* Neural Regen Res, 2020. **15**(8): p. 1388-1396.
225. Lo, E.H., T. Dalkara, and M.A. Moskowitz, *Mechanisms, challenges and opportunities in stroke.* Nat Rev Neurosci, 2003. **4**(5): p. 399-415.
226. Unal-Cevik, I., et al., *Apoptotic and necrotic death mechanisms are concomitantly activated in the same cell after cerebral ischemia.* Stroke, 2004. **35**(9): p. 2189-94.
227. Kilinc, M., et al., *Lysosomal rupture, necroapoptotic interactions and potential crosstalk between cysteine proteases in neurons shortly after focal ischemia.* Neurobiol Dis, 2010. **40**(1): p. 293-302.
228. Edinger, A.L. and C.B. Thompson, *Death by design: apoptosis, necrosis and autophagy.* Curr Opin Cell Biol, 2004. **16**(6): p. 663-9.
229. Szabo, C., P. Pacher, and R.A. Swanson, *Novel modulators of poly(ADP-ribose) polymerase.* Trends Pharmacol Sci, 2006. **27**(12): p. 626-30.
230. Vandenabeele, P., et al., *Molecular mechanisms of necroptosis: an ordered cellular explosion.* Nat Rev Mol Cell Biol, 2010. **11**(10): p. 700-14.
231. Sheng, R., et al., *Autophagy regulates endoplasmic reticulum stress in ischemic preconditioning.* Autophagy, 2012. **8**(3): p. 310-25.
232. Wu, H., et al., *Caspases: a molecular switch node in the crosstalk between autophagy and apoptosis.* Int J Biol Sci, 2014. **10**(9): p. 1072-83.
233. Holler, N., et al., *Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule.* Nat Immunol, 2000. **1**(6): p. 489-95.

234. Susin, S.A., et al., *Molecular characterization of mitochondrial apoptosis-inducing factor.* Nature, 1999. **397**(6718): p. 441-6.
235. Li, H., et al., *Caspase inhibitors reduce neuronal injury after focal but not global cerebral ischemia in rats.* Stroke, 2000. **31**(1): p. 176-82.
236. Wang, F., et al., *Fas (CD95) induces rapid, TLR4/IRAK4-dependent release of pro-inflammatory HMGB1 from macrophages.* J Inflamm (Lond), 2010. **7**: p. 30.
237. Namura, S., et al., *Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia.* J Neurosci, 1998. **18**(10): p. 3659-68.
238. Jin, K., et al., *Fas (CD95) may mediate delayed cell death in hippocampal CA1 sector after global cerebral ischemia.* J Cereb Blood Flow Metab, 2001. **21**(12): p. 1411-21.
239. Culmsee, C. and M.P. Mattson, *p53 in neuronal apoptosis.* Biochem Biophys Res Commun, 2005. **331**(3): p. 761-77.
240. Hoffman, W.H., et al., *Transcriptional repression of the anti-apoptotic survivin gene by wild type p53.* J Biol Chem, 2002. **277**(5): p. 3247-57.
241. Lodish, H., et al., *Cell death and its regulation*, in *Molecular cell biology*, K. Kahr, Editor. 2007, W.H. Freeman and Company: New York. p. 937.
242. Zhao, M., et al., *Methylene blue exerts a neuroprotective effect against traumatic brain injury by promoting autophagy and inhibiting microglial activation.* Mol Med Rep, 2016. **13**(1): p. 13-20.
243. Xu, H., et al., *Methylene blue attenuates neuroinflammation after subarachnoid hemorrhage in rats through the Akt/GSK-3beta/MEF2D signaling pathway.* Brain Behav Immun, 2017. **65**: p. 125-139.
244. Wang, Y.C., et al., *Chemogenetics-mediated acute inhibition of excitatory neuronal activity improves stroke outcome.* Exp Neurol, 2020. **326**: p. 113206.
245. Gao, J., et al., *Coupling between NMDA receptor and acid-sensing ion channel contributes to ischemic neuronal death.* Neuron, 2005. **48**(4): p. 635-46.
246. Pignataro, G., R.P. Simon, and Z.G. Xiong, *Prolonged activation of ASIC1a and the time window for neuroprotection in cerebral ischaemia.* Brain, 2007. **130**(Pt 1): p. 151-8.
247. Li, M.H., et al., *Modulation of Acid-sensing Ion Channel 1a by Intracellular pH and Its Role in Ischemic Stroke.* J Biol Chem, 2016. **291**(35): p. 18370-83.
248. Cai, C.C., et al., *Glycine Protects against Hypoxic-Ischemic Brain Injury by Regulating Mitochondria-Mediated Autophagy via the AMPK Pathway.* Oxid Med Cell Longev, 2019. **2019**: p. 4248529.
249. Tao, J., et al., *Neuroprotective effects of pinocembrin on ischemia/reperfusion-induced brain injury by inhibiting autophagy.* Biomed Pharmacother, 2018. **106**: p. 1003-1010.
250. Shi, Q., et al., *A natural diarylheptanoid protects cortical neurons against oxygen-glucose deprivation-induced autophagy and apoptosis.* J Pharm Pharmacol, 2019. **71**(7): p. 1110-1118.
251. Yan, B.C., et al., *Neuroprotective Effects of Gabapentin Against Cerebral Ischemia Reperfusion-Induced Neuronal Autophagic Injury via Regulation of the PI3K/Akt/mTOR Signaling Pathways.* J Neuropathol Exp Neurol, 2019. **78**(2): p. 157-171.
252. Pieroni, A., et al., *Studies on anti-complementary activity of extracts and isolated flavones from Ligustrum vulgare and Phillyrea latifolia leaves (Oleaceae).* J Ethnopharmacol, 2000. **70**(3): p. 213-7.
253. Xu, X., et al., *Species and cell-type properties of classically defined human and rodent neurons and glia.* Elife, 2018. **7**.