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The unique role of Angiogenin in neuroprotection and neurorepair after cerebral ischemia

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Abbreviations

AIF: Apoptosis-inducing Factor

ALS: Amyotrophic Lateral Sclerosis

AMPK: Adenosine Monophosphate-activated Protein Kinase

ANG: Angiogenin

AT2R: Angiotensin II Type 2 Receptor

BBB: Blood-Brain Barrier

Bcl-2:B cell lymphoma/leukemia 2

BDNF: Brain-derived Neurotrophic Factor

BETAS: Beta-hCG+Erythropoietin in Acute Stroke

CBF: Cerebral Blood Flow

CCA: Common Carotid Artery

CDKs: Cyclin-dependent Kinases

CNS: Central Nervous System

CPCGI: Compound Porcine Cerebroside and Ganglioside Injection

CXCR4/7: CXC motif Receptor 4/7

DAHP: 2,4-diamino-6-hydroxypyrimidine

DG: Dentate Gyrus

DISC: Death-inducing Signaling Complex

DPI: Diphenyleneiodonium

ECA: External Carotid Artery

ECs: Endothelial Cells

EGF: Epidermal Growth Factor

ELISA: Enzyme-linked Immunosorbent Assay

eNOS: endothelial Nitric Oxide Synthase

ER: Endoplasmic Reticulum

ERK: Extracellular signal-regulated Kinase

FADD: Fas-associated protein with Death Domain

FGF: Fibroblast Growth Factor

G-CSF: Granulocyte Colony-stimulating Factor

GSK-3β: Glycogen Synthase Kinase 3β

HGF: Hepatocyte Growth Factor

hr-ANG: human recombinant Angiogenin

ICA: Internal Carotid Artery

ICAD: Inhibitor of Caspase-activated DNase

ICAM-1: Intercellular Adhesion Molecule 1

IFN-γ: Interferon-γ

IL-8: Interleukin-8

iNOS: inducible Nitric Oxide Synthase

JNK: Jun N-terminal kinase

LPS: lipopolysaccharide

MAG: Myelin-associated Glycoprotein

MCA: Middle Cerebral Artery

MCAO: Middle Cerebral Artery Occlusion

MCP-1: Membrane Cofactor Protein 1

MMP: Matrix Metalloproteinases

MSCs: Mesenchymal Stromal Cells

NAD: Nicotinamide Adenine Dinucleotide

NF-κB: Nuclear Factor-κB

NMDA: N-methyl-D-aspartate

nNOS: Neuron Nitric Oxide Synthase

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NOX: Nicotinamide adenine dinucleotide phosphate oxidase

NSCs: Neural Stem Cells

OB: Olfactory Bulb

PAF: Platelet-activating Factor

PARP: Poly-ADP-ribose Polymerase

PD: Parkinson's Disease

PD-ECGF: Platelet-derived Endothelial Cell Growth Factor

PDGF: Platelet-derived Growth Factor

PlGF: Placental Growth Factor

qRT-PCR: Quantitative Reverse Transcriptase PCR

ROS: Reactive Oxygen Species

RPs: Ribosomal Proteins

rRNA: ribosomal RNA

SGZ: Subgranular Zone

S-NLC: Sesamol-nanostructured Lipid Carrier

STA: Superior Thyroid Artery

STARS: Subacute Therapy with Amphetamine and Rehabilitation for Stroke

SVZ: Subventricular Zone

t-BHP: tert-Butyl Hydroperoxide

TGF: Transforming Growth Factor

TLR4: Toll-like Receptor 4

TNF: Tumor Necrosis Factor

tPA:tissue Plasminogen Activator

tRFs: tRNA-derived small RNA Fragments

TTC:2,3,5-Triphenyltetrazolium Chloride

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

VEGF: Vascular Endothelial Growth Factor

WHO: World Health Organization

YB-1:Y-box binding protein 1

Abstract

Stroke is a leading cause of death and disability worldwide with major socioeconomic impact and healthcare costs. In this regard recent data shows that there are over 13 million new strokes annually, one in four people over age 25 will have a stroke in their lifetime, 80 million people are currently living with the consequences of a stroke and more than five million people die from stroke annually. Despite these threatening numbers, nowadays the only available treatments for acute stroke are the pharmacological reperfusion therapies to dissolve the clot/thrombus or the mechanical approaches to evacuate the occluding thrombus (thrombectomies). Fortunately, these are effective and saving-live therapies, but with strict inclusion protocols and a short therapeutic time-window of hours after the symptoms onset due to the increase in hemorrhagic complications if administrated late or the lack of efficacy. However, not all patients are candidates for reperfusion therapies including all hemorrhagic strokes, among others. After this acute phase of the disease stroke patients also have therapeutic opportunities with multidisciplinary neurorehabilitation programs, which have been integrated into the guidelines for stroke management for years.

With this scenario, it is essential to investigate novel stroke therapies designed to be potentially translated into the clinical practice as rapid as possible, with a therapeutic impact in both neuroprotection and neurorepair. In this context, the present thesis investigates the therapeutic potential of administering Angiogenin (ANG) in a mouse model of cerebral ischemia in a clinically-relevant therapeutic approach.

ANG is a ribonuclease recognized to regulate cell proliferation, survival, differentiation or migration by activating different signaling pathways, with protective effects in other

neurological diseases such as amyotrophic lateral sclerosis and Parkinson and known associations with the presence of ANG variants. The potential protective and proliferative actions of ANG protein on endothelial and neural cells has been reported by different research groups as well as its expression in the brain after ischemia and in blood circulation, suggesting a link between this ribonuclease and stroke disease. The present thesis assumes a new step in ANG research, by investigating its therapeutic actions in the ischemic brain in pre-clinical stroke models. With this purpose, bioactive human recombinant ANG (hr-ANG) has been used as post-stroke therapy in C57Bl/6 male mice in a clinically-relevant transient middle cerebral artery occlusion model induced by a nylon filament. First, ANG was intraperitoneally administered either acutely 90 minutes or subacutely at 24h of occlusion, and infarct lesion and stroke outcome evaluated 2 days after occlusion, and showing neuroprotective effects in both therapeutic approaches responding to 5 ug-ANG treatment with no major complications supporting the safety of the proposed therapy.

In the subacute therapeutic protocol we identified neuroprotection mechanisms involving the apoptosis signaling pathway since the effector active caspase-3 was reduced in the ischemic ipsilateral hemisphere at 24h of treatment in those animals presenting reduced infarct size, and further confirmed by the inhibition of upstream ischemia-induced active caspase-9 and Bax in the same ischemic hemisphere. Second, the 5ug-ANG therapy was administered during 2 weeks by multiple intraperitoneal doses after cerebral ischemia, and the impact on neurovascular remodeling was evaluated by quantifying vessel density, migrating neuroblasts and proliferative cells in the ischemic brain. Interestingly, increased migrating neuroblasts from the subventricular neurogenic niche towards the infarcted tissue were observed in the 5ug-ANG treatment group, but no differences in vascular remodeling were observed at two weeks.

Overall the present thesis supports the potential role of hr-ANG as a treatment for ischemic stroke, based on its previously-reported neuroprotective and neurorepair actions, proposing a therapeutic clinically-relevant time-window that could be further combined with approved thrombolytic treatments or neurorehabilitation programs.

1. Introduction

1.1 Presentation of the stroke disease

Stroke is a medical condition that reduces blood flow to the brain resulting in cell dysfunction or death [1-3]. The risk factors of stroke include high blood pressure, diabetes, smoking, atrial fibrillation and others. There are two types of stroke: ischemic and hemorrhagic **(Figure 1).** Ischemic stroke is caused by the interruption of the brain blood supply, and hemorrhagic stroke results from an abnormal vascular structure or blood vessel rupture [4, 5]. Most of the strokes correspond with the ischemic presentation (87%). Worldwide, stroke is the second leading cause of death and the third leading cause of disability and the World Health Organization (WHO) reports that there are 15million people suffering a stroke each year around the world, among them 5 million patients will die and 5 million patients will survive but with neurological deficits that limit their functional independence. Moreover, there are 33 million stroke survivors globally who need long-term care and post-stroke rehabilitation to recover the lost functions [6, 7].

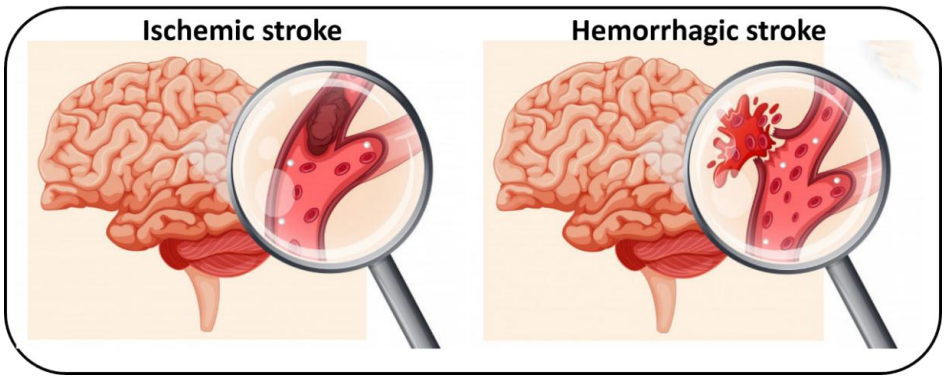

Figure 1. Ischemic and hemorrhagic stroke in the human brain. The figure is adapted from (vecteezy.com-human-brain-and-hemorrhagic-stroke).

Regarding treatments for ischemic stroke, nowadays the only available and approved treatments are the pharmacological reperfusion therapies to dissolve the clot/thrombus with tissue plasminogen activator (tPA) or the mechanical approaches to evacuate the occluding thrombus (thrombectomies). Although these are effective and saving-live therapies, in general, they can only be applied to ischemic patients within the first hours of the symptoms onset (up to 6-8 hours) due to the increase in hemorrhagic complications if administrated late [8-12], or inefficacy. Besides, not all patients are candidates for reperfusion therapies. For hemorrhagic stroke, the therapies mainly focus on stopping the bleeding and controlling intracranial pressure [13]. However, for patients who are not candidates for reperfusion therapies, or for those who survive a stroke with functional deficits, the only therapeutic options are the post-stroke rehabilitation programs, which have been integrated into the guidelines for stroke management for years [11, 14-19]. Rehabilitation aims to compensate for the sensory-motor deficits, to replace the loss of diminished functions, and to acquire maximum independence for daily life activities [20-24]. We know that rehabilitation is influenced by the individual neuroplasticity responses of each patient [25-27], but the biological mechanisms responsible for these functional improvements either by natural evolution or induced by rehabilitation therapy are still being investigated.

In this scenario, it is also needed to look for proper neuroprotective and neuroreparative strategies for stroke patients and investigate the mechanisms behind. Many advances have been achieved in this field helping to understand the molecular and cellular mechanisms of stroke, but no clinically effective neuroprotective or neuroreparative treatments are available at the moment. The work presented in this thesis using experimental cerebral ischemia models aims to investigate a novel neuroprotective and

neuroreparative strategy, its effects and corresponding protective mechanisms of action. For this, the following introduction will mainly focus on the ischemic stroke presentation of the disease.

1.2 The physiopathology of ischemic stroke

After ischemic stroke, not all cells die after the blood supply interruption. There might be two areas of hypoperfused tissue: one part is the so-called ischemic core, which is defined almost immediately after the vessels occlusion and where cells are going to die rapidly without any reperfusion intervention; another part is named the hypoperfused ischemic penumbra, which could end being part of the ischemic core or where the brain cells can be rescued with appropriate reperfusion or neuroprotection strategies [28, 29]. **(Figure 2)**.

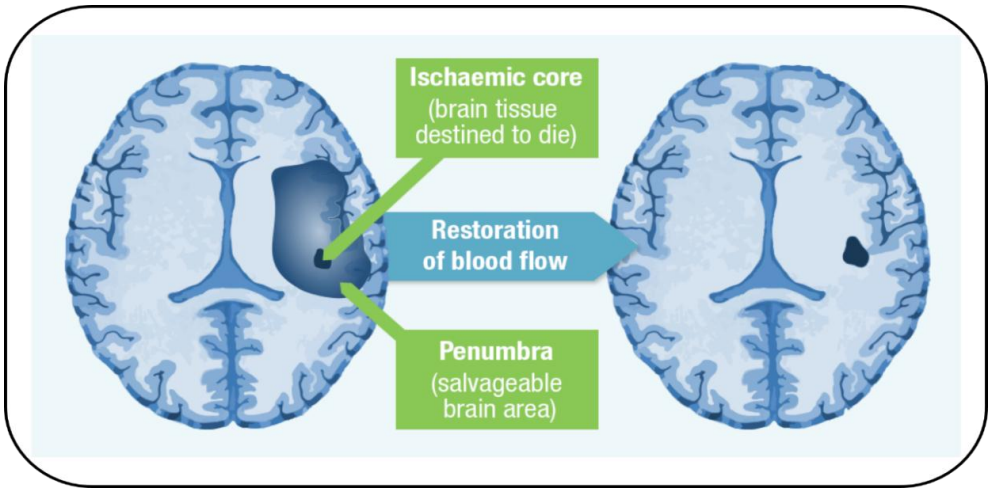

Figure 2. The ischemic core and penumbra. The figure is adapted from pathophysiology of stroke (strokeforum.com/overview/pathophysiology).

Cell death caused by ischemic stroke includes mechanisms of necrosis, apoptosis and autophagocytosis. The destiny of the brain tissue and cells is determined by complex mechanisms including energy failure, ionic imbalance, excitotoxicity, oxidative stress, nitrosative stress, peri-infarct depolarizations, inflammation and immunity. Briefly, following the ischemic stroke, there is an energy failure, which will increase the excitatory amino acids and intracellular calcium, these events further activate the free radical and peroxynitrite production, calpain, phospholipases, and poly (ADP-ribose) polymerase. Apoptosis pathways are also activated meanwhile. In the penumbra, peri-infarct depolarization aggravates the energy-consuming of ischemic neurons. The inflammation also contributes to tissue damage and cell death [30]. **(Figure 3).**

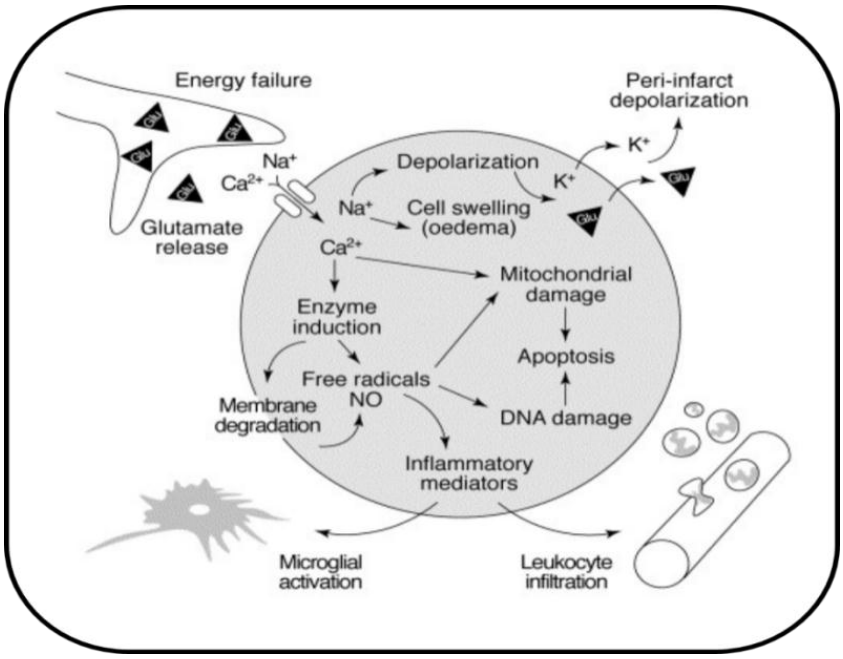

Figure 3. Representation of the ischemic cascade mechanisms after stroke. The figure is adapted from Dirnagl et al.1999 [31].

1.2.1 Iron imbalance and excitotoxicity

It is known that the brain is highly sensitive to changes in oxygen and glucose levels. The acute deprivation of oxygen and glucose caused by ischemic stroke leads to brain cells depolarization within a few minutes, which activates the voltage-dependent calcium channels [32]. On the other hand, the depolarization also increases the amount of glutamate binding to the ionotropic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors, which will also increase the calcium influx [33, 34]. The increased intracellular calcium ion mediates a series of cascades leading to a degradation of membrane and proteins which are crucial for cell survival. Besides, an excess of calcium enters into the mitochondria causing dysfunction and mitochondrial edema, which also contributes to cell death [33, 34]. Apart from calcium ions, the imbalances of other ions such as sodium and zinc also lead to excitotoxic cell death [35, 36].

1.2.2 Oxidative and nitrosative stress

The oxidative stress mainly occurs after reperfusion through generating reactive oxygen species (ROS) such as O_2^-, which leads to an excess of superoxide production and triggering mitochondrial permeability transition [37, 38]. Besides, the ROS could catalyze arachidonic acid conversing to prostanoid, and the hypoxanthine degradation [39]. All of these events lead to tissue damage and cell death.

Apart from the reactive free radicals, tissue damage could also be caused by nitrosative stress. Nitric oxide (NO) has dual functions during ischemic stroke, either to protect or destroy the tissues which are determined by the time and place of its production [40, 41]. There are three subtypes of NO synthase (NOS) (NOS I, NOS II, and NOS III)

controlling the synthesis of NO [42]. When there is oxidative stress, NO could react with superoxide anions to form peroxynitrite, which is extremely reactive and cytotoxic to the tissue [43]. During the process of ischemia, the first activated NOS is the neuron NOS I (nNOS), which is calcium-dependent and could be activated by calcium influx, this leads to a rapid increase of NO level [44]. Besides, the inducible NOS II (iNOS), which is calcium-insensitive, is not expressed in healthy brain tissue but is expressed in non-neuronal cells 12 hours after cerebral ischemia and lasting for 1week, contributing to produce a large amount of NO and leads to delayed brain tissue damage [45].

The generation of reactive free radicals and NO is also associated with DNA damage and nuclear enzyme poly-ADP-ribose polymerase (PARP) activation. The oxidative and nitrosative stress causes single-stranded DNA nicks, which active the PARP-1 and then contribute to generating poly-ADP chains by using quite a lot of β-nicotinamide adenine dinucleotide (NAD^+), this process consumes a large amount of ATP, all of this aggravates cell energy failure and causes cell death [46]. Unlike the nNOS and iNOS, the endothelial NOS III (eNOS) possesses protective functions for ischemic stroke since NO is important for the function of the blood vessels and the maintenance of cerebral blood flow [47], proving the reduction of cerebral infarct size in animal models [48, 49].

1.2.3 Inflammation and immunosuppression

The inflammation caused by cerebral ischemia can be described as the rapid activation of resident cells, mainly the microglia cells, followed by the infiltration of circulating inflammatory cells, such as neutrophils, T cells and monocytes/macrophages. In the acute phase of ischemic stroke (minutes to hours), the injured tissue rapidly releases ROS and pro-inflammatory mediators (cytokines and chemokines) [50]. These

mediators promote the expression of adhesion molecules on brain endothelial cells and leukocytes, contributing to the adhesion and migration of circulating leukocytes [51]. It has been shown that the adhesion molecules promote neutrophils entering into the brain, which contributes to cell death since by blocking the neutrophil invasion or the adhesion molecules expression the infarct size can be reduced [52]. In the subacute phase (hours to days), the infiltrated leukocytes release cytokines and chemokines, the overproduction of ROS or the induction/activation of inflammatory molecules such as Matrix Metalloproteinases (MMP), will further amplify the inflammatory response and ultimately contribute to the destruction of blood-brain barrier (BBB), brain edema, hemorrhagic transformation and neuronal death [53]. However, several pro-inflammatory factors have a dual role in the early and late phases of stroke. For example, it has been reported that the MMP-9 aggravates early ischemic brain damage, but promotes neuroregeneration and neurovascular remodeling in the later repair stage [53], or that MMP13 is linked to acute brain injury but needed for vessel remodeling during recovery [54, 55]. Therefore, it is very important to fully understand the time course of events causing inflammation in the ischemic brain. In addition, the activated microglia cells not only generate the pro-inflammatory mediators but also produce the neurotrophic and protective factors, such as transforming growth factor (TGF)-β, erythropoietin and metallothionein, playing an anti-inflammation role post-stroke [56, 57]. **(Figure 4).**

Ischemic stroke also can induce immunosuppression breaking the balance between the immune system and central nervous system (CNS). For example, the immunosuppression caused by cerebral ischemic stroke in a rodent model leads to spontaneous systemic bacterial infections [58].

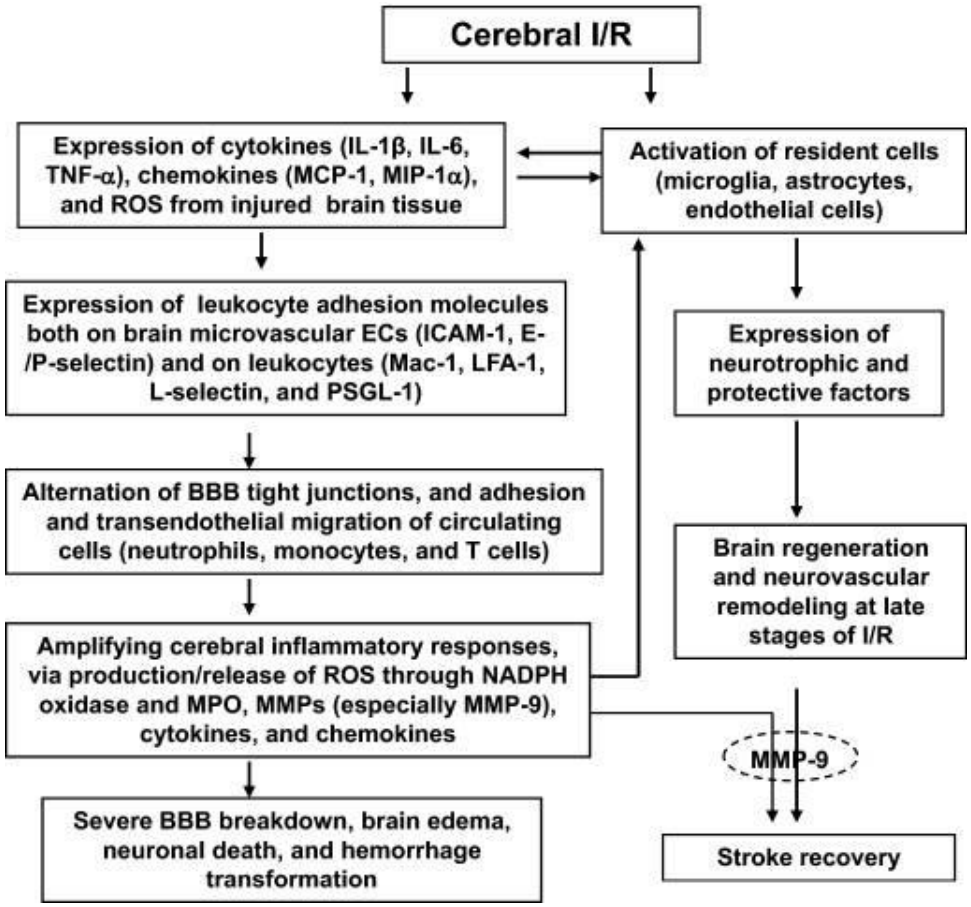

Figure 4. Potential inflammatory pathways that respond to cerebral ischemia. The figure is adapted from Jin et al.2010 [59].

1.2.4 Cell apoptosis

The primary injury of ischemic stroke typically leads to necrosis, which usually occurs in the core of infarction; while apoptosis typically occurs in the ischemic penumbra, develops in a delayed manner and is associated with secondary injury of ischemic stroke [60]. The caspase-dependent and independent apoptosis pathways have been described in ischemic neuronal death. Caspases are a series of aspartate-specific cysteine proteases, which are constitutively present in the brain cells, and could be

activated by extrinsic and intrinsic stimulation pathways [61], **(Figure 5).** Briefly, the extrinsic stimuli could activate the Fas and tumor necrosis factor (TNF)-α, which can bind to the death-inducing signaling complex (DISC), which is composed of the death receptor (a typical example is Fas receptor), Fas-associated protein with death domain (FADD) and caspase-8. The intrinsic stimulations include the generation of oxygen radicals, the damage of DNA, the increase of intracellular calcium and the activation of lysosomal proteases. Under the stimuli of these intrinsic activators, caspases could cleave a series of downstream targets including PARP, an inhibitor of caspase-activated DNase (ICAD), cytoskeletal proteins and other caspases. The cleaved caspase-3 is the most crucial and effector protein in brain cell apoptosis, especially in the penumbra region [62]. During the process of caspase activation, cytochrome c is essential because it could form a complex named apoptosome with the existing caspase-9 and ATP [63]. At the same time, Bcl-xL, one member of the Bcl-2 family, could inhibit the formation of the apoptosome, while cytosolic Bid, another member of the Bcl-2 family, contributes to the release of cytochrome c and the formation of apoptosome [64]. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) cells and the DNA fragments are considered apoptosis downstream biomarkers [65].

There is also a caspase-independent apoptosis pathway in ischemic stroke, the apoptosis-inducing factor (AIF) is the crucial activator, which could be promoted by PARP-1 activation. After AIF is released from the mitochondria, it translocates to the nucleus and causes chromatin condensation [66]. Other studies indicate that the activation and up-regulation of the cell cycle machinery, such as the cyclin-dependent kinases (CDKs) in ischemic stroke also contributes to cell death [67]. Although CDK-5 does not participate in cell cycle progression, it is involved in neuron transcription,

differentiation and functions, is activated by ischemic stroke and then converts p35 into p25 form, thus inducing cell death [68].

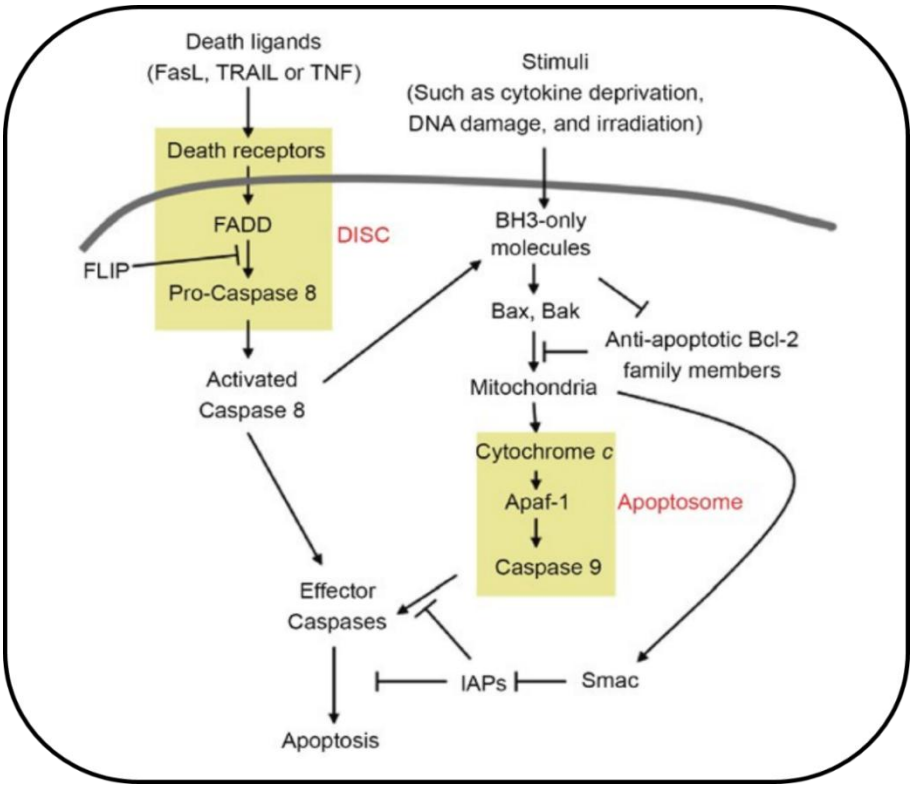

Figure 5. Apoptosis signaling pathways. The figure is adapted from Xu et al.2007 [61].

1.3 Current treatments for ischemic stroke

Ischemic stroke is a medical emergency that should be treated quickly to restore the blood flow to the hypoperfused brain area. The primary treatment is intravenous thrombolysis with tPA which could dissolve the clot, but must be intravenously administrated within the first 4.5 hours of the symptoms onset. The earlier is received the therapy, the better the prognosis. Timely treatment not only increases survival

chances but also may reduce complications. tPA can also be delivered directly to the ischemic area by inserting a catheter through an artery in the groin and threading it to the brain, but it is still under investigation. Certain risks should be considered before using tPA, such as the potential bleeding in the brain, to determine if tPA is suitable for the candidates [10]. If tPA does not achieve the arterial recanalization, clots could be removed by mechanical interventions, which is a more recent therapy making use of a stent retriever attached to a catheter which removes clots directly from the occluded blood vessel in the brain after endovascular insertion. It is particularly beneficial for patients with large vessel occlusions [9]. This mechanical thrombectomy should be administered within the first 8 hours of the symptoms onset. Although recent studies have proved that under certain conditions, it can be performed within the first 24 hours [69]. Other important acute management interventions are oxygenation and ventilation, blood pressure control, glycemic control, cerebral edema treatment or temperature control. Long-term treatments include antiaggregants or anticoagulants to prevent further stroke recurrence, as well as interventions such as carotid endarterectomy, angioplasty or stenting according to specific patient's etiologies [11].

Despite the hyperacute and acute emergency treatments, one-third of stroke patients will present relevant functional disabilities which can be later treated by personalized rehabilitation programs which help patients to recover functions and improve functional independence. Nowadays the rehabilitation therapy starts when the patients are in the hospital within the first days after stroke and continue in a rehabilitation hospital unit, in an outpatients clinic or in home-assisted programs according to patient's condition [14]. The patients' rehabilitation program will be adjusted to their medical condition, age, pre-stroke general health status and family support. The comprehensive rehabilitation

program might include motor-skill exercises**,** mobility training**,** constraint-induced therapy**,** range-of-motion therapy**,** cognitive disorders therapy, communication disorders therapy, medication therapy, psychological evaluation and treatment, involving a multidisciplinary medical team lead by physiatrists and including rehabilitation nurses, dietitians, physical therapists, occupational therapists, recreational therapists, speech therapists, social workers, psychologists or psychiatrists. The duration of stroke rehabilitation depends on the individual achievements and planned objectives, but is nowadays extended beyond the 6 months post-stroke [14].

1.4 Stroke Neuroprotection

No neuroprotectant treatments have been approved for stroke, however, multiple mechanisms implicated in the ischemic cascade have been considered as potential therapeutic targets to reduce or minimize the infarct lesion. The most relevant mechanisms and targets are reviewed in this section.

1.4.1 Ion channel blockers

Soon after the arterial blockage, the brain tissue enters in a state of energy deprivation, which causes the cell membrane energy-dependent ion channel or ion pump to be inactivated, leading to the depolarization of the membrane. The presynaptic membrane voltage-dependent calcium channel is activated and releases excitatory amino acids such as glutamate and aspartic acid, which ultimately produce cell excitotoxicity effects, which increases the intracellular calcium ion, sodium ion, chloride ion and water, further aggravating neuronal damage.

By inhibiting the influx of calcium ions, calcium channel blockers can not only reduce the release of excitatory amino acids in the presynaptic membrane of neurons and intracellular calcium overload but also dilate cerebral blood vessels and increase cerebral blood flow. This therapeutic strategy has been tested, for example with the antihypertensive drug nimodipine which can pass through the BBB and exert a neuroprotective effect for ischemic stroke [70]. In addition, it has been shown that a new type of marine fungus extract named Xyloketal B can reduce cell death caused by oxygen and glucose deprivation. Mechanistic studies have found that it works by reducing calcium ion influx and inhibiting cell apoptosis [71].

Glutamate and aspartic acid are neurotransmitters that can regulate fast excitatory synapses in the CNS, and the NMDA receptor is one of the main receptors for excitatory amino acids. Dizocilpine (MK-801) as a non-competitive NMDA receptor antagonist can block NMDA ion channels. Animal experimental studies have found that MK-801 can significantly reduce the volume of cerebral infarction [72]. Maslinic acid and MK -801 have a synergistic effect, which can extend the treatment time window of MK-801 from 1 h to 3 h [73], but further research is still needed.

1.4.2 Antioxidants

Multiple studies have shown that oxidative stress is closely related to the pathophysiological process of cerebral ischemia [37]. ROS and free radicals (including superoxide anions, hydroxyl free radicals and peroxynitrite are produced in large quantities after ischemic stroke, and further lead to inflammatory reactions, apoptosis and tissue damage. Under healthy conditions, these free radicals can be eliminated by peroxidase; however, after ischemic stroke, the balance between the production and

elimination of free radicals is disrupted, eventually leading to brain damage. In recent years, researchers have explored neuroprotective drugs targeting oxidative stress in ischemic stroke, some of them will be discussed.

1.4.2.1 Free radical scavengers

NXY-059 is one well-known free radical-trapping agent that has shown neuroprotection in animal models of stroke [74]. To determine its efficacy in humans after acute ischemic stroke, the Stroke-Acute Ischemic NXY Treatment (SAINT I) trial was conducted, showing that NXY-059 administration within the first six hours of acute ischemic stroke onset significantly reduced disability, but did not significantly improve neurologic function measured by the NIHSS score [75]. To confirm the efficacy, a second multicentric and larger trial (SAINT II) was conducted, however, it did not prove that the NXY-059 was effective [76]. Zang and his colleagues designed and synthesized a series of pyrone carbazole alkaloids, the compounds 10a, 7c and 11a derivatives showed cytoprotective effects both in the hydrogen peroxide-treated and the oxygen-glucose deprived cell model. Among them, compound 7c can effectively scavenge free radicals showing strong activity both in *in vitro* cell experiments and in the rat middle cerebral artery occlusion (MCAO) model. In addition, compared with the free radical scavenger edaravone, the antioxidant activity of this derivative is stronger [77]. With this the free radical scavenger drug-strategy is a potential neuroprotective treatment for ischemic stroke.

1.4.2.2 Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway agonists

The PI3K/Akt pathway is closely related to cell proliferation and survival, and its activation is also involved in the neuroprotective process of some antioxidants. The compound (di4-6S) is a small synthetic hyaluronan disaccharide that can increase the

content of glutathione in cells, and increase the cell survival rate both *in vitro* and *in vivo* in ischemia models, thereby plays an antioxidant neuroprotective role; in addition, di4-6S can increase the content of p-Akt in ischemic brain tissue, and its neuroprotective effects can be blocked by the PI3K/Akt pathway inhibitor LY294002 [78], indicating that the antioxidant neuroprotective effect of di4-6S is related to the activation of the PI3K/Akt pathway. Chen and his colleagues designed and synthesized a series of tetramethylpyrazine derivatives which could be used as new neuroprotective agents for ischemic stroke [79]. Among them, the compound 22a exerts a powerful free radical scavenging effect by activating the PI3K/Akt pathway, alleviates the neuronal damage induced by tert-Butyl hydroperoxide (t-BHP), and has a neuroprotective effect for the rat MCAO model [79]. Previous studies have also proved that the PI3K/Akt pathway agonist sesamol has antioxidant activity [80], but it can be quickly eliminated in the body, which limits its further application and research. The sesamol-nanostructured lipid carrier (S-NLC) could slow down the clearance rate of sesamol in the body, thereby it could play a more effective role since recently it has been shown that S-NLC alleviates oxidative stress in ischemic stroke by activating the PI3K pathway [81]. Therefore, the PI3K/Akt pathway agonist is presented as a potential drug for ischemic stroke.

1.4.2.3 Nicotinamide adenine dinucleotide phosphate oxidase (NOX) inhibitors

NOX mediates the production of superoxide in brain cells and plays an important role in the development of stroke and stroke-related cerebrovascular diseases. Inhibition of NOX has a neuroprotective effect on ischemic stroke [82]. Kim and his colleagues summarized the therapeutic effects of NOX inhibitors for ischemic stroke [80]. The antioxidants such as diphenyleneiodonium (DPI), apocynin, honokiol and plumbagin have neuroprotective effects for ischemic stroke through blocking the NOX. Wang and his colleagues [83] designed and synthesized a series of new compounds containing

tetramethylpyrazine and carnitine substructures, and investigated their potential and mechanisms of action in the treatment of stroke-related neuronal damage. Among these compounds, LR134 and LR143 have shown significantly neuroprotective effects in a rat MCAO model by reducing inflammatory reactions and NOX-mediated oxidative stress [83]. Therefore, NOX inhibitors are also one of the potential drugs for ischemic stroke.

1.4.2.4 Nitric oxide synthase (NOS) inhibitors

As explained, an important free radical in the pathophysiological process of ischemic stroke, nitric oxide, is mainly produced by iNOS. According to the latest study by Mahmood and his colleagues [84], salvianolic acid A exerts neuroprotective effects for cerebral ischemia-reperfusion injury by inhibiting the expression of endothelial eNOS and the formation of peroxynitrite. The cyanide photosensitizing dye platonin is also an effective antioxidant since it can inhibit the synthesis of nitric oxide and iNOS expression in macrophages stimulated by lipopolysaccharide (LPS), reduce BBB damage, and thereby playing a protective role for ischemic stroke [85]. It is clear that NOS inhibitors, especially iNOS inhibitors, have a protective effect for oxidative damage after ischemic stroke.

1.4.3 Anti-inflammatory drugs

Inflammation plays an important role in the progress of cerebral ischemia injury. After an ischemic stroke, the accumulation of white blood cells and the activation of microglia in the ischemic brain tissue lead to the generation of a variety of pro-inflammatory cytokines. Microglia plays an important role in brain inflammation, especially in the ischemic penumbra. In addition, endothelial cells, astrocytes and neurons also secrete pro-inflammatory cytokines after ischemic injury, causing further neuronal damage. Therefore, anti-inflammatory drugs may also be one of the therapeutic methods for

ischemic stroke.

1.4.3.1 Pro-inflammatory cytokine inhibitors

Transcription factors such as nuclear factor-κB (NF-κB) will be activated after ischemic stroke, leading to an increase of inflammatory factors. Studies have found that triptolide has anti-inflammatory and neuroprotective effects in the rat MCAO model by down-regulating the expression of NF-κB [86]. The calcium channel blocker Xyloketal B can also reduce the mRNA expression levels of pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor α (TNF-α), IL-6 and interferon γ (IFN-γ) by inhibiting the ROS/Toll-like receptor 4 (TLR4)/NF-κB pathway, thereby alleviating the neurological deficit in mouse MCAO model [87]. In the past, it was proposed that salvianolic acid B was an anti-apoptotic agent. However, recent studies indicate that salvianolic acid B also has a neuroprotective effect for ischemic stroke in rats through the CD40/NF-κB pathway, and can inhibit the expression of pro-inflammatory cytokines such as intercellular adhesion molecule 1 (ICAM-1), IL-1, IL-6, IL-8 and membrane cofactor protein 1 (MCP-1) [88].

1.4.3.2 Adenosine monophosphate-activated protein kinase (AMPK) activators

AMPK activators can significantly reduce the inflammatory response and inhibit inflammatory damage in various cerebral ischemia models. It has been reported that the natural product (+)-balasubramide, compound 3C, showed significant anti-neuro-inflammatory effects *in vitro* [89]. In cell experiments, it can down-regulate the expression of pro-inflammatory cytokines in mouse microglia BV2 and primary microglia stimulated by LPS; in animal experiments, it can reduce the infarct volume after cerebral ischemia. Furthermore, this anti-inflammatory effect can be blocked by AMPK siRNA or AMPK upstream CaMKKβ siRNA [90]. This study reveals that the

AMPK activator compound 3C can exert anti-inflammatory effects through the AMPK pathway, and also emphasizes the importance of AMPK in relieving neuroinflammation, but its clinical application needs to be further investigated.

1.4.3.3 Chemokine receptor blockers

Studies have shown that inhibiting the chemokine CXC motif receptor 4/7 (CXCR4/7) pathway can significantly improve neurological function after cerebral ischemia and reverse the immune response [91]. It is reported that a new type of CXCR4 blocker named CX549 has neuroprotective and neurotrophic effects, it can effectively reduce the activation of microglia, improve the neuron survival rate *in vitro* experiment, and inhibit the expression of inflammatory cytokines [92]. This study indicates that the CXCR4 may be a potential therapeutic drug for ischemic stroke.

1.4.4 Apoptosis inhibitors

Cerebral ischemia could cause cell apoptosis. Ischemia-related apoptotic factors include B cell lymphoma/leukemia 2 (Bcl-2) protein-family and caspase protease-family, as well as p53, NF-κB, PI3K/Akt and AMPK. The Bcl-2 and the caspase families play an important role in the regulation of apoptosis, and the balance between them plays a key regulatory role in apoptosis.

1.4.4.1 Caspase-3 inhibitor

Studies have shown that the regulation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 and caspase-3 affects neuronal apoptosis [93]. M826 is a caspase-3 inhibitor that was designed and synthesized by Merck Frosst Centre for Therapeutic Research. M826 has shown neuroprotective effects in a rat cerebral ischemic model by blocking the caspase-3 activation [94]. Tideglusib is a glycogen synthase kinase 3β (GSK-3β) inhibitor, which has the potential to prevent or

treat neonatal ischemic brain injury. Its specific mechanism includes inhibition of caspase-3 activity [95]. The caspase-3 inhibition is one of the main research goals for the anti-apoptotic treatment of ischemic stroke.

1.4.4.2 Bcl-2 agonists

Isoquercetin, Xyloketal B and 2,4-diamino-6-hydroxypyrimidine (DAHP) can exert anti-apoptosis functions by regulating the activity of Bcl-2 and caspase-3 [96]. Nerve growth factors used in the clinical practice to treat ischemic stroke and Parkinson's disease (PD), such as the compound porcine cerebroside and ganglioside injection(CPCGI), can also inhibit cell apoptosis in a rat MCAO model by increasing the ratio of Bcl-2/Bax [97].

1.4.4.3 Angiotensin II type 2 receptor (AT2R) inhibitors

Animal experiments have found that AT2R activation has a neuroprotective effect on ischemia. The AT2R-deficient mice have a larger infarct size compared with wild-type mice after cerebral ischemia, indicating that AT2R activation is very important for the protection of ischemic injury [98]. Compound 21 is the first identified non-peptide AT2R agonist which can improve the outcome of ischemic stroke and reduce the apoptosis of ischemic tissue. The cytokines involved in this process include the brain-derived neurotrophic factor (BDNF), IL-10, eNOS, p-Akt and caspase-3 [99].

1.4.5 Other treatment methods

Increasing the oxygen and energy supply of brain tissue post-stroke has also been proposed as a neuroprotection therapy for stroke; the typical example is the hyperbaric oxygen therapy. A large number of studies have shown that hyperbaric oxygen has a protective effect on ischemic brain injury [100]. However, a small group of prospective clinical studies also showed that hyperbaric oxygen did not show a significant protective

effect [101]. Therefore, researchers investigated atmospheric oxygen therapy. Animal experiments have found that atmospheric oxygen treatment can increase the partial pressure of oxygen (PaO2) and blood oxygen saturation in experimental animal models; at the same time, the PaO2 in the brain tissue is also increased, especially in the penumbra area, and the infarct size is finally reduced [102].

Reducing the oxygen and energy consumption post-stroke is another proposed neuroprotection strategy; the typical example is hypothermia therapy, which can not only protect against ischemic brain injury by reducing tissue energy requirements but also significantly inhibit the complications of reperfusion, including the reduction of endothelial injury and BBB damage, the inhibition of white blood cells brain infiltration, and the reduction of free radicals. However, how to implement hypothermia therapy and its standards for patients with ischemic stroke is still inconclusive [103].

Stem cell therapy has also been considered recently as a potential neuroprotection strategy for ischemic stroke. For example, it has been reported that the extracellular vesicles of human neural stem cells intravenously injected into a pig cerebral ischemia model exerts neuroprotective effects [104]. This research provides a new direction for stem cell therapy for ischemic stroke.

1.5 Stroke neurorepair

1.5.1 Spontaneous repair mechanisms

Ischemic stroke can trigger spontaneous repair, which can last for several weeks. For certain functions, especially language and cognition, it can last for several years. A better understanding of spontaneous repair mechanisms can provide useful strategies

for post-stroke neurorepair based on the boosting or enhancement of endogenous mechanisms and repair processes [105].

The responses of the brain after an ischemic stroke can be divided into three stages with different therapeutic strategies **(Figure 6)**: the initial brain injury occurring within the first minutes-hours after the ischemic event when reperfusion or neuroprotection treatments could rescue the tissue on risk. The second stage extends from the first days to weeks after the ischemic stroke responding to injury with spontaneous repair strategies offering a new time window to boost neurorepair. And the third stage is the chronic phase when the brain endogenous repair events stabilize, but specific interventions might be still useful to modify the brain structure and functions [106].

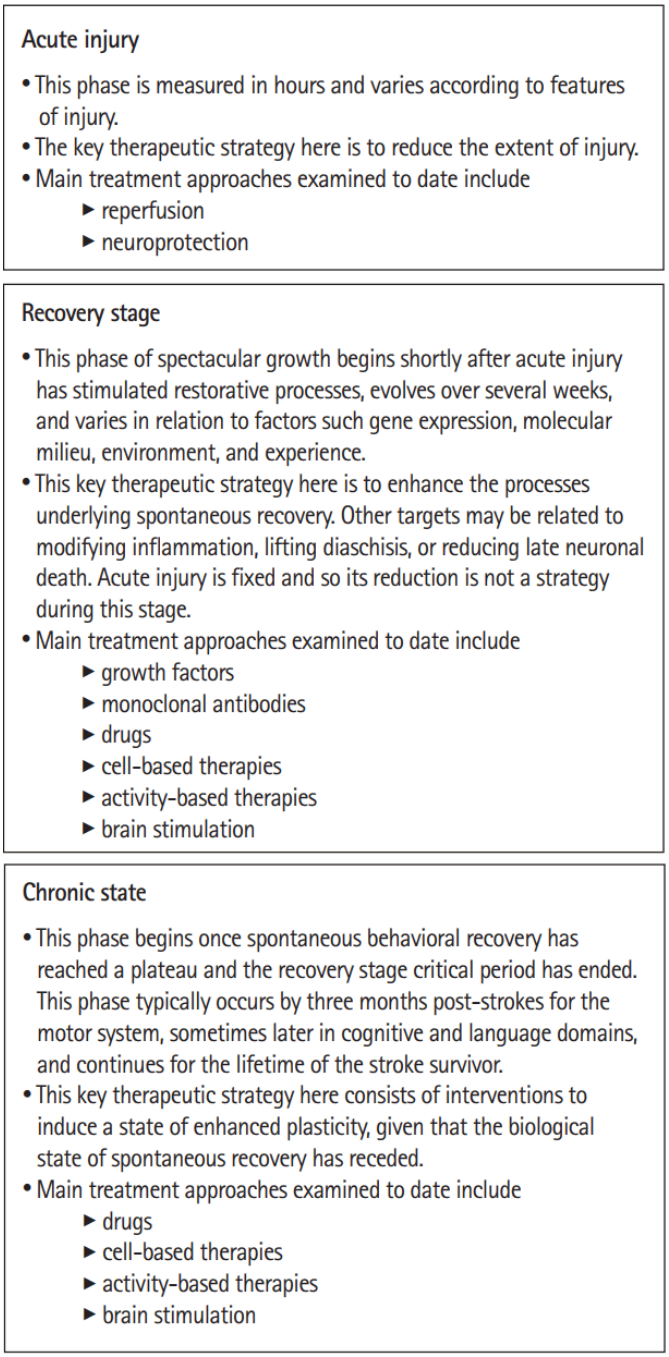

Figure 6. The three stages of spontaneous post-stroke evolution and t corresponding treatment opportunities.

The figure is adapted from Steven et al.2018 [106].

1.5.2 Therapies to promote the neurorepair

1.5.2.1 Growth factors

Growth factors are a promising therapy to promote neurorepair because they play an important role in normal CNS development, and have crucial functions for spontaneous repair including angiogenesis, cell proliferation, differentiation and migration, cell survival and apoptosis, synaptic plasticity, and immune regulation [107]. Preclinical studies have shown that administration of exogenous growth factors 24 hours or more of the ischemic stroke onset can significantly improve the prognosis [108]. Most studies have examined hematopoietic growth factors, for example, the granulocyte colony-stimulating factor (G-CSF), which is safe and well-tolerated, but it could not significantly improve the prognosis of stroke [109]. Other studies have found that a single growth factor administration such as epidermal growth factor [110] or β-hCG [111] followed by an erythropoietin therapy can promote the proliferation of neural stem cells. The Beta-hCG+Erythropoietin in Acute Stroke (BETAS) phase IIa study reported no major safety hazards, further reporting an improvement in the 90-day Barthel index score in most patients as well as for other specific domain scales [112].

1.5.2.2 Monoclonal antibodies

Monoclonal antibodies could bind to specific targets such as receptors or cell surface markers to modulate the targeted signaling pathways. In the neurorepair process after ischemic stroke, monoclonal antibodies can neutralize molecules that inhibit the growth of the CNS to create a microenvironment that is more suitable for neurorepair [113]. The existence of myelin-associated glycoprotein (MAG), oligomycin glycoprotein, and Nogo-A in the CNS could not provide a suitable environment for neuropair post-stroke, furthermore, the increase of these molecules could aggravate the ischemic stroke [114, 115]. Using monoclonal antibodies to block these molecules can promote axon growth. A

clinical study found that the monoclonal antibody GSK249320 tends to improve the gait speed of ischemic stroke patients, however the subsequent stage IIb double-blind trial could not confirm this recovery effect, although GSK249320 was well tolerated and presents low immunogenicity [116].

1.5.2.3 Drugs

Many small molecule drugs have been also investigated to improve the prognosis of ischemic stroke. Small molecules may have advantages in passing through the BBB. Many small molecules are non-polar (hydrophobic, so they can easily cross the phospholipid plasma membrane bilayer) and small in size, thus they can easily enter into the brain, one example is neurotransmitters. The studies in this area are mostly focused on monoaminergic drugs. For example, amphetamine, which can interact with multiple monoaminergic targets. It has been shown beneficial for ischemic stroke in small clinical trials [117], but research on Subacute Therapy with Amphetamine and Rehabilitation for Stroke(STARS) was unsatisfactory [118]. Dopamine is a neurotransmitter that can regulate a variety of neurological functions, including excitability, synaptic transmission, plasticity, protein transport and gene transcription. The role of dopamine in motor control is known, which contributes to cortical plasticity and plays a key role in motor skills learning [119]. Another monoaminergic neurotransmitter, serotonin, may also promote neurorepair and improve ischemic stroke recovery since has been shown that increasing serotonin can improve ischemic stroke recovery [120]. Research on norepinephrine for ischemic stroke recovery is still limited, but the feasibility and safety positive results in acute stroke patients support further studies [121]. Finally, drugs that regulate acetylcholinergic neurotransmission have been reported to promote neurorepair in rodent ischemic models, but the translation into clinical studies is still limited [122].

1.5.2.4 Cell-based therapies

Cell-based therapies are getting more and more attention nowadays. A variety of cell-based therapies are being studied, such as transformed tumor cells and stem cells. Stem cell therapies can be autologous, allogeneic or heterologous. Mesenchymal stromal cells (MSCs) have attracted widespread attention in the past decades, as a subtype of adult non-hematopoietic pluripotent cells. A large amount of preclinical evidence shows that MSCs can improve the prognosis of ischemic stroke [123], and the early clinical studies data are also satisfactory [124, 125]. However they have not been implemented yet as approved treatments for stroke since the biological efficacy and administered-cell characteristics could change over time, and long-term evaluation follow-up studies are still required.

1.5.2.5 Other therapies

As discussed above, the rehabilitation programs are a useful therapeutic strategy for recovery after stroke, but their specific actions on neurorepair mechanisms are still unknown. Novel equipment-assisted rehabilitation can help patients to recover, such as robotic and virtual reality devices and systems [126, 127]. These devices have potential advantages such as consistent and durable output, programmability, practicality for virtual reality applications, safety, high precision, and great potential for remote rehabilitation, so they can reach underserved areas. Brain stimulation may change brain functions and has the potential to promote neurorepair to improve the prognosis of ischemic stroke [128], but further research is needed in this field.

1.6 Angiogenin

With the presented scenario claiming at the urgent need of new treatments for ischemic stroke supporting the current reperfusion therapies, the present thesis has tested the therapeutic effects of Angiogenin (ANG) in experimental ischemic stroke. The following section focuses on the properties and functions of this unique ribonuclease.

ANG was first isolated from the human adenocarcinoma cell line (HT-29) and characterized as a tumor-derived angiogenic protein by Vallee and his colleagues in 1985 [129]. It was first known as a powerful angiogenic factor for angiogenesis, but further studies found that ANG, a small 123 amino acid protein, shares 33% of its sequence with the bovine pancreatic ribonuclease A [130]. ANG (also named RNase 5) has been classified as a member of the ribonuclease A superfamily [131], therefore it also has ribonuclease activity (the activity to catalyze the degradation of RNA into smaller components contributing to the metabolism of nucleic acids) which is weak compared to other RNases, but crucial for its bio-functions. This unique property determines its center roles in angiogenesis, cell proliferation and cell survival. Several structural and functional studies revealed that ANG presents a cell-binding site [132], a nuclear localization sequence [133], and a ribonucleolytic active site [134], each of them being necessary for its biological functions. ANG exists not only in a large number of tumor cells [135-142] but also in normal human cells [143] and fluids [144, 145] where it can induce cell proliferation [146] and cell survival [147, 148]. However, detrimental effects of ANG have been also reported. In this regard, Saxena and colleagues in 1992 reported that ANG could induce cytotoxicity, attributed to the tRNA degradation specifically induced by ANG [149]. A further study performed by Thomas et al. demonstrated a significant global decrease in tRNA levels upon treatment with ANG, but

the tRNA down-regulation was not responsible for the toxicity of ANG, it was the tRNA-derived small RNA fragments (tRFs)species up-regulated by ANG which produced significant cytotoxicity, observed as cell death in a dose-dependent manner [150]. But their findings contrast with previous studies reporting that certain tRFs could promote cell survival [151], becoming a topic of controversy nowadays.

1.6.1 Angiogenin characteristics and function

Angiogenesis is a process of new blood vessels growing from pre-existing ones via endothelial cells (ECs) migration, proliferation and organization [152, 153]. There are some angiogenesis mechanisms, such as the splitting of pre-existing vessels induced by intussusception, or the vessel expansion stimulated by circulating precursor cells [154, 155]. However, in the strictest sense, angiogenesis implies vessel sprouting which accounts for most of the vessel growth [152, 153]. Sprouting angiogenesis is a transient and reversible process controlled by the balance between pro-angiogenic and inhibitory factors of ECs. As a summary, the process includes: (1) stimulation of pro-angiogenic signals, (2) degradation of basal membrane, (3) activation and differentiation of ECs, migration of tip cell, proliferation of stalk cell and sprout extension, (4) formation of vessels loops established by tip cells and basement membrane synthesis by stalk cells, (5) lumen formation and perfusion, (6) vessels endothelium and wall maturation and network formation, (6) vessels quiescence and stabilization, [156-163]. Obviously, ECs are the cellular fundament of angiogenesis and play an important role in all crucial steps. Moreover, other authors have shown that the proliferation rate of tip cells has a greater effect on the spread and extent of vessel growth compared to the migration rate of stalk cells [164]. In this context, ECs proliferation is one of the most important events in angiogenesis.

There is evidence showing that ANG could promote ECs proliferation by translocating to the nucleus [133], further proved when by inhibiting its nuclear translocation it could stop ECs proliferation and angiogenesis [165]. Further studies demonstrated that the nuclear function of ANG in ECs is related to ribosomal RNA (rRNA) transcription [166], during this process, the ribonuclease activity of ANG plays an important role. This ANG-mediated rRNA transcription in ECs induces cell proliferation and angiogenesis [167] and mechanistic studies have clarified that ANG stimulates rRNA transcription by binding to rDNA [168] and the upstream control element of rDNA promoter [169]. On the other hand, ANG could also stimulate rRNA transcription indirectly through the mTOR signal-transduction pathway [170, 171]. Furthermore, ANG not only mediates the rRNA transcription but it is also involved in its processing [172]. For example, ANG could cleave tRNAs into 5′ and 3′ functional small noncoding RNAs (5′- and 3′-tiRNA) at the position of anticodon loop, which contributes to the cell survival under stress conditions [173], see **Figure 7.** Apart from the nuclear translocation mediated by the nuclear localization sequence, the integrity of the ribonucleolytic active site and the cell-binding site are also essential for its angiogenesis function [132, 174]. In this context, to induce ECs proliferation and stimulate angiogenesis, ANG should (1) maintain structural integrity, (2) interact with ECs through cell-binding site, (3) mediate nuclear translocation through nuclear localization sequence, (4) accumulate in the nucleolus, (5) bind to DNA, and (6) mediate rRNA transcription.

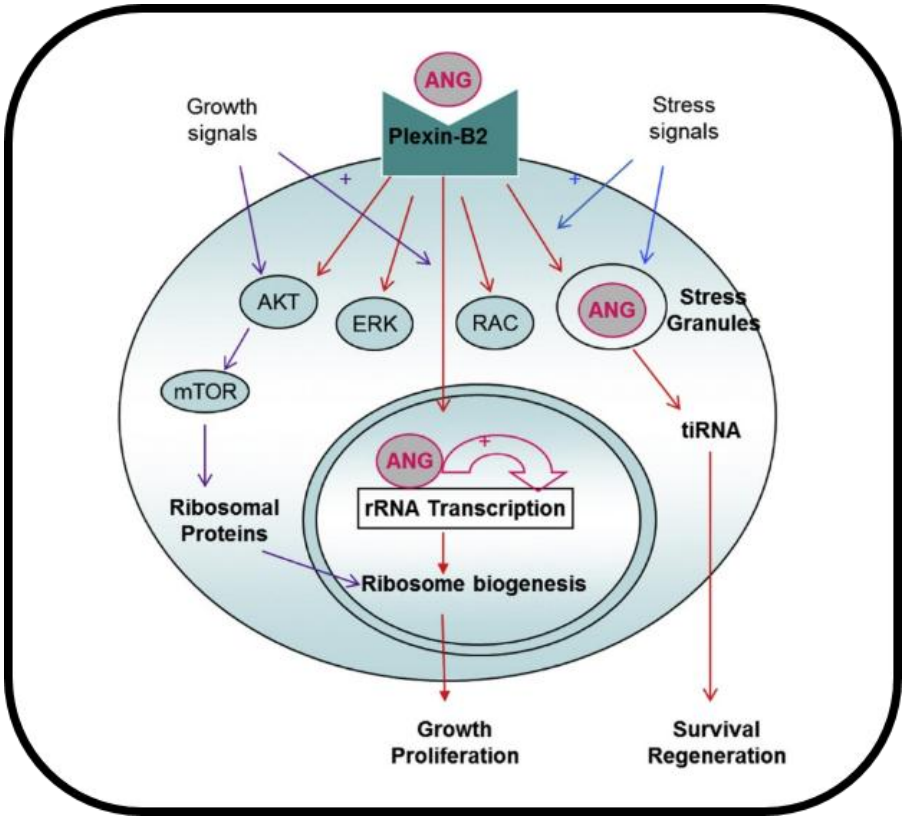

Figure 7. Potential ANG pathway for cell survival and proliferation. The figure is adapted from Xu et al.2017 [175].

Many other pro-angiogenic factors could also induce ECs proliferation and angiogenesis, but ANG-mediated rRNA transcription in ECs acts as a crossroad for numerous pro-angiogenic factors to stimulate angiogenesis [167]. At the moment, a large amount of pro-angiogenic factors have been found and investigated, such as vascular endothelial growth factor(VEGF) [176] , platelet-derived growth factor (PDGF) [177] , platelet-derived endothelial cell growth factor (PD-ECGF) [178], hepatocyte growth factor (HGF) [179], fibroblast growth factor (FGF) [180] , epidermal growth factor (EGF) [181] , placental growth factor (PlGF) [182], platelet-activating factor (PAF) [183] , interleukin-8 (IL8) [184] and ANG [167]. Most of them could promote angiogenesis by

supporting ECs and smooth muscle cell proliferation and migration [185]. During cell proliferation, along with the increase of cell proliferation rate, there is a corresponding increase in the protein synthesis rate [186] and ribosome synthesis [187, 188]. The ribosome synthesis is a process comprising rRNA transcription, processing and folding of the pre-rRNA and mature rRNA assembly with ribosomal proteins (RPs) [189]. During this process, the ultimately rate-limiting step of ribosome synthesis is the rRNA transcription [190], therefore induced-rRNA transcription in ECs is particularly important for its proliferation and angiogenesis, and ANG is a molecule that could regulate this step. Importantly, it has been shown that other pro-angiogenic factors (such as VEGF and bFGF) rely on ANG to induce rRNA transcription in ECs [167, 191, 192]. Moreover, inhibiting ANG does not only suppresses ANG-induced angiogenesis, it also inhibits angiogenesis induced by other pro-angiogenic factors [167]. Thus, ANG-mediated rRNA transcription in ECs may act as a crossroad in the angiogenesis process stimulated by numerous pro-angiogenic factors [167]. All of this indicates that ANG-mediated rRNA transcription in proliferating ECs is a key requirement for angiogenesis.

1.6.2 The role of Angiogenin in neuroprotection

The neuroprotection effects of ANG have been mainly described in neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS) [147] and Parkinson's disease [193] (PD). ALS is a motor neuron disease, which is associated with more than 20 gene mutations, such as those in the SOD1 gene or the loss-of-function mutations in the ANG gene. In terms of the mutations in the ANG gene, many of them affect the ribonuclease activity of ANG and its nuclear translocation, which leads to the inhibition of its angiogenic activity. Other mutations result in the inhibition of neurite pathfinding and the ending of neuroprotection.

A direct evidence supporting the functions of ANG in ALS is a study in which the treatment with human ANG (hANG) in SOD1 mutant mice not only extended their lifespan but also promoted motor neuron survival [147]. In this regard other investigations have shown that ANG can contribute to motor neuron survival under hypoxia conditions, excitotoxic injury, endoplasmic reticulum (ER) stress and trophic factor deprivation. For example, ANG could protect P19 EC cell-derived motor neurons from death under the hypoxia conditions [194]; ANG could protect cultured motor neurons from excitotoxic injury through the PI-3-kinase/Akt kinase pathway [147]; ANG also protected against cell death induced by ER stress and trophic factor deprivation [147]. Additionally, when motor neurons were treated with the inactive ANG variant, the neuroprotective effects described above were abolished, which indicates that the ribonuclease activity of ANG is crucial for the survival of motor neurons [147, 194]. However, it has been described that both increasing and abolishing the ribonuclease activity of ANG lead to neurites damage of motor neurons in ALS [195].

For the neuronal protection from oxidative stress, ANG can trigger the stress response program of the neurons. Briefly, ANG cleaves tRNAs into 5′ and 3′ functional small noncoding RNAs (5′- and 3′-tiRNA) at the position of the anticodon loop [171]. The tiRNAs combine with the Y-box binding protein 1 (YB-1), which is a translational silencer, inhibiting the translation initiation complex formation [196]. The tiRNAs can inhibit cap-independent and cap-dependent translation, which leads to the inhibition of global protein translation. They can also promote motor neuron survival also through the inhibition of apoptosis and the promotion of stress granule formation. Besides, the tRNA fragments could assume G-quadruplex (G4) structures which could promote the neuroprotective effects [197].

As a neuron-secreted factor, ANG also possesses neuroprotective effects through the paracrine signaling pathways after being endocytosed by astrocytes [198]. The conditioned medium derived from astrocytes treated with ANG promotes the survival of motor neurons under stress conditions.

Parkinson is a progressive movement disorder caused by the degeneration of the midbrain region which controls motor movement. Although there is evidence showing that PD is caused by the mutations of several single-genes, most of the PD forms are sporadic. The neuronal pathological hallmark of PD is the presence of Lewy bodies, which are formed mainly with the aggregated α-synuclein that has an important pathogenic role in PD [199]. PD and ALS are both defined as movement disorders diseases, but different neurons are degenerated in each disease: the dopaminergic neurons of the substantia nigra are degenerated in PD, while motor neurons are degenerated in ALS. The role of ANG in PD arose from the observation that some ALS patients with ANG variants showed PD symptoms [200]. Moreover, several ALS patients have a higher risk to develop PD. Other studies have shown that the overexpression of human α-synuclein in mice leads to an alteration of 200 genes expression, especially the mAng 1 gene expression which was 7.5-fold lower than in wild-type littermates [201], as a consequence, the mANG 1 protein was significantly reduced in this mouse PD model. Besides, in the cellular models (SH-SY5Y and M17) of PD induced by the neurotoxins rotenone and MPP+, ANG could protect against this dopaminergic neuronal cell death, and reduce the activation of caspase-3 [193].

Previous results from the Neurovascular Research Laboratory at VHIR have shown that the ANG level in the post-stroke ipsilateral brain hemisphere was higher than in the contralateral hemisphere, and increased after rehabilitation therapy in blood samples

related to good outcomes, suggesting that stroke could increase the expression of ANG being protective for the ischemic brain [202]. A more recent study has further shown that ANG is overexpressed after physical exercise in the SVZ of the ischemic hemisphere, which enhances the hypothesis that ANG can be protective for the ischemic brain, and suggests that ANG could interact with neurogenesis [203]. However, the studies of the potential neuroprotective and repair actions of ANG after stroke are lacking nowadays, though they are supported by the protective neuronal survival from oxidative stress in ALS [204] and the inhibition of neuronal apoptosis in PD by actions of ANG [193].

1.6.3 The role of Angiogenin in neurorepair

The definition of neurorepair is the restoration of the structure or the functions of the CNS after injury, such as in ischemic stroke. During this process, neurogenesis plays an important role. Neurogenesis is the process by which the neurons are produced by Neural Stem Cells (NSCs). For a long period, it was thought that there were no newborn neurons in the adult mammalian brain, but the first evidence of adult neurogenesis was presented by Joseph Altman in 1962, when his team found that there were new neurons formed in the cerebral cortex of adult rats [205]. And then, demonstrating that the adult neurogenesis could occur in the hippocampal dentate gyrus (DG), later in 1963 [206]. With a further study in 1969, he revealed that new neurons could also be born in the subventricular zone (SVZ) of adults, and then migrate into the olfactory bulb (OB), where they mature into local interneurons [207]. After years of research, it is accepted that adult neurogenesis occurs in two regions of the adult brain: in the subgranular zone (SGZ) of the hippocampal DG and in the SVZ of the lateral ventricles [208]. In the adult mammalian brain, it has been shown that during SGZ neurogenesis, the NSCs give birth to granule cells participating in memory formation and learning [209], while in the SVZ

the NSCs migrate through the rostral migratory stream towards the OB where they disperse radially and differentiate into functional interneurons [210, 211]. In adult humans, however, several researchers indicate that postnatal olfactory bulb neurogenesis in humans is much lower than in other mammals, very limited newborn neurons in the SVZ integrate into the OB [212] but integrate into the striatum close to the SVZ [213].

In stroke disease, neurogenesis is not only enhanced in the SVZ and the SGZ where new neurons are normally formed, it is also enhanced in the stroke injured striatum and cortex by increasing the migration and differentiation of neuroblasts from the SVZ and the SGZ attempting to repair itself [214]. Stroke-induced neurogenesis in areas of the adult brain where new neurons are not physiologically formed has been reported by numerous studies, showing that stroke could enhance the neuroblasts formation in the SVZ and stimulate these newborn neuroblasts to migrate into the injured striatum and differentiate into mature neurons [215-217]. In terms of neurogenesis in the cerebral cortex following stroke, studies in rodents demonstrate that stroke could trigger neuroblast migration from the SVZ to the cortical infarct where they differentiate into mature neurons [218-220]. In adult humans, however, only the existence of DNA fragmentation and DNA repair has been reported in cortical neurons shortly after stroke [221]. There is no direct evidence of cortical neurogenesis in adult humans following stroke.

Recent research has shown that angiogenesis plays an important role in the process of neurogenesis after ischemic stroke, as angiogenesis and neurogenesis are coupled in the post-stroke brain [222, 223]. Further studies demonstrate that there is a specialized microenvironment named neurovascular niche where the interaction between

angiogenesis and neurogenesis occurs [224-226]. Angiogenesis contributes to neurogenesis by participating in the proliferation of neural stem/progenitor cells, ad in the migration and differentiation of neuroblasts. It has been shown that the vasculature enhances the proliferation of neural stem/progenitor cells by expressing several endothelial cells-derived extracellular factors, such as VEGF and FGF-2 [222], among many other factors. Following the proliferation of NSCs, neuroblasts migrate from the SVZ to the peri-infarct area, where post-stroke angiogenesis occurs [222, 227]. Furthermore, neuroblasts migrate through blood vessels of peri-infarct areas where vascular remodeling occurs after stroke [224]. One explanation for this phenomenon is that the vasculature could supply oxygen, nutrients and soluble factors to support the migration of neuroblasts [228]; another explanation is that the vasculature could serve as a scaffold for neuroblasts migration [229, 230]. It has been reported that when angiogenesis is inhibited, the numbers of neuroblasts in the peri-infarct tissue are significantly reduced [224, 231]. Also that after participating in NSCs proliferation and migration, angiogenesis can also regulate the differentiation of NSCs by providing oxygen to alleviate the hypoxia [232]. Other authors have shown that angiogenesis was also associated with the functional improvement observed in ischemic animals subjected to daily physical exercise, and that this functional improvement was abolished by the administration of endostatin (an angiogenesis inhibitor) [233]. All of this demonstrates the important role of angiogenesis in post-stroke neurogenesis, indicating that angiogenesis could be a therapeutic target for neurogenesis to re-establish the function affected by stroke.

The central role of ANG in angiogenesis suggests that ANG could have an important role in post-stroke neurogenesis, becoming a therapeutic target for stroke. However, the

role of ANG in post-stroke neurogenesis has been less investigated. Some studies have shown that ANG could induce P19 cells neurospheres formation [175, 234], suggesting its implication in neuronal differentiation. Moreover, ANG could induce mouse embryonic stem cells to differentiate into GFAP-positive progenitor neurons [234]. and a recent study from the Neurovascular Research Laboratory at VHIR has shown thatANG increases the NSC yields of SVZ-derived neurosphere cultures, suggesting its potential role in neuronal proliferation [203].

2. Objectives

2.1. To prove the feasibility and safety of hr-ANG administration after ischemic stroke in a pre-clinical mouse model.

2.2. To determine the therapeutic actions of early hr-ANG treatment after ischemic stroke in a pre-clinical mouse model.

2.3. To determine the mechanisms of action of hr-ANG treatment in the post-stroke brain.

3. Methods

3.1 Ribonucleolytic activity zymogram

Zymogram electrophoresis was used to confirm ribonuclease activity of the hr-ANG used in the present investigation. Purified hr-ANG produced in *E.Coli* (265AN050/CF, R&D Systems, USA) was dissolved in sterile PBS. A total amount of 5µg, 10ug, 15ug hr-ANG and a PBS negative control samples were subjected to standard SDS-PAGE electrophoresis. Briefly, hr-ANG samples were mixed with 2x Laemmli Sample Buffer (BIO-RAD, USA) in non-reducing agent conditions. Gels used contained 15% polyacrylamide co-polymerized with 0.3mg/mL poly(C) (P4903-25MG, Sigma-Aldrich, USA), which is a substrate for ANG and RNase A. After electrophoresis, SDS was removed from the gel by washing (4 x 15 min) with 10 mM Tris-HCl buffer (pH 8.0) containing 2-propanol (20% v/v), followed by washing (2 x 15 min) with 10 mM Tris-HCl buffer (pH 8.0). Then the gel was incubated into 0.1 M Tris-HCl buffer (pH 8.0) overnight at room temperature (RT) on a shaker. After washing (1 x 10 min) with 10 mM Tris-HCl buffer (pH 8.0), the gel was stained with 10 mM Tris-HCl buffer (pH 8.0) containing 0.2% toluidine blue (89640-5G, Sigma-Aldrich, USA) for 30 min and followed by washes with 10 mM Tris-HCl buffer (pH 8.0) until an optimal contrast was achieved. Consequently, regions in the gel corresponding to proteins with ribonuclease activity appear as a clear band in a dark purple background in visible light. All the materials and reagents used were ribonuclease-free certified. A molecular weight marker (# 161-0374, BIO-RAD, USA) was also run for reference values.

3.2 Animals

All experimental procedures in animals were approved by the Animal Ethics Committee of Vall d'Hebron Institut de Recerca (CEEA 21/16) according with the Spanish legislation and the Directives of the European Union. C57BL/6 male mice (8 to 10 weeks old) were purchased from Janvier Laboratory (Saint Berthevin Cedex, France). Mice were housed in a temperature/humidity-controlled room and maintained on a 12-hour light-dark cycle and given water and food ad libitum. Buprenorphine (0.05 mg/kg, s.c, Divasa Farma-Vic S.A, Spain) was administered to all ischemic mice to minimize pain and discomfort. Isoflurane (4% for induction, 1-2% for maintenance in medicinal air, Abbot Laboratories, Spain) was given via facemask during all surgical procedures described below. All experiments were conducted by animal randomization to the treatment group and hr-ANG was administered in a *blind manner*. In this thesis a total of 158 mice were used to complete the whole study:

First, to prove the administration route and safety of hr-ANG administration after ischemic stroke in a pre-clinical mouse model, 45 mice were needed. Among them, 7 were excluded because of the failure of blood collection. Second, to determine the therapeutic actions and mechanisms of early hr-ANG treatment after ischemic stroke in a pre-clinical mouse model, 113 mice were needed. Among them, 16 were excluded because of death during the experimental protocol (n=14), uncertain treatment (n=1), or failure of brain collection (n=1).

3.3 Human recombinant Angiogenin administration protocols

Purified hr-ANG was dissolved in sterile PBS according to the manufacturer's instructions, aliquoted in stock solution at (100ug/mL) and stored at -80ºC until further use.

To prove the best route administration and treatment safety of hr-ANG administration, the protocol A was performed, in which naïve mice were given 1ug or 5ug via i.p. or i.v. injections or vehicle (saline) in 210µl as shown in **Figure 8 A,** and further euthanized at different time-points (1h, 6h or 24h) when brain and blood samples were collected following protocols details in section 3.6.

To determine the therapeutic actions and mechanisms of early hr-ANG treatment after ischemic stroke in a pre-clinical mouse model, 4 experimental protocols (B, C, D, E) were performed with the middle cerebral artery occlusion for 75 minutes and reperfusion model (surgical procedures described in section 3.4); see **Figure 8 B to E**. In protocol B, to test the dose-response on brain injury, mice were given hr-ANG (5ug or 10ug, i.p. injection) or vehicle 24h after reperfusion and euthanized 48h after ischemia. In protocol C, to study early molecular responses to hr-ANG treatment, mice were given hr-ANG (5ug or 10ug, i.p. injection) or vehicle 24h after reperfusion and euthanized 27h after ischemia (and 3h after treatment). In protocol D, an alternative neuroprotective therapeutic time point was tested and mice were given hr-ANG (5ug, i.p. injection) or vehicle acutely 15min after reperfusion and euthanized 48h after ischemia. Finally, to study long-term effects, in protocol E mice were given hr-ANG (5ug, i.p. injection) or vehicle 24h after reperfusion followed by additional administration every 3 days, and euthanasia 13 days after ischemia.

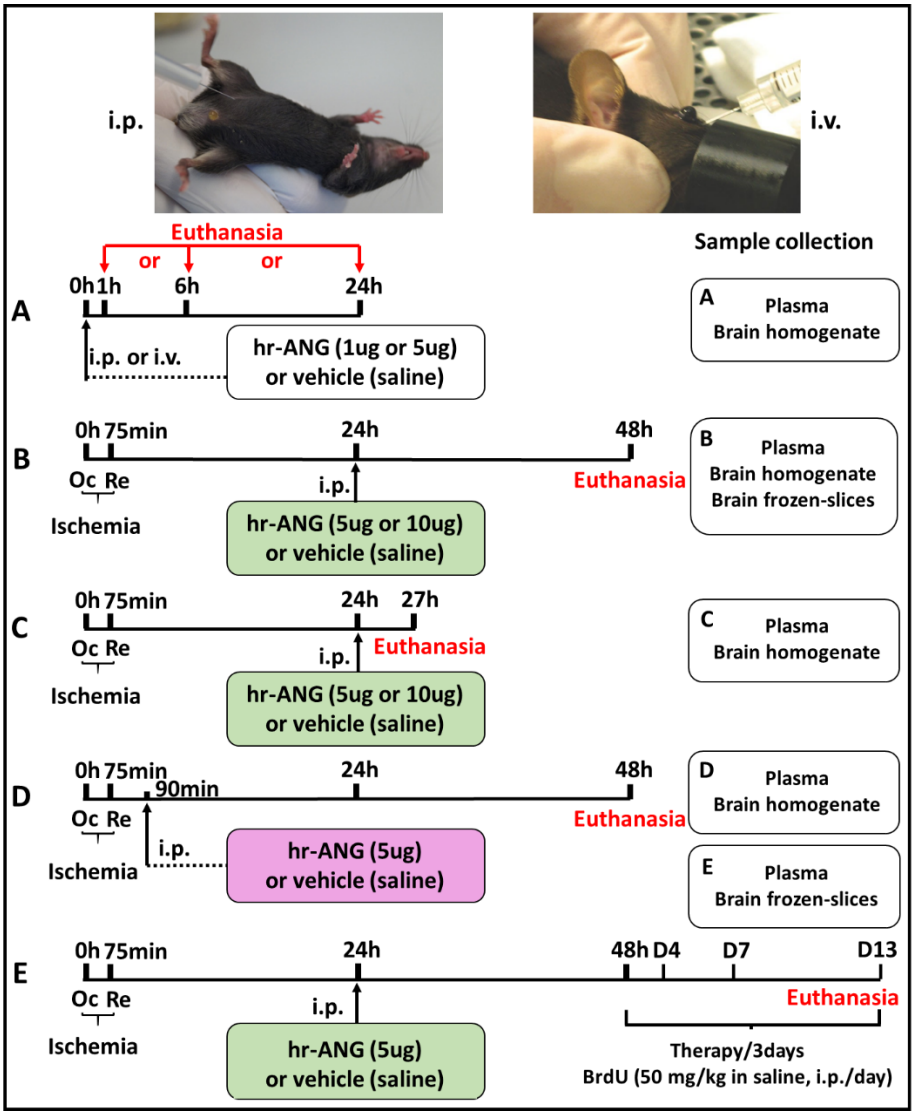

Figure 8. **Hr-ANG administration protocols conducted in the present study. (A)** Naïve mice were treated hr-ANG or saline i.p. or i.v. and euthanized at different time-points post-injection. Mice in B, C, D and E were induced for 75min occlusion of the proximal middle cerebral artery: in **(B)** were treated with either hr-ANG or vehicle i.p. 24h after occlusion and euthanized 48h after occlusion, in **(C)** mice were given hr-ANG or vehicle i.p. 24h after occlusion and euthanized 27h after occlusion, in **(D)** mice were treated with 5ug or vehicle i.p. 15min after reperfusion and euthanized 48h after ischemia, and in **(E)** mice treated with either hr-ANG or vehicle i.p. 24h after reperfusion received additional treatment every 3 days, and were euthanized 13 days after occlusion. i.p., intraperitoneal; i.v., intravenous; h, hour; D, day; Oc, occlusion; Re, reperdusion. The images of i.p. and i.v. (retro-orbital route) injection in mice are adapted from Tal et al. 2011 [235].

3.4 Middle cerebral artery occlusion and reperfusion model

The surgical procedures were conducted under body temperature control using a rectal probe connected to a thermoregulatory pad and a cortical cerebral blood flow (CBF) monitoring for the arterial occlusion and reperfusion. Briefly, mice were anesthetized with isofluorane (4% for induction, 1-2% for maintenance in medicinal air, Abbot Laboratories, Spain) via face-mask, and eyes were protected using an ophthalmic ointment (LipolacTM, Angelini Farmaceutica, Spain). The regional cortical CBF was monitored close to the region irrigated by the middle cerebral artery(MCA) by affixing a laser Doppler probe (Moor Instruments, UK) to the skull. Then the mice were placed in a supine position, a 1 cm long midline incision on the neck was made followed by the exposition of the surgical field and the identification of the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). Arteries were carefully dissected free from surrounding nerves and fascia. Later, the superior thyroid artery (STA) branches of the ECA/ICA were coagulated permanently. The ECA was further ligated distally as well as the bifurcation of the CCA with an 8-0 silk suture (Laboratorio Aragó, S.L) and a vascular clamp was placed at the ICA level. The ECA was cut followed by the insertion of an intraluminal filament (Doccol 602256PK10Re) through the ECA stump into the ICA, afterward, the clamp from the ICA was removed and the filament was inserted for a distance of 9-10 mm to occlude the proximal MCA. The CBF was continuously monitored by the laser-doppler flowmetry instrument with a probe placed distally in the MCA cortical territory. The proper occlusion was achieved when a decrease in the blood flow was observed during the filament insertion. Finally, the incision on the neck was gently sutured and the mice were allowed to recover from anesthesia at 37ºC in a recovery box. The occlusion of the MCA territory was maintained for 75min, and then the mice were re-anesthetized for reperfusion, the filament was

withdrawn from the ICA and the transient CCA ligation was removed, while the ECA remained permanently occluded. The CBF was monitored during occlusion and reperfusion following this criteria: only mice with an 80% reduction of CBF after filament insertion and a 75% recovery after filament removal were included in the study. The neck incision was sutured and mice were returned to cages to allow controlled recovery from anesthesia. Buprenorphine (0.05 mg/kg, s.c, Divasa Farma-Vic S.A, Spain) was administered to minimize pain and discomfort after surgery and during the next 48 hours. The mice were euthanized according to different protocols detailed in section 3.3, and corresponding samples were collected as detailed in section 3.6.

3.5 Neuroscore test

The neurological score was used to evaluate the neurological deficits after cerebral ischemia. Mice in protocols B and D were scored at 24h and 48h after ischemia, in protocol C were scored 24h and 27h after ischemia and in protocol E were scored 24h after ischemia followed by daily neurological score evaluation until euthanasia. The mice were scored with a neurological function scale adapted from previous studies [236] (xx) as detailed in **Table 1** Scoring different functional areas.

Table 1. Neurological function scale. The scale is adapted from Wayne et al. 1997 [236]

	0	1	2	3	4
1. Hair	Normal	Localized disorder, especially around eyes and nose	Generalized disorder, ruffled and/or dirty fur	-	-
2. Ears	Normal position	Transient lope-eared	Steady lope-eared	-	-
3. Eyes	Normal	Rheumy or dark discharge	Closed	-	-
4. Posture	Normal	Hunched unstable	Upright head and/or body rests on ground	Lies on side, can assume prone position with strain	-
5. Spontaneous activity	Normal	Calm, quiet, explores slowly	Inert somnolent, not exploring	No spontaneous movements during1 minute	-
6. Body symmetry (open beach top)	Normal	Slight asymmetry	Moderate asymmetry	-	-
7. Gait (open beach top)	Normal	Stiff, inflexible	Limping	Trembling, drifting, falling	Does not walk spontaneou sly
8. Climbing gripping surface, 45° angle)	Normal	Climbs with difficulty, limb weakness present	Holds onto slope, does not slip or climb	Slides down slope, unsuccessful effort to prevent fall	-
9. Circling behaviour (open beach top)	No circling behaviour	Predominantly one-sided turns	Circles to one side, although not constantly	Circles constantly to one side	-
10. Forelimb symmetry (mouse suspended by its tail)	Normal	Light asymmetry	Marked asymmetry	Prominent asymmetry	Slight asymmetry, no body/limb movement
11. Compulsory circlingnt **(limbs on bench, rear suspended by tail**	Absent	Tendency to turn to one side	Circles only to one side	Pivots to one side sluggishly and does not rotate in a full circle	-
12. Whisker response (light touch from Behind)	Normal symmetrica l response	Light asymmetry	Prominent asymmetry	Absent response ipsilaterally, diminished contralaterally	Absent response bilaterally
13. Gripping test of the forepaws	Normal	less power of the impaired forepaw	cannot grip with the impaired forepaw	cannot grip the grid	-

3.6 Brain and plasma collection and processing

All mice were deeply anesthetized at their respective times and blood samples were collected through cardiac puncture in EDTA collection tubes and centrifuged at 12000 rpm at 4 °C for 12 min. The supernatant plasma fraction was collected and stored at −80 °C until further use. Then, mice were transcardially perfused with cold saline to remove blood from brain vessels and, immediately after perfusion, brains were quickly removed. The intact brains of treated naïve mice from protocol A were carefully dissected to isolate the right and left hemispheres separately and collected into Eppendorf tubes, which were flash-frozen in liquid nitrogen and stored at −80 °C until further use. The brains of ischemic mice from protocols B, C and D were subjected to TTC stain as described in section 3.7. The TTC-stained sections were then carefully dissected to isolate the right (ipsilateral, ip) and left (contralateral, cl) hemispheres separately, each of them was collected into an Eppendorf tube, which were flash-frozen in liquid nitrogen and stored at −80 °C until further use. A sub-group of brains from protocol B and those from protocol E were obtained for immunofluorescence studies as described below: after intracardiac perfusion with saline brains were removed, fixed with 4% PFA overnight followed by 30% sucrose for cryoprotection, embedded in OCT (Tissue-Tek, Fisher Scientific, US). Later brain were cut into 12um-thick coronal slices in a cryostat and frozen at -80ºC until use.

3.7 Infarct volume and intracerebral hemorrhage evaluation.

The ischemic mice brains from protocols B, C and D were sectioned into 1mm-thick coronal sections using an acrylic matrix in cold conditions and further stained with 2.5% of 2,3,4-triphenyl-2H-tetrazolium chloride (TTC; Sigma, MO, USA) in saline for 15

minutes at RT. Then the TTC solution was replaced by cold saline and images of the complete stained slides were acquired by a CanoScan 4200F (Canon, Japan) for further analysis. The TTC-stained sections were then carefully dissected to isolate the right (ipsilateral, ip) and left (contralateral, cl) hemispheres separately, each hemisphere was then collected into an Eppendorf tube, flash-frozen in liquid nitrogen and stored at −80 °C until further use. Infarct volume was measured by the ImageJ free software, and calculated by integration of the white lesion areas and considering the average of anterior and posterior views. Infarct percentage was defined as infarct volume divided by the total hemisphere volume. The presence of intracerebral hemorrhage was evaluated by 3 different methods: (1) Classification by visual score as previously described [237] as no-hemorrhagic transformation=0; hemorrhagic infarction type 1 (HI-1) =1; hemorrhagic infarction type 2 (HI-2) =2; parenchymal hemorrhagic type 1(PH-1) =3; parenchymal hemorrhagic type 2(PH-2) =4; (2) Measuring the parenchymal hemorrhage area by integration of hemorrhagic area with ImageJ free software and expressed in square millimeters (mm^2) and (3) classification by counting the total number of hemorrhagic events. Both infarct volume measurement and intracerebral hemorrhage evaluation were conducted in a blinded manner for treatment group.

3.8 Protein and RNA extraction

Brains obtained from naïve mice were homogenized and lysed with freshly prepared ice-cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl2, 0.05% BRIJ-35, 0.02% NaN3, 1% Triton X-100, 1% phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich, USA) and 0.5% aprotinin (Sigma-Aldrich, USA). Homogenates were centrifuged at 12,000 rpm at 4°C for 12 min and the protein fraction in the supernatants

assessed by the bicinchoninic acid assay (BCA, Thermo Fisher Scientific Inc., USA). The TTC-stained brain sections of the ipsilateral or the contralateral hemispheres from protocols B, C and D were homogenized and total fractions of both protein and RNA were isolated using the MirVana™ Paris™ Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. The protein fractions were assessed by the BCA assay, and the RNA quality and quantity were measured by the Nanodrop Spectrophotometer. RNA and protein fractions were kept at −80 °C until further use.

3.9 Angiogenin ELISA

The enzyme-linked immunosorbent assay (ELISA) was used to determine the administered hr-ANG and endogenous mouse-ANG levels in the plasma and the brain protein fractions described above. Total protein was determined by duplicate in each sample by the BCA assay. The plasma of treated naïve mice from protocol A and ischemic mice from protocol B were tested with the human Angiogenin Quantikine ELISA Kit (DAN00, R&D SYSTEMS, MN, USA), while their brain samples were tested with the human Angiogenin SimpleStep ELISA® Kit (ab219629, abcam, UK) which is more sensitive. Additionally, the brain samples of protocol B were also tested with the mouse Angiogenin SimpleStep ELISA® Kit (ab208349, abcam, UK). All ELISA plates were performed following the manufacturer’s instructions. In brief, 200ul of the diluted plasma sample (1:20) and 50ul of the diluted brain sample (1:1) were assayed per duplicate and the mean value was used for analysis. Optical densities were measured by Synergy TM Mx microplate reader (BioTek Instruments Inc, USA). Samples with a coefficient of variation (CV) higher than 20% were excluded from the analysis. The plasma ANG level was expressed in pg/ml, whereas the brain ANG level was adjusted

by total protein and expressed in pg of ANG/ug total protein.

3.10 Western Blot

The TTC-stained brain sections from protocols B, C and D were processed as described in section 3.11 to obtain the protein content and assessed by the BCA assay. A total amount of 30µg of protein was mixed with 4x Laemmli Buffer (BIO-RAD, USA) and 5% of 2-mercaptoethanol, heated at 95ºC for 5 minutes, subjected to electrophoresis (100V, 100 min) in 10% polyacrylamide electrophoresis gels (for AKT, phospho-AKT, Erk1/2 and phospho-Erk1/2 western blots) or electrophoresis (100V, 120 min) in 12% polyacrylamide electrophoresis gels (for caspase-3, cleaved caspase-3, cleaved caspase-9, Bax and Bcl-2 western blots) and transferred (1.3A, 25V, 10 min) into nitrocellulose membranes (BIO-RAD, USA) using the Trans-Blot Turbo (BIO-RAD, USA). Then, membranes were blocked for 1 hour with 10% non-fat milk (in PBS, 0.1% Tween 20, Sigma-Aldrich, USA) and incubated overnight at 4ºC on a shaker with the following antibodies: anti-AKT (1:1000, 9272, Cell Signaling, USA), anti-phospho-AKT (1:500, 4060, Cell Signaling, USA), anti-Erk1/2 (1:2000, 4695, Cell Signaling, USA), anti-phospho-Erk1/2 (1:500, 9101, Cell Signaling, USA), anti-caspase-3 (1:500, 9662, Cell Signaling, USA), anti-cleaved caspase-3 (1:500, STJ97448, St John's laboratory, UK), anti-cleaved caspase-9 (1:250, 9509, Cell Signaling, USA), anti-Bax (1:1000, 2772, Cell Signaling, USA), anti-Bcl-2 (1:300, 3498, Cell Signaling, USA), anti-Tubulin (1:2000, T6199, Sigma-Aldrich, USA). The membranes were then washed 3 times with PBST (PBS-0.1% Tween 20) and incubated with corresponding secondary antibodies at 1:2000 for 1 hour at RT with gentle agitation. The content of IgG in the brain of mice from protocol B was also investigated by using 10% polyacrylamide electrophoresis gels

following the same protocol, while only incubated with the secondary antibody (sheep-anti-mouse IgG Horseradish Peroxidase, 1:1000, NA931, GE Healthcare, USA) overnight at 4ºC on a shaker. Finally, all membranes were washed 3 times with PBST (PBS-0.1%Tween 20) and briefly incubated with Pierce® ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., USA) to visualize the chemiluminescence signal with the Odyssey® Fc Imaging System (LI-COR, Inc., USA). Molecular weight marker (# 161-0374, BIO-RAD, USA) was also run for reference values.

3.11 Quantitative reverse transcriptase PCR (qRT-PCR) for 45S ribosomal RNA

The TTC stained sections from protocols B and C were used to extract total RNA as described above in section 3.11. The quality and quantity of RNA were measured by the Nanodrop Spectrophotometer method and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). A thermocycler (Thermal Cycler 2720, Applied Biosystems, USA) was used for the retrotranscription (10 min at 25 °C, 120 min at 37 °C, and 5 sec at 85 °C). Finally, the samples were preserved at 4 °C until use or frozen at −80 °C. RT-PCR reaction was performed using a mixture of 5 µL of TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific Inc.), 0.5 µL of TaqMan® Gene Expression Assay (Gapdh: Mm99999915_g1, 45S rRNA: Mm03985792_s1; Thermo Fisher Scientific Inc.), 3.5 µL of RNase-free water and 1 µL of cDNA sample. A sample calibrator consisting of cDNA of naïve mouse brain was in all plates to compare samples between different assays. Samples were run in triplicate using 384-well plates on 7900HT Fast Real-Time PCR

System (Applied Biosystems, Foster City, CA, USA). Gene expression results were expressed in RQ (relative quantification) using the Livak (ΔΔct) method.

3.12 Brain Immunofluorescence

Protocol E was designed to evaluate ANG effects on neurogenesis and angiogenesis 2 weeks after MCAO including the daily intraperitoneal administration of 5-Bromo-2′-deoxyuridine (BrdU, 50 mg/kg in saline, B9285, Sigma-Aldrich, MO, USA) beginning 48 h after MCAo and until euthanasia to label dividing cells. At the end of the study and before euthanasia mice were injected intravenously (retro-orbitally) with Dylight 594-labeled tomato lectin (80 µg/mouse, DL-1177, Vector Laboratories, USA), which was allowed to circulate for 10min to label functional blood vessels, followed by a saline intracardiac perfusion. Additionally, the brains from protocol E were also fixed with 4% PFA overnight followed by 30% sucrose for cryoprotection, embedded in OCT, cut into 12um-thick coronal slices in a cryostat and frozen at -80ºC until use. Later, 3 consecutive coronal slices of the SVZ and 3 from the hippocampal areas were subjected to the immunofluorescence protocol. Briefly, the slices were placed at RT for 30 min, washed 3 times (5min in 0.1% PBS-Tween, 5min 0.3%-PBS-Triton X-100 and 5min 0.1% PBS-Tween), further incubated for 1 hour with 2 M HCl-PBS followed by 10 min in 0.1 M borate buffer and 5 min in 0.1% PBS-Tween for detection of nuclear BrdU of dividing cells. The Sections were then blocked with 0.1% PBS-Tween containing 1% BSA (Sigma-Aldrich, MO, USA) and 5% goat serum (Merck Millipore, MA, USA) for 1 hour, followed by incubating overnight at 4ºC with the following antibodies: 1:200 rabbit anti-DCX (ab207175, Abcam, UK), 1:100 rat anti-BrdU (ab6326, Abcam, UK). The slices were then washed 3 times for 5min at RT with 0.1% PBS-Tween followed by incubating

for 1 hour at RT with the following secondary antibodies: Alexa fluor 488 goat anti-rabbit IgG (1:500), Alexa fluor 647 goat anti-rat IgG (1:500). The slices were then washed 3 times for 5 min at RT with 0.1% PBS-Tween, mounted in Vectashield™ with DAPI (Vector Laboratories, USA). The whole coronal slices (including SVZ or hippocampus) were imaged with the Pannoramic 250 FLASH (3D HISTECH). The total area with DCX+, Brdu+ or Lectin + fluorescence in of ROIs defined in the cortical and subcortical areas was quantified and analyzed with the ImageJ free software and expressed as mm^2.

3.13 Statistical Analyses

Both SPSS 20.0 package and Graph Pad Software were used for all statistical analyses. The Graph Pad Software was used for graph representations. The normality of continuous variables was assessed by the Shapiro-Wilk test ($N<30$). Normally-distributed variables were analyzed by ANOVA (followed by Tukey post hoc), or unpaired t-test. Whereas non-normally distributed variables were analyzed by Kruskal-Wallis test and/or Mann-Whitney U-test. The contingency tables were analyzed by Pearson's chi-square test. Correlations were evaluated using Pearson's coefficient for normally distributed variables. Bar graphs represent mean±SD and the results with a p-value less than 0.05 were considered statistically significant.

4. Results

4.1 The administered hr-ANG is bioactive, presents better plasmatic bioavailability when administered intraperitoneally and reaches the brain tissue.

First, we examined the bioactivity of the administrated hr-ANG by its ribonuclease activity, which is crucial for its bio-functions. As shown in **Figure 9A**, all tested amounts (5ug, 10ug and 15ug total hr-ANG) displayed catalytic activity for the poly(C) substrate present in the zymogram gel, and the catalytic efficiency was dose-dependent as shown by the band area quantifications as shown in **Figure 9B**; the PBS served as a negative control without catalytic activity for the hr-ANG band (≈14kDa).

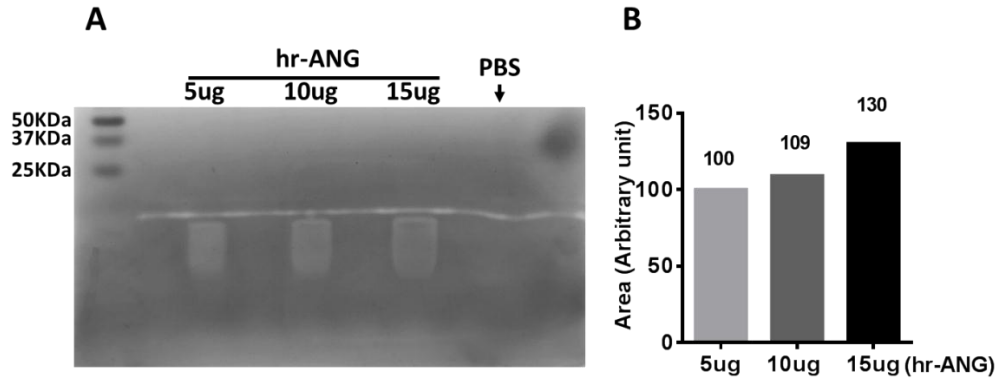

Figure 9. Bioactivity of the administered hr-ANG. (**A**) Representative image of ribonucleolytic activity in a Poly(C) zymogram, the white bands in a dark background indicate the regions in the gel corresponding to proteins with ribonuclease activity matching with the hr-ANG molecular weight (≈14KDa). (**B**) Bar graph representing the catalytic activity of the hr-ANG band area of the tested amounts (5ug, 10ug and 15ug total hr-ANG). Area is represented as percentage vs. 5ug Arbitrary Units.

Then, we investigated the best administration route for the hr-ANG administration in naïve mice following (protocol A) as shown in **Figure 10A** by administering increasing hr-ANG doses intraperitoneally or intravenously. The bioavailability in blood was analyzed by ELISA, see **Figure 10B**, showing that the administrated hr-ANG (1ug or 5ug) could be detected in plasma at different time-points (1h, 6h and 24h) post-administration (i.p. and i.v.), but significantly decreasing over time ($p= 0.029$ for the 5ug i.p. 1h vs 24h; $p= 0.015$ and $p= 0.057$ for the 1ug i.p. 1h vs 6h and 24h respectively). Moreover, the study also showed higher plasma hr-ANG levels when administered i.p. vs, i.v. (at 6h and 24h, $p= 0.019$ and $p = 0.007$ respectively for the 1 ug dose). Importantly no mice died after the administration during the follow-up period.

To prove the *in vivo* bioactive actions fo the administered hr-ANG we further examined several cell survival-pathway molecules known to be regulated by the ribonuclease actions of ANG (such as AKT, ERK and their phosphorylated forms) in brains of naïve mice treated with hr-ANG (1ug, i.p.) or vehicle following protocol A at the corresponding time-points (1h, 6h and 24h) by Western-Blot (see **Figure 10C**). The results showed no changes in the p-AKT/AKT ratio ($p=0.992$, see **Figure 10D**) whereas p-ERK/ERK ratio transiently increased at 1h ($p=0.035$ vs. naïve), to further decrease to naïve levels at 6h ($p=0.017$ vs. 1h), which were maintained at 24h; see **Figure 10E.**

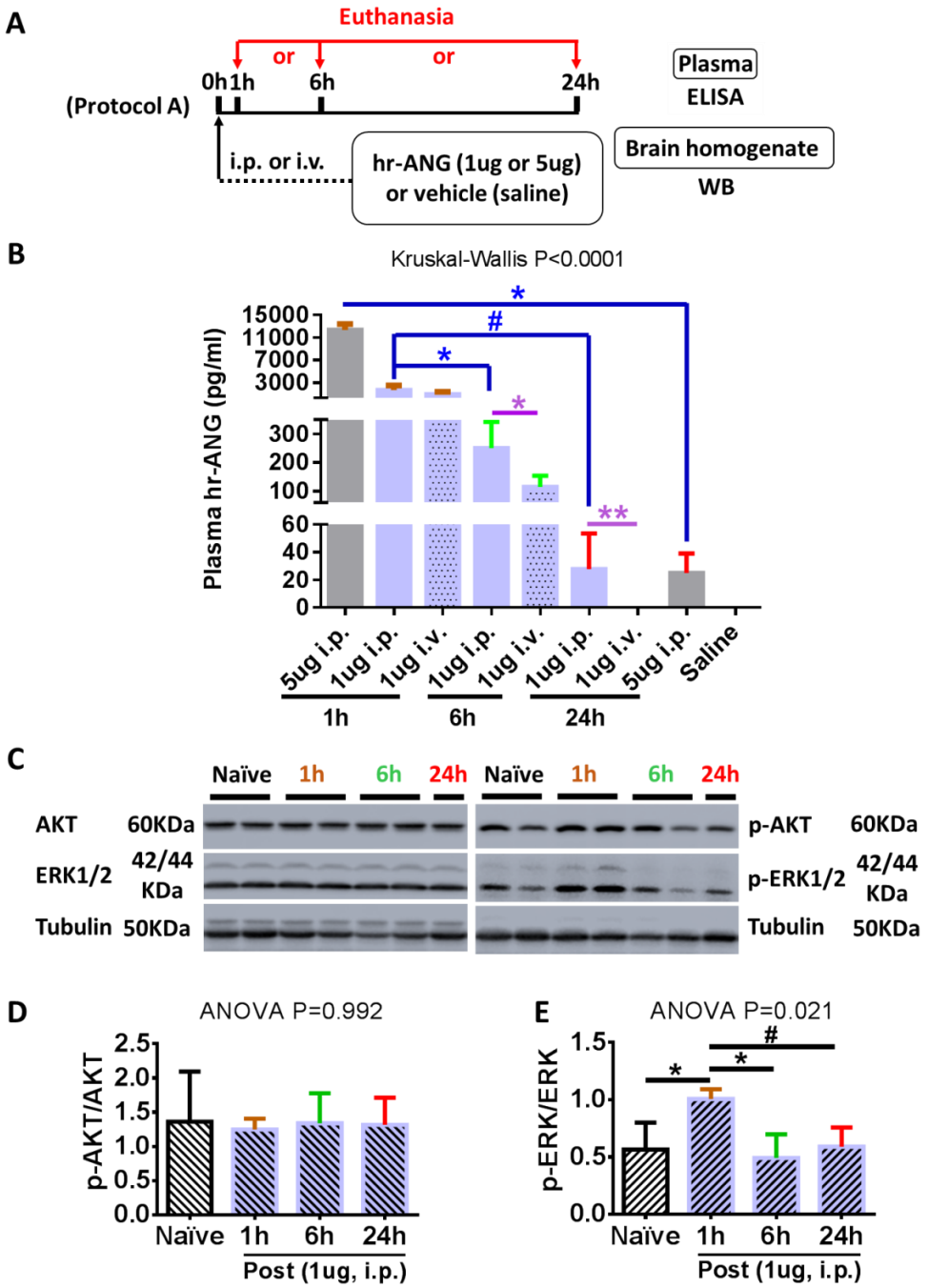

Figure 10. The plasma kinetics and *in vivo* brain bioactivity of the administered hr-ANG in naïve mice. (**A**) Timeline of the experimental procedure of the pharmacokinetics study. (**B**)Bar graph representing the plasma hr-ANG levels at different time-points (1h, 6h or 24h) post-administration (i.p. or i.v.) in naïve mice (n=4/group). (**C**) Representative images of Western-Blot of AKT, p-AKT, ERK1/2 and p-ERK1/2. (**D**) and (**E**)

Bar graphs representing the brain p-AKT/AKT and p-ERK/ERK ratios respectively at different time-points (1h, 6h and 24h post-administration of 1ug hr-ANG, i.p.) in naïve mice. Data is presented as mean±SD; $^{\#}p < 0.1$, *p < 0.05 and **p < 0.01 as indicated by the horizontal lines;$^{\#}$p, *p,*p and **p represent no-adjusted p-values; h, hour; i.p., intraperitoneal; i.v., intravenous; hr-ANG, human recombinant angiogenin.

We then examined the bioavailability of hr-ANG both in plasma and brain tissue after cerebral ischemia after hr-ANG administration at 24h following the experimental protocol B described in **Figure 11A.** The administration of both 5ug and 10ug hr-ANG caused a significant increase in plasma compared with vehicle-treated mice ($p = 0.025$ and $p < 0.0001$ respectively) as shown in **Figure 11B**. Additionally, whereas endogenous mouse brain ANG did not change between groups ($p = 0.141$, **Figure 11C**) the mice receiving hr-ANG therapy presented human ANG in the ipsilateral ischemic hemisphere, being significant for the 10ug group when compared to vehicle ($p = 0.008$) as shown in **Figure 11D**. Finally, the amount of hr-ANG protein detected in the ipsilateral hemisphere was found associated with the extension of intracranial hemorrhages (in saline-perfused brains) ($R=0.831$, $p= 0.002$)as shown in **Figure 11E**.

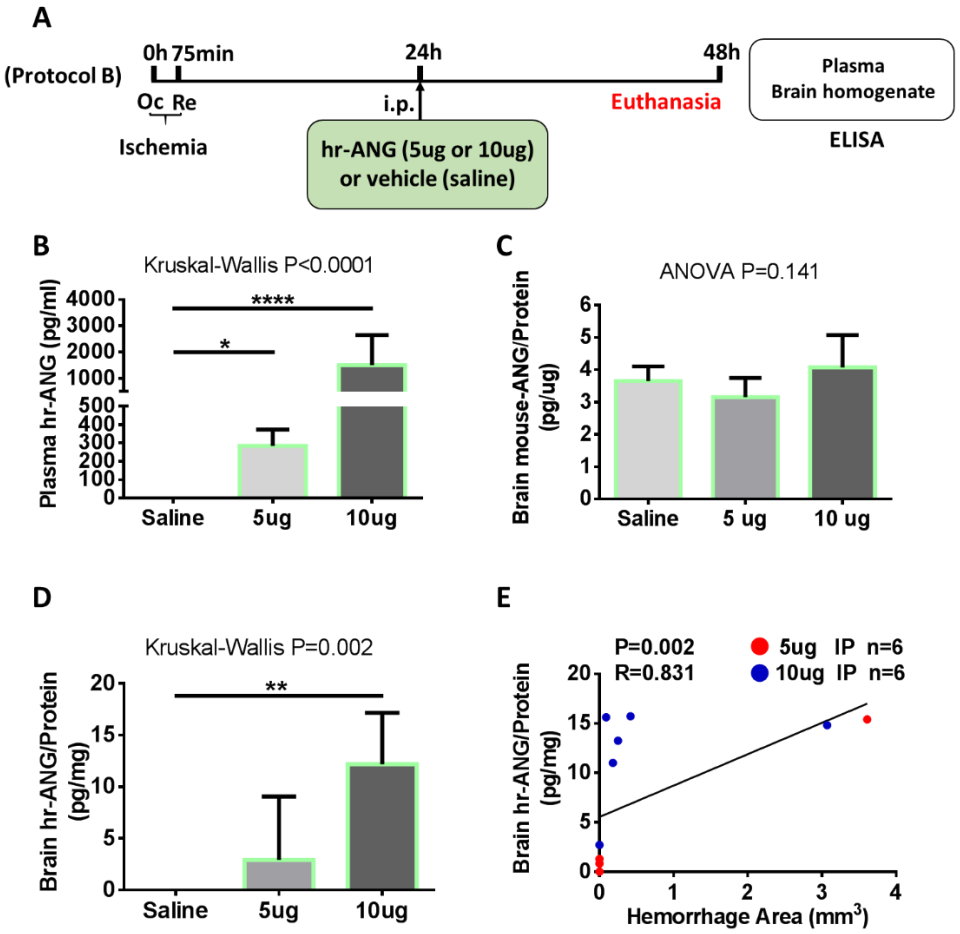

Figure 11. Study of the plasma and brain ANG levels after hr-ANG therapy. (**A**) Timeline of the experimental procedure. (**B**) Bar graph representing plasma hr-ANG levels at 24h post-administration (saline-treated, n=8; 5ug hr-ANG-treated, n=9; 10ug hr-ANG-treated, n=6). (**C**)Bar graph representing the ipsilateral mouse-ANG brain levels at 24h post-injection (saline-treated, n=5; 5ug hr-ANG-treated, n=6; 10ug hr-ANG-treated, n=5). (**D**) Bar graph representing the ipsilateral hr-ANG brain levels at 24h post-administration (saline-treated, n=4; 5ug hr-ANG-treated, n=6; 10ug hr-ANG-treated, n=6). (**E**) Scatter plot showing the correlation of hr-ANG levels with the hemorrhage area in the ipsilateral brain hemisphere at 24h post-injection (n=6/group). Bar graphs represent mean±SD; *p < 0.05, **p < 0.01 and ****p < 0.0001 as indicated by the horizontal lines;h, hour; min, minute; i.p., intraperitoneal; Oc, occlusion; Re, reperfusion; hr-ANG, human recombinant angiogenin.

4.2 The hr-ANG treatment reduces infarct volume dose-dependently.

Following protocol B, as shown in **Figure 12A**, subacute administration (24h post-ischemia) of 5ug hr-ANG significantly reduced total and cortical infarct percentage at 48h post-ischemia compared with vehicle-treated mice (38.4±13.4 vs 51.5±10.0, p = 0.029 and 24.2±12.0 vs 34.9±7.9, p = 0.039 respectively). However, the 10 ug dose did not show any change on infarct percentage (total, p= 0.470, cortical, p= 0.386 and subcortical p= 0.986**)**.

The protocol C as shown in **Figure 12B** was conducted to investigate early cell survival mechanisms (3h post-treatment) as explained in the next section 4.4, when infarct size was still similar among groups (total, p = 0.421; cortical, p = 0.489; subcortical, p = 0.427).

Later the acute hr-ANG therapy was investigated following protocol D, as shown in **Figure 13A,** when 5ug of hr-ANG was administered 15 minutes after reperfusion which did not reduce the total and cortical infarct percentage (total, p = 0.061; cortical, p = 0.196), but significantly reduced subcortical infarct size at 48h post-ischemia compared with vehicle-treated mice (12.9±2.1 vs 15.1±2.9, p = 0.029).

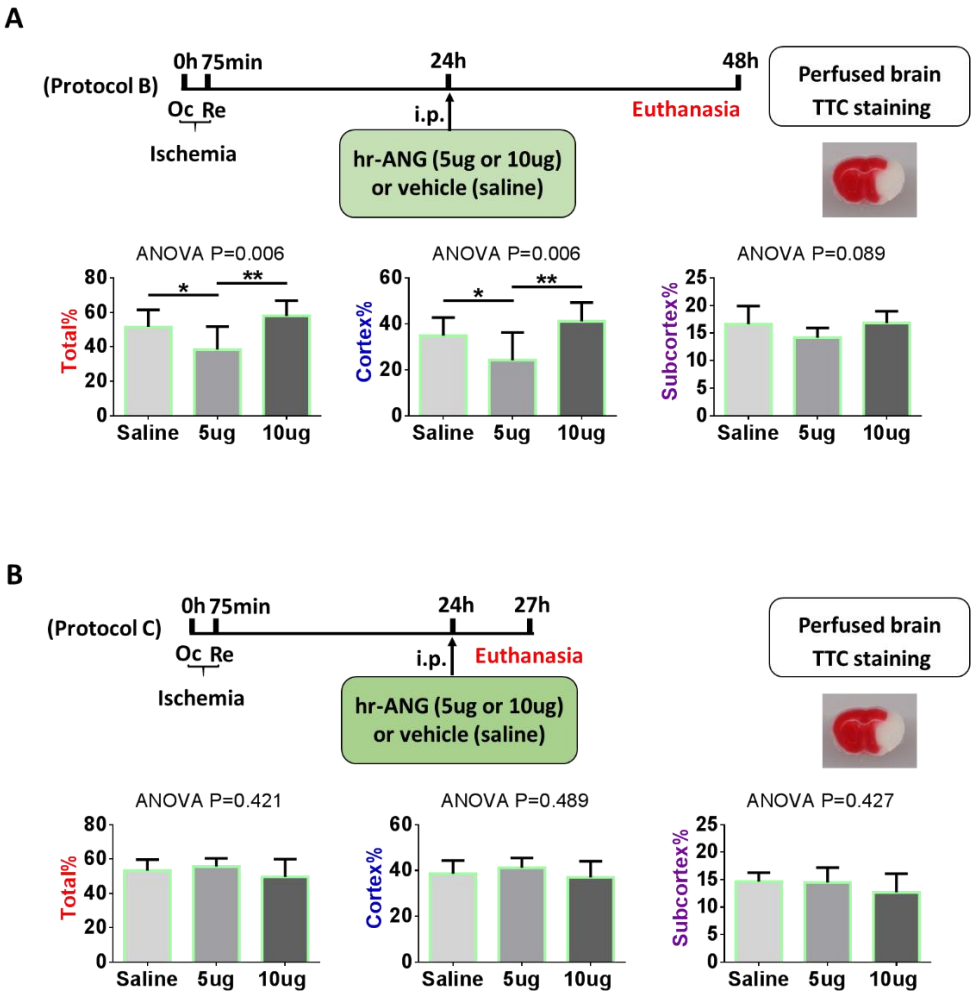

Figure 12. Study of the neuroprotective effects of subacute hr-ANG treatment on infarct size. (**A**) Timeline of the experimental procedures and bar graphs representing the total, cortical and subcortical infarct size after the subacute treatment (n=13 for saline, n=9 for 5ug hr-ANG and n=6 for 10ug hr-ANG). (**B**)Timeline of the experimental procedures and bar graphs representing the total, cortical and subcortical infarct size early after subacute treatment (n=6 for saline, n=6 for 5ug hr-ANG and n=5 for 10ug hr-ANG). Bar graphs represent mean±SD; *p < 0.05 and **p < 0.01 as indicated by the horizontal lines;h, hour; min, minute; i.p., intraperitoneal; Oc, occlusion; Re, reperfusion; hr-ANG, human recombinant angiogenin; TTC, 2,3,5-Triphenyltetrazolium Chloride

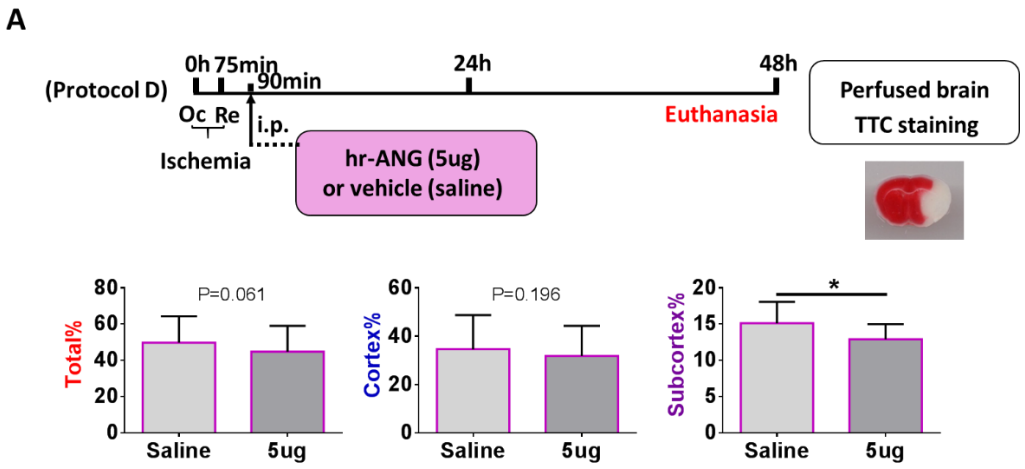

Figure 13. Study of the neuroprotective effects of acute hr-ANG treatment on infarct size. (**A**) Timeline of the experimental procedures; bar graphs representing the total, cortical and subcortical infarct size 24 hours after acute treatment (n=16 for saline and n=13 for 5ug hr-ANG). Bar graphs represent mean±SD; *p < 0.05 as indicated by the horizontal lines;h, hour; min, minute; i.p., intraperitoneal; Oc, occlusion; Re, reperfusion; hr-ANG, human recombinant angiogenin; TTC, 2,3,5-Triphenyltetrazolium Chloride.

4.3 The proposed Angiogenin acute and subacute therapy after cerebral ischemia is safe.

The subacute and acute therapy with hr-ANG did not show increased mortality, with no statistical differences between groups at 48 h of ischemia (p= 1 and p= 0.648, respectively); see **Figure 14 A** and **B**. Regarding the presence of intracranial hemorrhages, both subacute and acute therapy did not increase the hemorrhage areas (p = 0.228 and p= 0.517 respectively) as shown in **Figure 14 C** and **D**. On the other hand, the subacute therapy of 5ug hr-ANG (but not 10ug) trend to reduce the number of mice presenting intracerebral hemorrhages (p=0.055) and significantly reduced the presence of intracerebral plasma IgG (investigated by Western-Blot, see **Figure 14 H**) (P=0.033) as shown in **Figure 14 E** and **G**, but not when administered acutely (p=1) as shown in **Figure 14 F**.

Regarding the neurological outcome, similar scores were observed 24 post-ischemia between groups in the subacute therapy protocol (pre-therapy, p = 0.418), see **Figure 15 A,** with no further improvements at 24 post-therapy (p = 0.151) as shown in **Figure 15 B**. On the other hand, the acute therapy with 5ug of hr-ANG administered 15 minutes after reperfusion improved the neuroscore results at 24h (p = 0.002) but was not maintained at 48h (p = 0.378), as shown in **Figure 15 C** and **D** respectively.

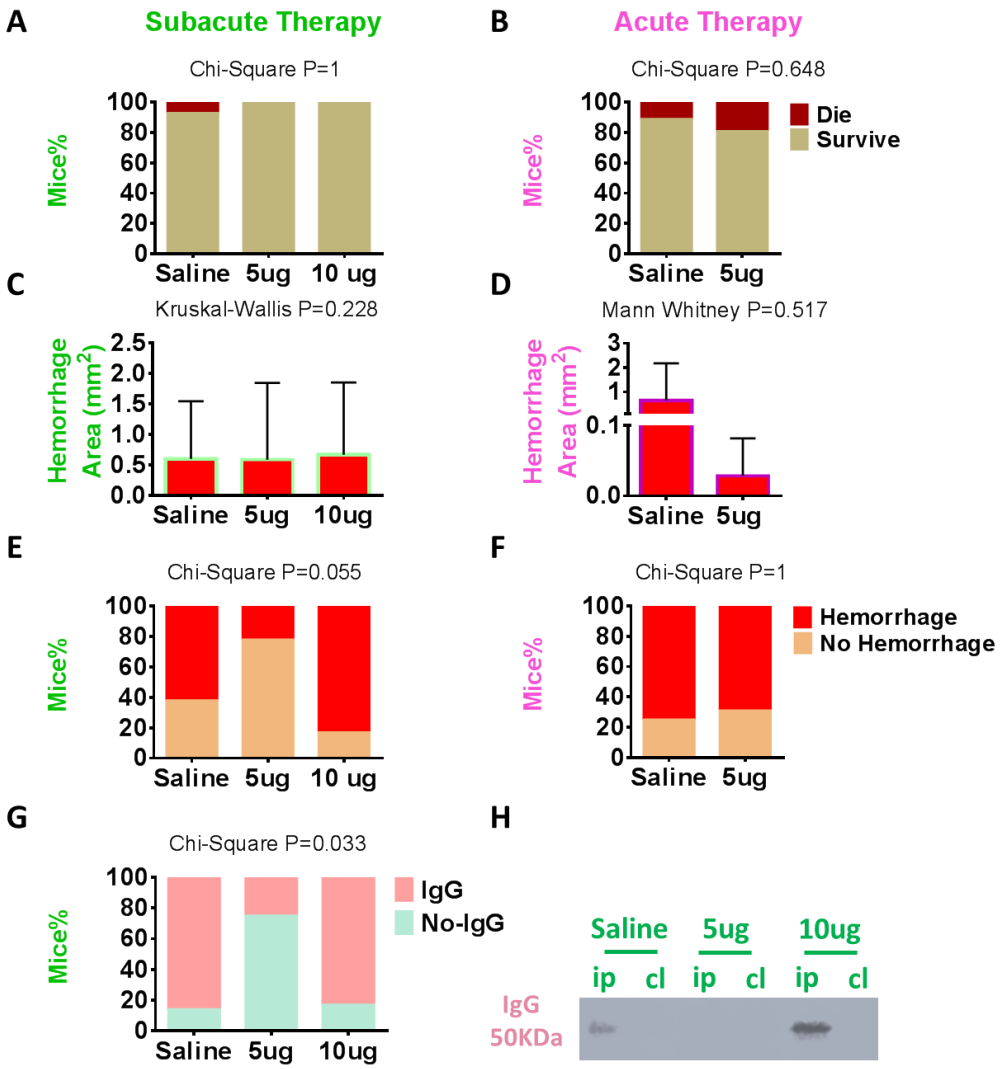

Figure 14. Safety study of the hr-ANG therapy based on the mortality and intracranial hemorrhages. (**A**) (**B**) Bar graphs representing the mortality of subacute and acute therapy, respectively. (**C**) (**D**) Bar graphs representing the brain hemorrhagic areas in the subacute and acute therapy, respectively. (**E**) (**F**) Bar graphs representing the hemorrhagic rates of subacute and acute therapy, respectively. (**G**) Bar graphs representing percentage of mice presenting intracranial IgGafter subacute therapy. (**H**) Representative images of Western-Blot of IgG. Number of mice in (**A**) (**C**) (**E**) saline (n=13), 5ug (n=9) and 10ug (n=6); in (**B**) (**D**) (**F**) saline (n=16) and 5ug (n=13); in (**G**) saline (n=7), 5ug (n=8) and 10ug (n=6). Bar graphs present mean±SD; IgG, immunoglobulin G; ip, ipsilateral; cl, contralateral.

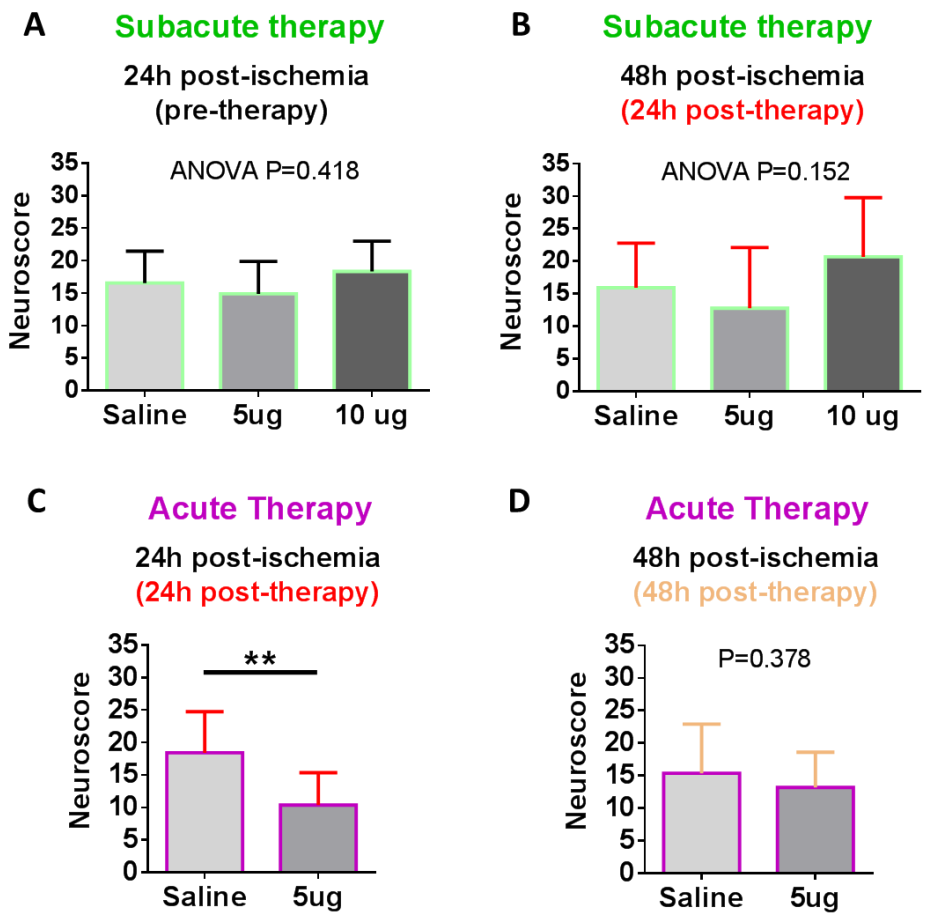

Figure 15. Safety study of the hr-ANG treatment based on the neurological score. (**A**) Bar graph representing the neurological score at 24h post-ischemia. (**B**) Bar graph representing the neurological score post-ischemia 24h after subacute therapy. (**C**) (**D**) Bar graphs representing the neurological score post-ischemia 24 and 48 hours, respectively, after acute (15 min) therapy. Number of mice in (**A**) (**B**) saline (n=13), 5ug (n=9) and 10ug (n=6); in(**C**) (**D**) saline (n=16) and 5ug (n=13). Bar graphs present mean±SD; **p < 0.01 as indicated by the horizontal line;h, hour.

4.4 Ischemia-induced brain cell apoptosis is inhibited by the subacute hr-ANG therapy.

First, we examined potential changes in 45S ribosomal RNA levels (which is essential for ribosomal biogenesis and cell survival), responding to RNAse activity after the

therapy with hr-ANG, in the experimental protocols B and C as shown in **Figure 16 A.** Our results show that subacute therapy of 5ug hr-ANG did not influence 45S ribosomal RNA levels neither at 3h nor at 24h post-therapy in the ischemic hemisphere when compared with vehicle-treated mice (p = 0.787 and p= 0.410 respectively). Also, that the 45S ribosomal RNA levels did not change from 3h to 24h post-therapy neither in vehicle (p= 0.741) nor in 5ug hr-ANG-treated mice (p= 0.452), as shown in **Figure 16 B**.

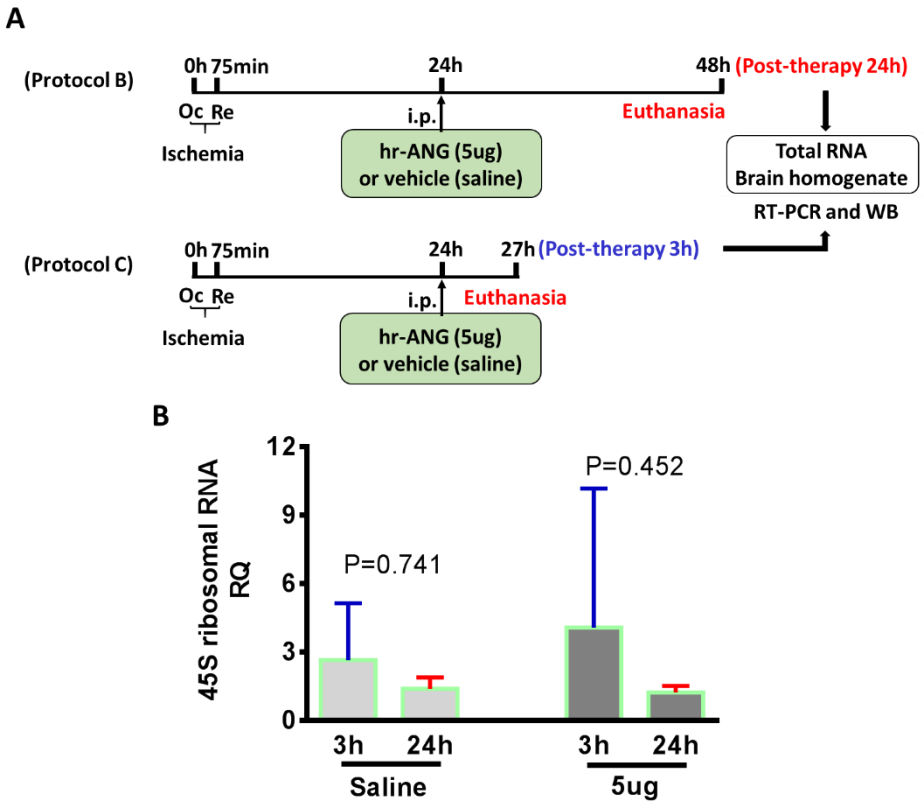

Figure 16. RT-PCR study for 45S ribosomal RNA synthesis. (**A**) Timeline of the experimental procedures. (**B**) Bar graphs representing the ipsilateral 45S ribosomal RNA levels at 3h (protocol C) and 24h (protocol B) post-therapy as well as the comparation over time (3h vs 24); saline (3h) n=6, saline (24h) n=8, 5ug hr-ANG (3h) n=6, hr-ANG (24h) n=7. Bar graphs represent mean±SD;h, hour; min, minute; i.p., intraperitoneal; Oc, occlusion; Re, reperfusion; hr-ANG, human recombinant angiogenin.

Then we investigated the protein expression of well-known cell survival-pathway molecules such as AKT, ERK and their phosphorylated forms by Western-Blot, see **Figure 17 A** and **C**.

Our results show that subacute therapy of 5ug hr-ANG did not increase ipsilateral ischemic p-AKT/AKT ratio at 3h ($p > 0.999$) nor at 24h ($p > 0.999$) post-therapy compared with vehicle-treated mice (saline). Moreover, the ipsilateral ischemic hemisphere presented p-AKT/AKT ratio significantly decreasing from 3h to 24h post-therapy in both vehicle ($p = 0.048$) and in 5ug hr-ANG-treated mice ($p = 0.032$), see **Figure 17 B.**

Regarding ERK signaling pathway, although 5ug hr-ANG did not increase in the ipsilateral ischemic p-ERK/ERK ratio at 3h ($p= 0.895$) nor at 24h ($p= 0.828$) post-therapy compared with vehicle-treated mice, the p-ERK/ERK ratio was significantly enhanced at 3h post-therapy only in the 5ug hr-ANG mice ($p = 0.015$) compared with naïve mice.And the ipsilateral ischemic hemisphere also presented a p-ERK/ERK ratio significantly decreasing from 3h to 24h post-therapy both in vehicle ($p = 0.01$) and in 5ug hr-ANG-treated mice ($p < 0.0001$), see **Figure 17 D.**

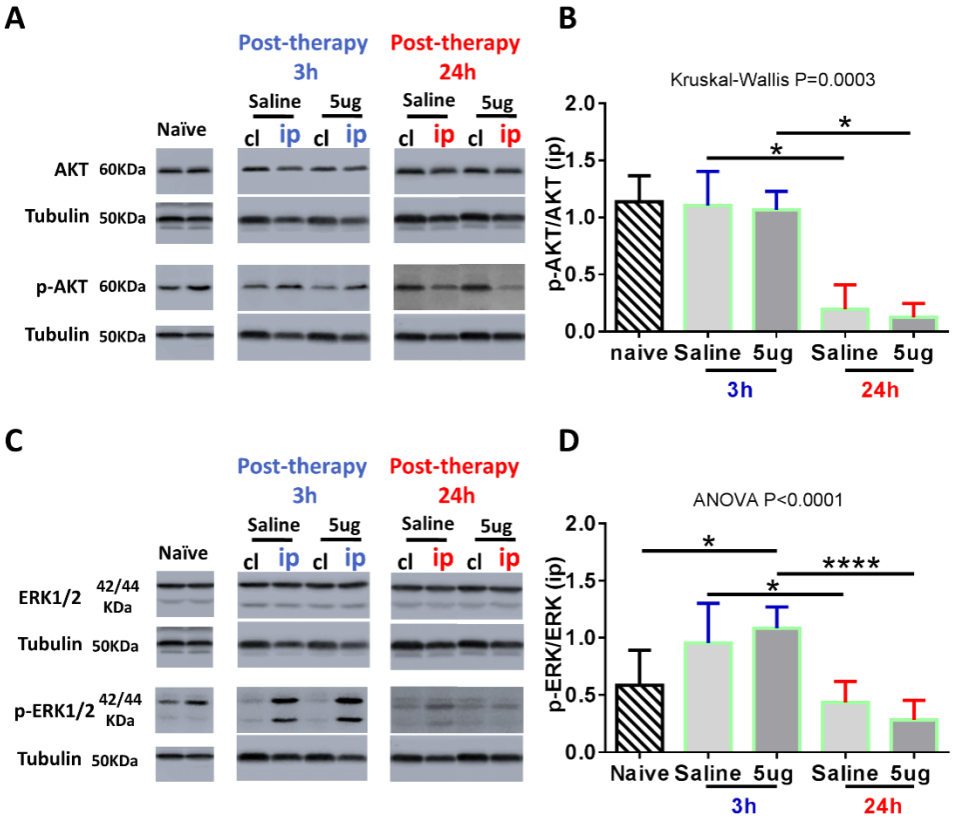

Figure 17. Western-Blot of AKT and ERK signaling pathway for cell survival after subacute hr-ANG therapy. (**A**) Representative images of Western-Blot of AKT and p-AKT. (**B**) Bar graphs representing the ipsilateral p-AKT/AKT ratio of naïve mice as well as the ischemic mice at 3h (protocol C) and 24h (protocol B) post-therapy (n=6/group). (**C**) Representative images of Western-Blot of ERK1/2 and p-ERK1/2. (**D**) Bar graphs representing the ipsilateral p-ERK1/2 ratio of naïve mice as well as the ischemic mice at 3h (protocol C) and 24h (protocol B) post-therapy (n=6/group). Bar graphs represent mean±SD; *p < 0.05, ****p < 0.0001 as indicated by the horizontal lines; ip, ipsilateral; cl, contralateral; h, hour.

Finally, we focused on the apoptosis signaling pathway by the molecular protein expression by Western-Blot, see **Figure 18 A**. As shown in **Figure 18 B**, the active caspase-3 levels increased in the ischemic ipsilateral hemisphere from 27h to 48h post-ischemia both in vehicle ($p = 0.003$) and 5ug hr-ANG-treated ($p = 0.004$) mice, as a consequence of the ischemic insult.

However, subacute administration (at 24h) of 5ug hr-ANG significantly reduced the ipsilateral active caspase-3 levels at 24h post-therapy compared with vehicle-treated mice ($p = 0.048$). We also investigated the upstream molecules of this effector active caspase-3 to deepen in the inhibitory mechanisms of stroke-induced apoptosis by hr-ANG therapy.As shown in **Figure 18 C, D** and **E**, the ipsilateral ischemic levels of caspase-3, active caspase-9 and Bax were significantly increased from 27h to 48h post-ischemia only in vehicle-treated ($p = 0.009$, $p = 0.01$ and $p = 0.049$ respectively), but not in the 5ug hr-ANG-treated mice($p = 0.119$, $p = 0.558$ and $p = 0.191$ respectively). Regarding Bcl-2, as shown in **Figure 18 F**, it was undetectable at 3h post-therapy, increasing at 24h post-therapy with no differences between vehicle and 5ug hr-ANG treatments ($p = 0.490$). And the pro-apoptotic Bax/Bcl-2 ratio was also similar at 24h post-therapy between vehicle and 5ug hr-ANG treatments ($p = 0.410$) as shown in **Figure 18 G**.

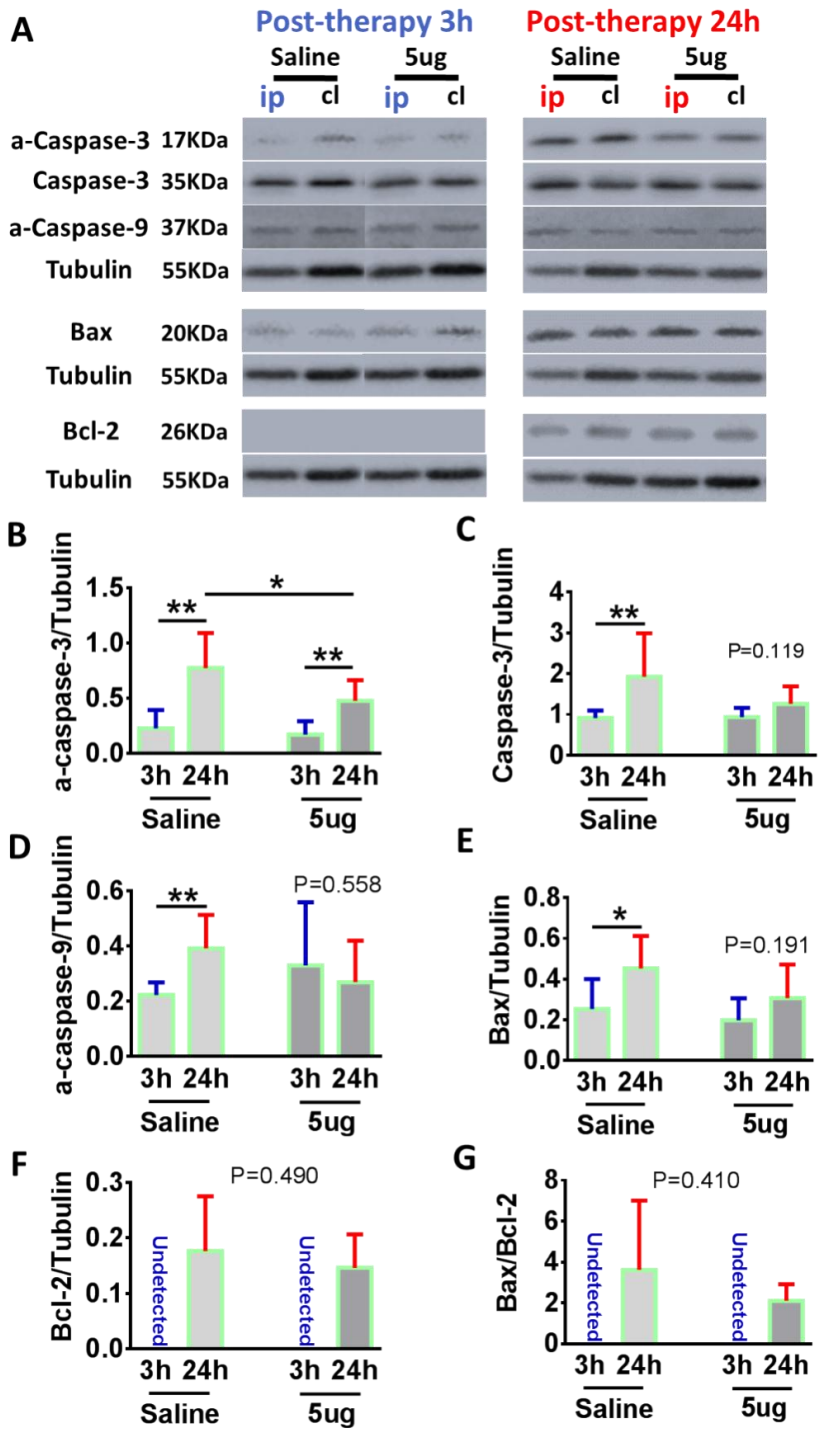

Figure 18. Western-Blot study for cell apoptosis in the ischemic brain after subacute hr-ANG therapy.

(**A**) Representative images of Western-Blot of active caspase-3, caspase-3, active caspase-9, Bax, Bcl-2 and

Tubulin. (**B**) (**C**) (**D**) (**E**) (**F**) (**G**) Bar graphs representing the ischemic ipsilateral active caspase-3, caspase-3, active caspase-9, Bax and Bcl-2 levels as well as Bax/Bcl-2 ratio at 3h (protocol C) and 24h (protocol B) after subacute therapy as well as the time-line (3h vs 24); (n=6-8/group). Bar graphs represent mean±SD; *p < 0.05 and **p < 0.01 as indicated by the horizontal lines;ip, ipsilateral; cl, contralateral; a-caspase-3, active caspase-3; a-caspase-9, active caspase-9;h, hour.

Finally, to investigate the mechanisms of reducing subcortical infarct size at 48h post-ischemia by acute (15 minutes post-reperfusion) therapy with 5ug hr-ANG (protocol D), we also examined the same molecules of cell survival and apoptosis signaling pathways by Western-Blot, see **Figure 19 B** and **Figure 20 A**. However, the results did show any differences between 5ug hr-ANG and vehicle treatments neither for p-AKT/AKT ($p = 0.093$) nor p-ERK/ERK ($p = 0.241$), see **Figure 19 C** and **D.** Similar results were observed for active caspase-3 ($p = 0.512$), caspase-3 ($p = 0.758$), Bax ($p = 0.740$), Bcl-2 ($p = 0.803$) and Bax/Bcl-2 ratio ($p > 0.999$); see **Figure 20 B, C, D, E and F**.

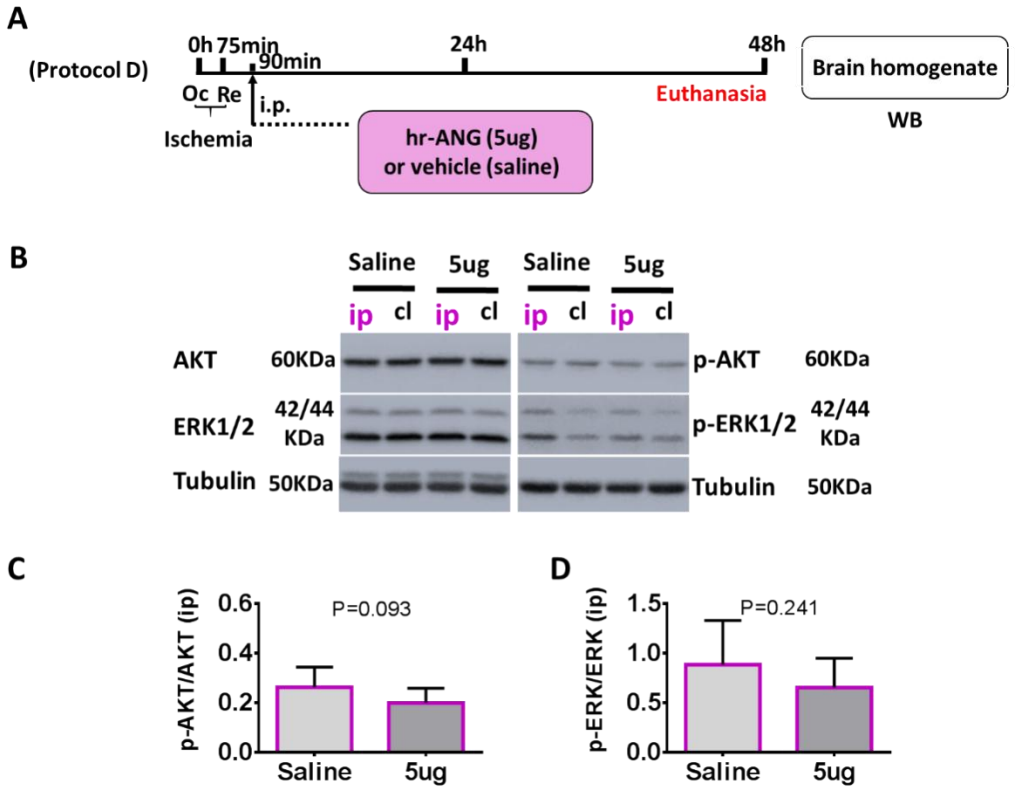

Figure 19. Western-Blot study of AKT and ERK signaling pathway for cell survival after acute hr-ANG therapy. (**A**) Timeline of the experimental procedure. (**B**) Representative images of Western-Blot of AKT, p-AKT, ERK1/2, p-ERK1/2 and Tubulin. (**C**) (**D**) Bar graphs representing the ipsilateral hemisphere p-AKT/AKT and p-ERK/ERK ratios after acute therapy respectively (n=8/group). Bar graphs represent mean±SD;h, hour; min, minute; i.p., intraperitoneal; Oc, occlusion; Re, reperfusion; hr-ANG, human recombinant angiogenin; WB, Western-Blot; ip, ipsilateral; cl, contralateral; h, hour.

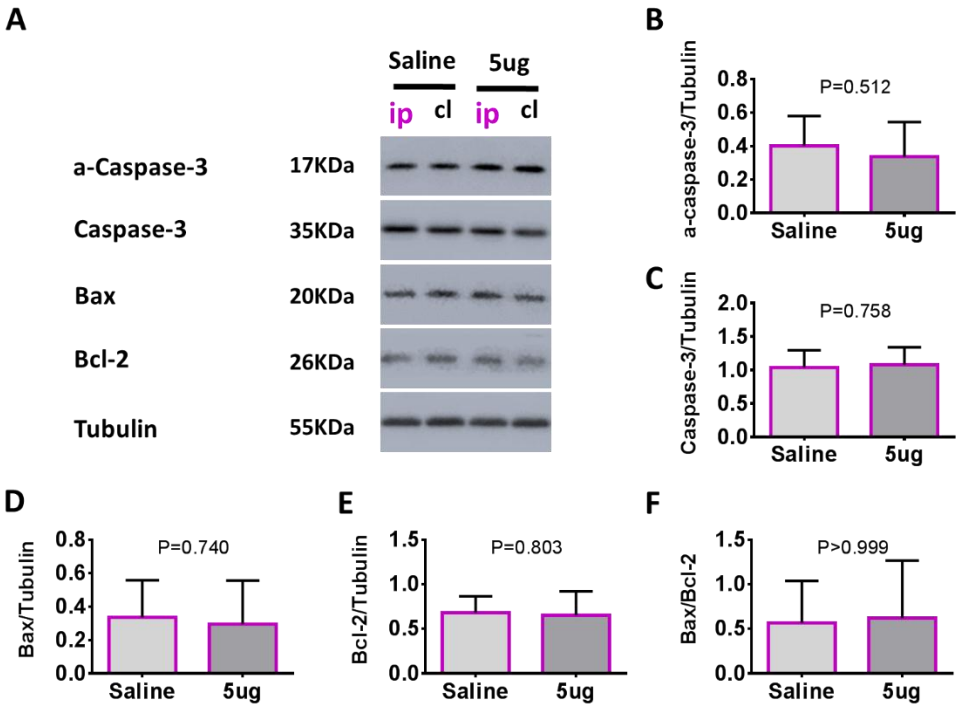

Figure 20. Western-Blot for apoptosis signaling pathway for cell survival after acute hr-ANG therapy. (**A**) Representative images of Western-Blot of active caspase-3, caspase-3, Bax, Bcl-2 and Tubulin. (**B**) (**C**) (**D**) (**E**) (**F**) Bar graphs representing the ischemic ipsilateral active caspase-3, caspase-3, Bax and Bcl-2 levels as well as Bax/Bcl-2 ratio after acute therapy respectively (n=8/group). Bar graphs represent mean±SD;ip, ipsilateral; cl, contralateral; a-caspase-3, active caspase-3; h, hour.

4.5 Subventricular-zone migrating neuroblasts are increased by the subacute hr-ANG treatment, but not vessel density.

The long-term effects of hr-ANG therapy on endogenous repair mechanisms were studied following protocol E when neuroprotective subacute therapy of 5 ug of hr-ANG was continued over two weeks as shown in **Figure 21 A.**

The proposed treatment increased migrating DCX-positive cells (a marker of neurogenesis) from the neurogenic subventricular niche of the ischemic hemisphere at two weeks compared with the vehicle treatment ($p< 0.001$) as shown in **Figure 21 B**; representative micrographs of ipsilateral subventricular zone with DCX-positive cells are also shown in **Figure 21 B**.

However, angiogenesis was not enhanced in the ipsilateral hemisphere of mice receiving 5ug Hr-ANG treatment since our results show that vessel density was similar between groups at two weeks of cerebral ischemia ($p= 0.376$) as shown in **Figure 21 C**; representative micrographs of ipsilateral hemispheres of lectin-positive vessels are also shown in **Figure 21 C**.

Regarding the cell proliferation, our results show that the 5ug hr-ANG therapy did not increase the general population of Brdu-positive cells (a marker of cell proliferation) in the ischemic ipsilateral hemisphere at two weeks compared with the vehicle treatment ($p= 0.625$) as shown in **Figure 21 D**; representative micrographs of ipsilateral hemispheres Brdu-positive cells are also shown in **Figure 21 D**.

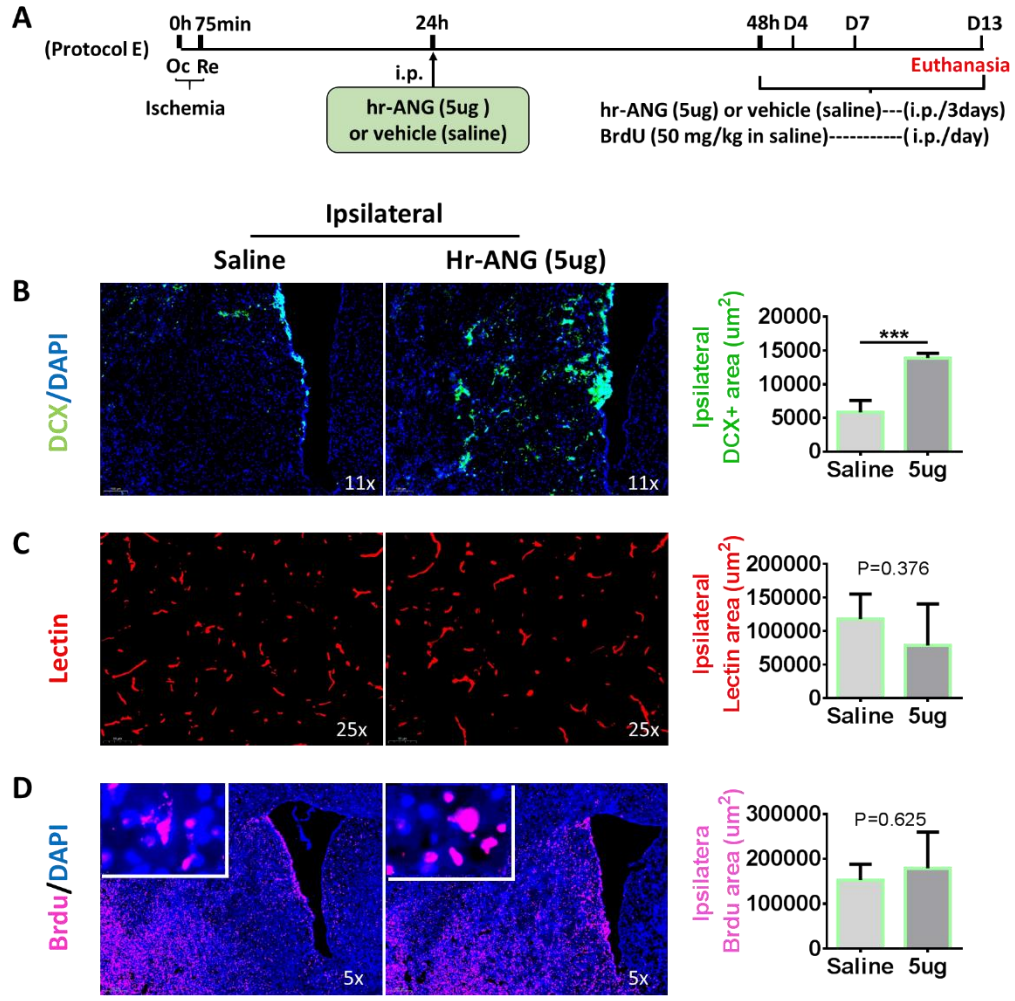

Figure 21. Peri-infarct neurogenesis and angiogenesis at two weeks after subacute hr-ANG treatment. (**A**) Timeline of the experimental procedure. (**B**) Representative micrographs of DCX (green) staining merged with DAPI; bar graph representing the ipsilateral DCX+ area. (**C**) Representative micrographs of lectin immunofluorescence (red) for vessels; bar graph representing the ipsilateral lectin+ area. (**D**) Representative micrographs of Brdu (pink) staining merged with DAPI; bar graph representing the ipsilateral Brdu+ area. Number of animals in (**B**) (**C**) (**D**) (n=3 for saline, n=4 for 5ug hr-ANG). Bar graphs represent mean±SD; ***p < 0.001 as indicated by the horizontal lines;h, hour; min, minute; D, day; i.p., intraperitoneal; Oc, occlusion; Re, reperfusion; hr-ANG, human recombinant angiogenin.

5. Discussion

5.1 Bioactive brain delivery of hr-ANG after cerebral ischemia is feasible.

ANG is considered a member of the ribonuclease A superfamily, but exhibits very weak ribonucleolytic activity compared to the RNase A, about 10^{-5} to 10^{-6} fold less as described by others [238] which is attributed to the blockage of the B1 center (the pyrimidine base-binding center) [239]. Unlike RNase A, which has no base specificity, ANG usually cleaves the 3′-side of cytidylic or uridylic acid residues when the pyrimidine is followed by adenine, but not all the potential cleavage sites [238, 240]. In addition, ANG shows preferential cleavage of single-stranded RNA as the substrate and the cleavage specificity depends on its secondary structure [240], and importantly although ANG has been shown to bind DNA in vivo, it does not cleave DNA [168]. Despite this low ribonucleolytic activity, most of the mutations in the ANG gene associated with ALS disease do not significantly alter the secondary structure or stability of ANG, but rather disrupt its ribonucleolytic activity or subcellular distribution [241, 242], suggesting the critical role of the ribonucleolytic activity of ANG for its biological functions in the CNS, especially in neurodegenerative conditions.

Taking into consideration this well-described function for ANG protein, we first verified if the administrated hr-ANG treatment used in this thesis presented existing the ribonucleolytic activity, as a well-known ANG function, which is confirmed by our zymogram results ex-vivo, showing that all tested amounts (5ug, 10ug and 15ug of total hr-ANG) displayed dose-dependant catalytic activity for the poly(C) substrate (a double-stranded homopolymer used as a model RNA) present in the zymogram gel. In

this regard, other studies have performed similar zymograms to determine the ribonucleolytic activity of RNase A and ANG [243, 244], including E.coli-derived Angiogenin protein [244], as the one administrated hr-ANG in the present study.

Other important studies have shown that exogenous ANG administration in several *vitro* and *vivo* pre-clinical studies could be neuroprotective and safe [147, 245]. For example in the ALS $SOD1^{G93A}$ mouse model, therapeutic administration of hr-ANG (1ug, i.p.) delayed motoneuron degeneration and disease progression [245], while using the same mouse model a dose-response study did not reveal an improvement in survival or motor function in mice treated with 10ug hr-ANG compared to 1 ug treatment [245, 246]. Considering these previous results, the present study characterized the plasma kinetics and *in vivo* brain bioactivity after systemic hr-ANG administration in naïve mice treated with the 1ug or 5ug (i.p. or i.v.) administration doses. Consistent with previous research [245], both hr-ANG doses induced a rapid and significant increase of human ANG plasma levels at 1h post-administration (i.p. and i.v.) followed by a significant decrease over time, but still detectable at 24 h. Moreover, we found higher plasma hr-ANG levels when administered i.p. which could be due to the rapid elimination rate after i.v. administration, for example, it has been reported that the i.p. administration of cefuroxime lysine achieved a higher plasma level and a longer elimination half-life (t1/2) than i.v. administration in rats [247]. Besides, i.p. administration is easy to administer in rodents compared to other available routes and is less stressful for the rodents [248]. According to these data, the present study established the i.p. administration as the appropriate administration route in all the pre-clinical protocols for the ischemia model. Actually, these additional studies in the cerebral ischemia model also confirmed the presence of human ANG in mice receiving hr-ANG subacutely (5ug or 10ug), remaining

detectable at 24 h post-therapy.

Although exogenous ANG has been reported to reduce endogenous ANG in endothelial cells by other authors [166], in the present study the endogenous mouse brain ANG did not change between groups post-therapy in our study. However, whether the administered therapeutic hr-ANG can pass the BBB is unknown. In the present study, we could not directly detect the administrated hr-ANG in perfused naïve mice brain by ELISA in our study (data not show), but our indirect bioactivity study shows an activation of the ERK signaling pathway in the naïve brains after hr-ANG administration, suggesting that ANG reached the brain tissue by activating well-described cell-survival pathways related to ANG [249]. Although in our study it is possible that the amount of hr-ANG reaching the brain in naïve mice with intact BBB is lower than the detection limit of the used technique, but it could be sufficient to induce ERK phosphorylation. On the other hand, we could detect the administrated hr-ANGin perfused ischemic hemispheres, especially in those from mice receiving 10ug hr-ANG therapy, although it could be due to the BBB leakage caused by ischemic stroke [250] since we found that the amount of hr-ANG protein detected in the ipsilateral hemisphere was associated with the extension of intracranial hemorrhages. Further studies to verify the uptake and location of the administrated hr-ANG in the naïve and ischemic mice brain are required to fully elucidate the mechanisms of brain delivery of the therapeutic ANG.

5.2. Safe hr-ANG therapy results in neuroprotective actions after cerebral ischemia.

Stroke is a devastating disease affecting 1 out of 4 persons over the age of 25 (Global Burden of Disease study) with an extraordinary high socioeconomic impact worldwide.

Fortunately, nowadays the only available and approved treatments for ischemic stroke are the pharmacological reperfusion therapies to dissolve the clot/thrombus with Tpa [251] or the mechanical approaches to evacuate the occluding thrombus (thrombectomies) [252]. Although these are effective and saving-live therapies, in general, they can only be given to ischemic patients within the first hours of the symptoms onset (up to 6-8 hours) due to the increase in hemorrhagic complications if perform late [252]. For patients who are not candidates for reperfusion therapies, or for those who survive a stroke but with functional deficits, the only therapeutic options are the post-stroke rehabilitation programs attempting to maximally compensate the lost functions [20]. Many advances have been achieved in this field helping to understand the molecular and cellular mechanisms of ischemic stroke [31, 32] and numerous studies have been performed to investigate novel neuroprotective treatments, some of them reaching the clinical trials phase (such as the free radical-trapping agent NXY-059 [75]) or neuroreparative therapies (for example growth factors [108]). However, no clinically effective neuroprotective or neuroreparative therapy has been approved nowadays. In this context, the proposal of novel therapies tested in clinically-relevant experimental studies is urgently needed.

ANG has been reported to be involved in various diseases such as cancer [253] or neurodegenerative diseases such as ALS and PD [147, 245] related to the multiple actions of this unique ribonuclease. The neuroprotection effects of ANG have been mainly described delaying motoneuron degeneration and disease progression in ALS disease [147, 245] or protecting against the dopaminergic neuronal cell death in PD disease [193]. Moreover, recent results from the Neurovascular Research Laboratory at VHIR have shown that the endogenous ANG protein levels in the post-stroke ipsilateral

brain hemisphere is higher than in the contralateral hemisphere, and increased after rehabilitation therapy in blood samples related to good outcomes, suggesting that stroke could increase the expression of ANG being protective for the ischemic brain [202]. A more recent study has further shown that ANG is overexpressed after physical exercise in the SVZ of the ischemic hemisphere, which enhances the hypothesis that ANG can be protective for the ischemic brain, and suggests that ANG could interact with neurogenesis. However, other studies investigating the potential neuroprotective and repair actions of ANG after stroke are lacking nowadays. In this thesis it is reported for the first time that administered hr-ANG could reduce the infarct size at short time, being protective for the ischemic brain. Specifically, the study reports that 5ug (but not 10ug) of hr-ANG therapy given acutely post-reperfusion or subacutely one day after occlusion reduced infarct size at 48h post-ischemia. Moreover, the detailed infarct lesion study shows that the subacute therapy significantly reduced the total and cortical infarct size, whereas the acute therapy significantly reduced the subcortical infarct size. This observations could be due to the cell death progression in the MCAo model which follows a well-described time-line progression from early infarction in the striatum to delayed infarction in the dorsolateral cortex overlying the striatum [254, 255]. Striatal infarction has been described to be mostly necrotic and occurs rapidly after the vessel occlusion [254, 255]. On the other hand, in proximal occlusions cortical infarction is more delayed in time, involving a prolonged and biphasic opening of the BBB, and containing a greater degree of apoptotic cell death than in the striatal infarction [254-257]. In initial subcortical infarct lesions, few neurons show morphological signs of necrosis during the initial 4h, significantly increase at 6h and the severity is aggravated at12h post-ischemia in the ischemic hemisphere, but the percentage of necrotic neurons is much lower in the cortex than subcortex [254]. Other researchers have reported that a large amount of

apoptosis primarily localizes to the inner boundary zone of the cortical infarct [255]. Thus, in our study, it is possible that the hr-ANG acute therapy was at the proper timepoint to protect against the early subcortical (striatal) infarction, while it did not protect against the delayed cortical infraction occuring at later time-points as the bioavailable hr-ANG decreases overtime in several hours. However, the subacute hr-ANG therapy mainly protected against cortical infarction as the subcortical injury was already irreversible at the moment of hr-ANG administration. These different mechanisms of cell death after cerebral ischemia and progression following MCAO also could explain the results of the mechanistic investigations in this study since only subacute (but not acute) hr-ANG neuroprotection has been found associated to neuroprotective actions on cell survival (ERK) and apoptosis (Caspases) pathways when rescuing the cortical (but not subcortical) cells. Consistent with our results, many hypoxia-induced genes involved in neuroprotection are induced solely in the cortex after MCAO, such as heat shock protein 70, Bcl-2, Bcl-XL, and Bax [258-260].

However, we can not ignore that some detrimental effects of ANG also have been reported by other authors, arguing that ANG contributes to the tRNA (transfer RNA) degradation, and this tRNA-derived small RNA fragments (tRFs) species could induce cytotoxicity [149, 150], which contrasts with previous studies reporting that certain tRFs could promote cell survival [151]. Overall alerting of potential harmful effects of the tested hr-ANG therapy in our study.

Besides, as an angiogenic factor, ANG could promote degradation of the basement membrane and extracellular matrix [261], which could potentially result in hemorrhage complications [262] and the risk of intracranial hemorrhages. With these safety concerns in mind, the present results point at the safety of the proposed ANG acute and subacute

therapies after cerebral ischemia. The presented results did not observe significant mortality or intracranial hemorrhagic events between treatments and vehicle groups after acute or subacute therapy at 48 h post-ischemia. Moreover, we report for the first time that ANG could reduce the hemorrhagic events after ischemic stroke at the neuroprotective dose of 5ugwhen given subacutely (but not 10ug), without an associated improvement of neurological outcomes; whereas only mice receiving acute treatment post-reperfusion showed transient improvements of the neurological outcome at 24 post-therapy(corresponding with the partial protection in the subcortical injury), but not later at 48h.

Disruption of the BBB is a key feature of intracranial hemorrhage formation after ischemic stroke [263, 264]. Previous studies have demonstrated two time courses (early and delayed) of disruption of BBB and further hemorrhage formation in ischemic stroke [264]. The early disruption of the BBB and hemorrhage formation occurs within the first 24 hours of ischemic stroke onset and the delayed occurs days later [264]. Thus, in our study, it is possible that the acute therapy had protected against the early hemorrhage formation within the first 24h post-ischemia, but could not continuously protect against potentially delayed hemorrhages from 24h to 48hpost-ischemia occurring in this MCAO model as described by others [237] as the treated hr-ANG significantly decreased over time as shown in our plasma ANG kinetics study. A transiently improved functional outcome during the first 24 hours after the acute therapy could support this hypothesis because intracranial hemorrhage is one of the factors strongly associated with neurological outcomes [265] which in the neuroscore tests are strongly associated to subcortical functional areas and is [266] being less sensitive for cortical injury matching with the lack of neurological improvement observed in our sub-cortical neuroprotection

observed in the subacute hr-ANG administration.

Regarding our mechanistic studies, as 10ug hr-ANG therapy did not show any efficacy nor harmful effects, for this reason, the mechanistic studies focused on the neuroprotective actions achieved by the 5ug hr-ANG dose therapy. We acknowledge the existence of this dose-dependent lack of efficacy, which alerts on the importance of conducting dose-response studies in both pre-clinical and even clinical studies in the future related to ANG therapy, not only in ischemic stroke but also in other neurodegenerative diseases, cancer and others. Further studies to investigate the dose-dependent efficacy of ANG are certainly required.

At the genetic level, ANG-stimulated rRNA transcriptions are involved in cell growth, cell survival and normal physiological function maintenance.ANG has been shown to undergo nuclear translocation in endothelial cells [133], cancer cells [267] and motor neurons [241] being very fast and occurring through receptor-mediated endocytosis [133]. Once in then ucleus, ANG accumulates in the nucleolus [133], where ribosome biogenesis takes place as a key step for cellular function. Nuclear ANG has been shown to bind to the promoter region of rDNA [268] and stimulates rRNA transcription [269], thus acting as a transcription factor to promote rRNA transcription and the production of new ribosomes, which is needed for normal cell growth and for maintaining a normal cell function [241, 270, 271]. The rate-limiting step in ribosome biogenesis is rRNA transcription [190], which would be an important aspect of cell growth and survival control as proteins are required essentially for all cellular activities. ANG-stimulated rRNA transcription has been shown to be involved in the maintenance of normal physiological function of motor neurons in ALS disease [241, 272], and a defect in this pathway, as in ANG mutations in ALS leads to insufficient synthesis of ribosomes,

thereby affecting motor neuron viability and survival [241]. According to these data, we examined potential changes in 45S ribosomal RNA levels in the ischemic hemisphere after the subacute therapy with hr-ANG (which is the rRNA expression directly stimulated by ANG) [269]. In contrast with the previous studies [241, 269, 272], the present study can not prove that the subacute therapy of hr-ANG influenced 45S ribosomal RNA synthesis neither at 3h nor at 24h post-therapy in the ischemic hemisphere. This lack of results could be due to the 45S ribosomal RNA rapid processing after its transcription [273], which occurred before the 3h post-treatment study time-point. Besides, exome also can rapidly and completely degrade45S ribosomal RNA [274], leading to mild or no accumulation of the 45S ribosomal RNA. Therefore, to prove the actions of ANG therapy on the ribosomal biogenesis probably require extensive studies covering early time-points to elucidate the true actions on this molecular pathway.

At the protein level it has been already mentioned that ANG can activate the AKT and ERK cell survival-pathway and increase the p-AKT [193] and p-ERK [249] levels rapidly. An unknown 170-kDa transmembrane protein has been described to serve as the receptor for extracellular ANG to trigger a series of signaling responses [275]. According to these data, the present study investigates the protein expression of AKT, ERK and their phosphorylated forms in the ischemic hemisphere after the subacute therapy with 5 µg of hr-ANG. In contrast with a previous study [193], we did not find that hr-ANG increased ipsilaterally the p-AKT/AKT ratio at 3h nor at 24h post-therapy compared with vehicle-treated mice. Moreover, we observed that the p-AKT/AKT ratio was significantly decreased from 3h to 24h post-therapy both in vehicle and hr-ANG-treated mice, and regarding ERK signaling pathway, although we did not find an increase in the ipsilateral

p-ERK/ERK ratio at 3h nor at 24h post-therapy. However, we observe that the p-ERK/ERK ratio was significantly enhanced at 3h post-therapy only in the hr-ANG therapy mice compared with naïve mice, suggesting that the subacute proposed therapy activated the ERK cell survival-pathway in the ischemic brain, which is consistent with a previous study [249]. On the other hand, the p-ERK/ERK ratio was significantly decreased from 3h to 24h post-therapy both in vehicle and hr-ANG-treated mice suggesting that the early increase observed after ANG treatment was not sustained over time.

Some of these results are different from previous studies [249, 276], perhaps due to unproper timepoints for the tissue obtention (too late after hr-ANG therapy) and because both AKT and ERK signaling pathways are triggered not only by ANG, but also by the ischemic stroke insult, potentially masking the ANG effects [277, 278]. A transient increase in AKT phosphorylation has been reported in rodents after permanent and transient MCAO: after permanent focal cerebral ischemia in Wistar rats, the AKTphosphorylation is significantly increased at 6h in the ischemic hemispheres [277], after permanent focal cerebral ischemia in Sprague-Dawley rats, the AKT phosphorylation is transiently increased at 1h and 5h, but returns to the baseline level at 24h in the ischemic penumbra, while p-AKT level does not change significantly in the ischemic core [279], after transient (60min) focal cerebral ischemia in C57BL/6 mice, the AKT phosphorylation is dramatically increased at 5 h after stroke, and then returns to baseline levels at 24h further decreasing at 48h below baseline AKT phosphorylated levels in the ischemic hemisphere [280]. The results observed in the present thesis show a decrease in Akt phosphorylation in the ischemic hemisphere over time (3h vs 24h) which is consistent with these previous publications, but without any effect by ANG

administration which might be explained by the transiently rapid increase of AKT phosphorylation described by other authors occurring before our timepoint investigations. In this regard, it has been reported that in SH-SY5Y dopaminergic cells the p-AKT level is significantly increased after ANG application as early as 30 seconds and then starts to decrease at 15min, but remain elevated level until 12h [193]. Thus, it is possible that hr-ANG administration might have triggered a rapid increase of AKT phosphorylation in the brain but followed by a rapid decrease before we obtained the tissue. Besides, the sustained increase observed in SH-SY5Y cells in vitro could be a response to stable ANG provided in the cell culture media maintaining the level for several hours, whereas in our hands the hr-ANG level in mouse plasma decreases rapidly over time (e.g. 10 times less at 6h vs 1h post i.p administration) which may lead to a more rapid decrease of the p-AKT level in the studied brain tissue. Another possibility is that the hr-ANG did not activate the AKT signaling pathway in our *in vivo* study. Actually, the verification of the bioactive actions in naïve mice of the hr-ANG used in this thesis did not show changes in the p-AKT/AKT pathway.

The other molecular pathway related to cell survival investigated in this thesis is the ERK1/2 phosphorylation. Transiently increase in ERK1/2 phosphorylation has also been reported in rodents after global ischemia, permanent and transient focal MCAO [278, 281]. After permanent focal MCAO in Sprague–Dawley rats, the p-ERK1/2 levels reach a maximum peak approximately at 1h of ischemia and remain significantly elevated at 6h in the penumbra tissue [281]. The permanent focal MCAO in CD-1 mice also shows the p-ERK1/2 levels are increased as early as 30min with a maximum peak at 2h, but decreasing to the control levels at 6h and remaining at the baseline level until 24h in ischemic hemisphere [282]. Moreover, different regional expression has been reported

in a study after transient (90min) focal cerebral ischemia in Sprague–Dawley rats, where brain immunostaining showed that the ERK1/2 phosphorylation is increased at 6h and 24h after MCAO within neurons and glia in the ischemic penumbra regions, whereas ERK1/2 phosphorylation is decreased in infarcted tissue [281]. Functional assays have shown similar data with increased ERK1/2 enzyme activity at 6 and 24h after transient MCAO, by the ERK1/2 kinase activity assay [283]. In the present study the progress of ERK phosphorylation in the ischemic tissue is consistent with these data. Some observations have reported the presence of increased p-ERK1/2 levels in cells that survive, while decreased levels in those cells that die post-ischemia may suggest that activation of the ERK1/2 pathway is required to prevent cells succumbing to ischemia-induced death, as ERK1/2 pathway is well known for promoting cell survival [284], which has been reported to protect the brain from damage in ischemic stroke in several studies [285, 286]. At the same time, ANG could trigger a rapid increase of ERK phosphorylation followed by a rapid decrease. It has been reported that exogenous human ANG induces ERK1/2 phosphorylation in HUVECs in a time-dependent manner, the p-ERK1/2 level increases rapidly, reaches a maximum at 1min, remains elevated for at least 30min, and starts to decline after 60 min [249]. Thus, it is possible that hr-ANG might have triggered a rapid increase of ERKphosphorylationbefore we examined the tissue and followed by a rapid decrease, leading to no significant differences compared with the vehicle-treated mice in our study. In this regard the p-ERK/ERK ratio was significantly enhanced at 3h post-therapy only in the ischemic hr-ANG treated mice compared with naïve mice, suggesting that hr-ANG activated the ERK signaling pathway post-stroke in our study. Actually, in the hr-ANG bioactivity study in naïve mice, we also found the p-ERK/ERK ratio transiently increased at 1h, further decreasing to non-ANG-treatment levels at 6h and maintained at 24h, which further supports that

hr-ANG could activate the ERK signaling pathway at very early time-points.

Finally, cell apoptosis has been considered as part of the ischemic cascade and reported in ischemic stroke for decades [60, 62]. Two apoptosis pathways have been characterized including the extrinsic or death-receptor pathway, and the intrinsic or mitochondrial pathway [61]. A well-accepted concept in the cell-death apoptosis research is that both pathways are mediated by an intracellular proteolytic caspase-dependant cascade and that procaspases are activated by binding to adaptor proteins [61]. The extrinsic stimuli could activate the Fas and tumor necrosis factor (TNF)-α, which can bind to the death-inducing signaling complex (DISC), which is composed of the death receptor (a typical example is Fas receptor), Fas-associated protein with death domain (FADD) and caspase-8 [61]. The intrinsic stimulation induces caspases to cleave a series of downstream targets including PARP, caspase-activated deoxyribonuclease (ICAD), cytoskeletal proteins and other caspases. During the process of caspase activation, cytochrome c is essential because it could form a complex named apoptosome with the existing caspase-9 and ATP [63]. At the end of the apoptotic mechanism, the cleaved caspase-3 is the most crucial and effector protein in cell death [62]. Also, the Bcl-2 family of intracellular proteins regulates the activation of procaspases. This family is comprised of both anti-apoptotic proteins such as Bcl-2 itself and Bcl-XL, and pro-apoptotic members including Bad, Bax, and Bak [287]. Several studies have shown the anti-apoptotic role of ANG in several diseases, such as cancer, neurodegeneration or the human alloreactive immune response. In cancer, ANG can participate in cancer development since it interacts with p53 inhibiting cancer cells apoptosis [288], in ALS disease ANG is protective since it reduces the ER stress-induced motoneuron apoptosis [147], in PD disease ANG is also protective since

it inhibits the apoptosis of dopaminergic cell lines [193], and in the human alloreactive immune response ANG protects against CD4+ T‑cell apoptosis.

However, studies of the potential anti-apoptosis actions of ANG after stroke are lacking nowadays. According to this thesis we report for the first time that ANG could reduce stroke-induced cell apoptosis at a relevant time point when infarct is still expanding proving the protective role of ANG administered after cerebral ischemia. Specifically, it is reported that ANG reduces the ischemia-induced levels of caspase proteins of the intrinsic pathway (active Caspase-9 and downstream active Caspase-3) as well as the pro-apoptotic expression of Bax: active caspase-3 level was significantly reduced at 24h post-therapy by hr-ANG treatment and the upstream ischemia-induced caspase-3, active caspase-9 and Bax increased from 3h to 24h post-therapy but being abolished only in the hr-ANG treated mice. Importantly, the therapeutic ANG was administered at the precise moment when apoptosis increased after cerebral ischemia, which could explain the subacute protection of the brain from expanding in the cortical areas.

5.3 Neurogenesis is enhanced after long-term hr-ANG treatment arising from the SVZ-niche.

Neurogenesis normally occurs in the SGZ of the hippocampal DG and the SVZ of the lateral ventricles in the adult brain [208] in both physiological and pathological conditions by neuroblast proliferation, migration and differentiation. Stroke could enhance the neuroblasts formation in the SVZ and stimulate these newborn neuroblasts to migrate into the injured striatum and cortex and differentiate into mature neurons [289]. In this scenario, it is also known that angiogenesis plays an important role in the process of neurogenesis after ischemic stroke, since it takes part of a specialized

microenvironment named neurovascular niche where the interaction between angiogenesis and neurogenesis occurs [224]. One explanation for this phenomenon is that the vasculature could supply oxygen, nutrients and soluble factors to support the migration of neuroblasts [228] but also that the vasculature could serve as a scaffold for neuroblasts migration towards the areas of repair [290]. Importantly, when angiogenesis is inhibited, the numbers of neuroblasts in the peri-infarct tissue are significantly reduced [224]. Therefore, the central role of ANG in angiogenesis suggests that ANG could also have an important role in regulating post-stroke neurogenesis, becoming a therapeutic target for stroke, but little is known. Some studies have shown that ANG could induce P19 cells to neurosphere formation [234], suggesting its implication in neuronal differentiation. Others have reported that ANG could induce mouse embryonic stem cells to differentiate into GFAP-positive progenitor neurons [234], and a recent study from the Neurovascular Research Laboratory at VHIR has shown that ANG increases the NSC yields of SVZ-derived neurosphere cultures from adult mice, suggesting its potential role in neuron precursors proliferation. With this background, we have investigated the long-term effects of subacute therapy of 5ug hr-ANG on endogenous repair in the ischemic brain. The present study shows for the first time that hr-ANG increased the migrating pools of DCX-positive cells (a neuroblast marker) arising from the neurogenic subventricular niche of the ischemic hemisphere and moving towards the injured tissue at two weeks of ischemia, suggesting a potential role of ANG in neurogenesis. However, we did not find an enhancement of angiogenesis at the studied time-point, suggesting that the hr-ANG therapy of our study could interact directly with neuroblasts in a mechanism independent from vascular remodeling. As it has been reported that Plexin-B2, which is a functional ANG receptor in a variety of physiological and pathological contexts [175], contributes to neurogenesis and promotes migration of

SVZ-derived neuroblasts [291]. Finally, we did not find the hr-ANG therapy increasing the general cell proliferation as the BrdU+ cells were not different among vehicle and hr-ANG treatment at two weeks post-ischemia. In this regard precise cellular investigations studying cell proliferation after ischemia and/or ANG treatment are needed, since it could be possible that other non-neurogenic cell populations could be also influenced by ANG (such as supporting glial cells) [292, 293], making it difficult to distinguish between neurogenic and non-neurogenic repair responses.

It is important to highlight that the results of this thesis propose a novel therapy for ischemic stroke in a clinically-relevant time window compatible with current life-saving pharmacological and mechanical thrombolytic therapies, which could not only protect the brain from the ischemia injury but could also impact neurorepair.

6. Conclusions

1. The proposed hr-ANG therapy is feasible and safe after ischemic stroke, providing bioactive hr-ANG several hours after administration.

2. The acute (90 minutes after occlusion) therapy with 5ug hr-ANG presents neuroprotective effects by improving neurological function at short-term and reducing subcortical infarct size.

3. The subacute (24h after occlusion) therapy with 5ug hr-ANG also provides neuroprotection by reducing total and cortical infarct size, as well as intracranial hemorrhagic events.

4. The subacute 5ug hr-ANG therapy promotes neurogenesis by increasing neuroblast migrating cells in the neurogenic subventricular niche of the ischemic hemisphere, but not vessel density.

5. The observed subacute neuroprotection could be targeting the stroke-induced cell apoptosis intrinsic pathway by reducing the expression of pro-apoptotic molecules such as Caspases and Bax

7. References

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8. Annex

8.1 CV

8.2 Publications

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EDUCATION AND TRAINING

- **PhD student in Neuroscience program (2017-2021),** Vall Hebrón Research Institute (VHIR), Universitat Autònoma de Barcelona (UAB), Barcelona
- **Master in Neurosurgery (2014-2017),** Department of Neurosurgery, the First Hospital of Jilin University, Jilin University.
- **Bachelor in clinical Medicine (2009-2014),** Shanxi medical University.

PUBLICATIONS

Gabriel-Salazar M, **Lei T**, Grayston A, Costa C, Medina-Gutiérrez E, Comabella M, Montaner J, Rosell A. Angiogenin in the Neurogenic Subventricular Zone After Stroke. Front Neurol. 2021 Jun 21;12:662235. doi: 10.3389/fneur.2021.662235. PMID: 34234733; PMCID: PMC8256153.

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Sun Y, Liu X, **Lei T**, Cao J, Huang H, Yu J. Primary intracerebral fibrosarcoma with intraventricular hemorrhage: a case report and literature review. Int J Clin Exp Med 2017;10(9):13888-13893.

SCIENTIFIC PRESENTATIONS

- Ting Lei et al. “Therapeutic actions of angiogenin therapy on stroke neuroprotection and neurorepair”. Oral and poster presentation in European Stroke Organization Conference. September 2021. Online.
- Ting Lei et al. “Human recombinant Angiogenin treatment as a neuroprotective treatment for cerebral ischemia”. Poster presentation in Vall d’Hebron Annual Conferences. December 2019. Barcelona.
- Ting Lei et al. “Human recombinant Angiogenin treatment as a neuroprotective treatment for cerebral ischemia”. Oral communication in VII INc Scientific Conferences. October 2019. Sant Feliu de Guíxols.

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ORIGINAL RESEARCH
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Angiogenin in the Neurogenic Subventricular Zone After Stroke

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Ischemic stroke is a leading cause of death and disability worldwide with effective acute thrombolytic treatments. However, brain repair mechanisms related to spontaneous or rehabilitation-induced recovery are still under investigation, and little is known about the molecules involved. The present study examines the potential role of angiogenin (ANG), a known regulator of cell function and metabolism linked to neurological disorders, focusing in the neurogenic subventricular zone (SVZ). Angiogenin expression was examined in the mouse SVZ and in SVZ-derived neural stem cells (NSCs), which were exposed to exogenous ANG treatment during neurosphere formation as well as in other neuron-like cells (SH-SY5Y). Additionally, male C57Bl/6 mice underwent a distal permanent occlusion of the middle cerebral artery to study endogenous and exercise-induced expression of SVZ-ANG and neuroblast migration. Our results show that SVZ areas are rich in ANG, primarily expressed in DCX+ neuroblasts but not in nestin+NSCs. *In vitro*, treatment with ANG increased the number of SVZ-derived NSCs forming neurospheres but could not modify SH-SY5Y neurite differentiation. Finally, physical exercise rapidly increased the amount of endogenous ANG in the ipsilateral SVZ niche after ischemia, where DCX-migrating cells increased as part of the post-stroke neurogenesis process. Our findings position for the first time ANG in the SVZ during post-stroke recovery, which could be linked to neurogenesis.

Keywords: stroke, angiogenin, neural stem/progenitor cells, neurogenesis, neurorepair, exercise

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INTRODUCTION

Stroke affects 15 million people worldwide annually, and it is a leading cause of long-term disability in industrialized countries (1, 2). Thrombolytic and endovascular thrombectomy are the only available treatments during the acute phase of ischemic stroke to reduce mortality and minimize functional and motor disabilities (3–5). However, the narrow time window limits these strategies, and only a small number of patients benefit from them. Although these vessel-recanalization strategies are effective, a large percentage of stroke survivors still suffer from motor disabilities and neurological deficits. With this scenario, the only proven effective treatment for disabled stroke patients is rehabilitation, which aims to compensate for the affected sensory-motor function and improve life quality and independence for daily activities (6, 7). In spite of the proven benefits

of multidisciplinary rehabilitation programs, these do not guarantee complete recovery for all patients, and individuals exhibit variable responses to similar treatments (8). In this regard, the biological responses responsible for the individual functional improvements have been investigated to identify brain plasticity mechanisms and targets to modulate the natural evolution of brain repair by rehabilitation (9–11), but are not fully elucidated. In this regard, a few pre-clinical studies have associated rehabilitation with restorative brain plasticity, including mechanisms of neuroangiogenesis (12–16); however, current knowledge on the molecules modulated by rehabilitation and potentially associated with brain plasticity is incomplete.

To further explore the molecular implications of tissue repair, we have focused on angiogenin (ANG), a ribonuclease protein that promotes cell proliferation and migration (17, 18) and related to excitotoxic motoneuron death in angiogenin loss-of-function mutations associated with ALS (19). We have previously shown ANG role in secretome-based therapies on brain endothelial cells (20), demonstrated the ANG upregulation in the blood of stroke patients under rehabilitation related to better outcomes at long term, and the angiogenin mRNA overexpression in the infarct tissue of ischemic mice also after rehabilitation (21). However, previous studies reported that neamine treatment (a blocking agent of the ANG activity) was neuroprotective after stroke in a rat model of type I diabetic rats and that the failure of bone-marrow-derived cell therapy after stroke in the same model of diabetic rats was potentially linked to an increase in periinfarct and vascular ANG in infiltrating macrophages (22, 23). Non-diabetic animals did not show ANG expression linked to vascular dysfunction (23). Its implications in neurogenesis are still unknown. In the present study, we aimed at investigating ANG in the subventricular zone (SVZ) niche after stroke by studying its tissue expression, effects on SVZ-derived neural stem cells (NSC), and its modulation after cerebral ischemia and rehabilitation.

MATERIALS AND METHODS

Brain Tissue Samples

All animals used in the present investigation are C57BL/6 male mice (6–12 weeks old). To investigate SVZ neurogenesis and ANG cellular expression, we used brain tissue slices from a previous protocol in which cerebral ischemia was induced also by the permanent electrocauterization of the distal branch of the middle cerebral artery and mice were submitted to physical exercise rehabilitation ($n = 6$) or non-rehabilitation ($n = 6$) (21). New mice were also used to obtain SVZ tissues in naive animals ($n = 4$) or after ischemia/rehabilitation (treadmill, $n = 6$) or ischemia/non-rehabilitation ($n = 6$). Finally, frozen NSC obtained from mouse SVZ cultures following published protocols (24) were also used. The experimental protocols were approved (Protocol Number 21.16) and supervised by the Animal Ethics Committee of Vall d'Hebron Institut de Recerca according with the Spanish legislation and the Directives of the European Union. ARRIVE guidelines were followed.

Mouse Habituation and Permanent Focal Cerebral Ischemia Model

Briefly, C57BL/6 mice were purchased from Janvier Laboratories (Saint Berthevin, France). Mice were housed in a temperature-/humidity-controlled room and maintained on a 12-h light–dark cycle and given water and food *ad libitum*. The habituation protocol for the treadmill was conducted to all mice before ischemia to avoid neophobic behaviors during exercise therapy. Briefly, the week before ischemia and for 3 consecutive days, all mice were placed in a stationary treadmill apparatus 10 min/day (from 3:00 to 6:00 p.m.).

The distal occlusion of the middle cerebral artery (MCAo) was conducted under body temperature and cortical cerebral flood flow (CBF) monitoring as described (25). Animals were anesthetized with isoflurane (Abbot Laboratories, Spain) for a maximum of 30 min via face mask (4% for induction and 1–2% for maintenance in Medicinal Air, 79% N_2/21% O_2), and eyes were protected using an ophthalmic ointment (Lipolac^{TM}, Angelini Farmaceutica, Barcelona, Spain). A small craniotomy was performed between the eye and ear area to expose the distal part of the MCA after temporal muscle retraction. The MCA was compressed using a 30-G needle and indirectly electrocauterized by heating the compressing needle. CBF was monitored using a laser-doppler flowmetry (Moor Instruments, Devon, UK), and only animals with a reduction in CBF below 80% were included. Buprenorphine (0.05 mg/kg) was administered subcutaneously during surgery, the skin was sutured, and mice were allowed to recovery from anesthesia under body temperature control.

Pre-clinical Treadmill Rehabilitation

For the study of SVZ neurogenesis, rehabilitation began 48 h after MCAo and consisted of 12 days of treadmill exercise or non-exercise (No-RHB). For treadmill, mice received 30 min of exercise by increasing the speed every 10 min (10, 15, and 20 cm/s) without any aversive stimulus (such as the electric shock), and a plastic barrier was placed between the shock grid and the treadmill line to prevent animals from resting on the top of the grid during the rehabilitation protocol. The No-RHB group was placed at the treadmill apparatus (0 cm/s) for 30 min the same days of treatment, but only free movements were allowed.

For the ANG molecular analysis of the SVZ, a new group of mice were habituated to the treadmill as described above, and 48 h after pMCAO, mice received treadmill rehabilitation or no rehabilitation for 3 consecutive days. The day after the last session, mice were euthanized for brain processing.

Infarct Volume Assessment

During the euthanasia procedure, brains were removed by intracardiac perfusion with cold saline and under deep anesthesia as described. Brains were cut into 1-mm-thick coronal sections and stained with 2.5% of 2,3,4-triphenyl-2H-tetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) for 10–15 min at room temperature when TTC solution was replaced by cold saline, and images were acquired for infarct quantification by the ImageJ free software as described previously correcting for brain edema (21).

Angiogenin ELISA

To determine the angiogenin levels in the SVZ, we dissected two middle sections of TTC-stained brains corresponding to the SVZ area along the wall of the lateral ventricles of the ipsilateral and contralateral hemispheres. Tissues were snap frozen in dry ice and stored at −80°C. Brain homogenates were prepared with 150 μl ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% BRIJ-35, 0.02% NaN_3, 1% Triton X-100, 1% phenylmethanesulfonyl fluoride, and 0.5% aprotinin), and protein content was collected from the supernatant after centrifugation at 15,000 g for 12 min at 4°C. Total protein was determined by duplicate in each sample by the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., Waltham, MA, USA). Finally, Mouse Angiogenin SimpleStep ELISA® Kit (ab208349, Abcam, Cambridge, UK) was used following manufacturer's instructions (sample dilution 1/5, and coefficient of variation of replicates <25%). Data are expressed as picograms of angiogenin per microgram of total protein per sample.

Immunohistochemistry

Mice of the SVZ neurogenesis study received daily intraperitoneal injections of 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg in saline, B9285, Sigma-Aldrich, St. Louis, MO, USA) beginning 48 h after MCAo until euthanasia to label proliferating cells. For euthanasia, transcardial perfusion with cold paraformaldehyde (4% PFA) was performed under deep anesthesia (isoflurane). Brains were removed and fixed with 4% PFA for 2 h, followed by 30% sucrose for cryoprotection, embedded in optimal cutting temperature (OCT) (Tissue-Tek, Fisher Scientific, Waltham, MA, USA), and frozen at −80°C until use. Slices (12-μm thick) were cut in a cryostat, placed at room temperature for 30 min, washed three times [0.1% phosphate-buffered saline (PBS)-Tween, 0.3% PBS–Triton X-100, and 0.1% PBS–Tween] and further incubated for 1 h with 2 M HCl-PBS followed by 10 min in 0.1 M borate buffer and 5 min in 0.1% PBS–Tween for the detection of nuclear BrdU of dividing cells. Sections were blocked using 0.1% PBS–Tween containing 1% BSA (Sigma-Aldrich, St. Louis, MO, USA) and 5% goat serum (Merck Millipore, Billerica, MA, USA) for 1 h. Then, slices were incubated with the following antibodies, namely, 1:400 rabbit anti-DCX (ab18723, Abcam, Cambridge, UK), 1:100 mouse anti-DCX (sc-271390, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:100 rat anti-BrdU (ab6326, Abcam, Cambridge, UK) or 1:100 rabbit anti-angiogenin (NBP2-41185, Novus, Centennial, CO, USA), or 1:100 mouse anti-nestin (556309; BD Biosciences, San Jose, CA, USA), and washed three times with 0.1% PBS–Tween prior to secondary antibody incubation. Alexa fluor 488 goat anti-rabbit IgG, Alexa fluor 488 goat anti-rat IgG, Alexa fluor 647 goat anti-rabbit IgG, or Alexa fluor 633 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) were used as secondary antibodies at 1:500 for 1 h at room temperature and washed with 0.1% PBS–Tween. Finally, sections were mounted in Vectashield™ with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and visualized using an Olympus BX61 (Olympus, Tokyo, Japan) or confocal laser scanning (FV1000, Olympus, Japan) microscopes.

Two brain slices per animal were imaged for the entire dorsolateral SVZ for the analyses. The total area of DCX+ fluorescence and double-positive cells (DCX+/BrdU+) was calculated using ImageJ software by an investigator who was blinded to the treatment group.

Neural Stem Cells

NSCs were obtained from the SVZ as previously described (24), **Figure 1A**. Frozen NSCs were thaw at 37°C and cultured in a mixture of 1:1 Dulbecco's modified Eagle's medium (DMEM) and F12 (Gibco, Thermo Fisher, Waltham, MA, USA) supplemented with 0.25% of P/S, 8 μg/ml of heparin (H-3149; Sigma-Aldrich, St. Louis, MO, USA), 0.02 μg/ml of hFGF-B (PHG0024; Thermo Fisher, Waltham, MA, USA), 0.02 μg/ml of hEGF (PHG0314; Thermo Fisher, Waltham, MA, USA), 2% of B27 (12587010; Thermo Fisher, San Jose, CA, USA), and 1% of L-glutamine (25030149; Thermo Fisher, San Jose, CA, USA). After 2 days in culture, 3D-proliferating structures known as neurospheres were observed (**Figure 1C**).

For phenotyping purposes, growing neurospheres were stained for nestin and doublecortin (DCX) at day 2. Briefly, neurospheres were fixed with 4% PFA for 10 min followed by three washes with 1× Dulbecco's phosphate-buffered saline (DPBS) and blocking with DPBS–Tween with 1% BSA (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. The primary antibodies mouse anti-nestin (1:200, BD-556309, BD Biosciences, San Jose, CA, USA) and rabbit anti-DCX (1:200, ab18723, Abcam, Cambridge, UK) were incubated overnight. Cells were washed three times, and the secondary antibodies Alexa Fluor 568 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG, respectively, were added for 1 h. Finally, cells were mounted in Fluoroshield™ with DAPI, and pictures were acquired using the Leica DM IRBE (Leica Microsystems, Wetzlar, Germany).

NSC Counts and Neurosphere Growth

Cell cultures of NSCs forming neurospheres were assessed using the trypan blue exclusion method and a hemocytometer to quantify NSCs numbers after angiogenin treatment (**Figure 2A**). Single NSCs were seeded at a density of 30,000 cells/ml and cultured in uncoated 12-well plates to allow neurosphere formation. Cells were treated on day 0, day 3, or both with 100 or 200 ng/ml of angiogenin. We also treated cells with 100 μM neomycin (a selective inhibitor of angiogenin, which blocks its nuclear translocation) on day 3. Three images per well were captured at ×100 magnification on day 5 of treatment using the Olympus IX71 microscope. ImageJ software was used to measure the neurosphere diameter. Afterwards, the neurospheres were collected and centrifuged at 1,500 rpm for 5 min; the pellet was resuspended in 300 μl of NSCs media and pipetted to obtain single NSCs, which were quantified by the trypan blue method with a hemocytometer. To evaluate the effect of each independent experiment, data are expressed as a percentage of the experimental control condition.

SH-SY5Y Culture and Neurite Outgrowth

The human neuroblastoma cell line SH-SY5Y was purchased from ATCC (ATCC® CRL-2266) since they exhibit a neuron-like phenotype with outgrowth neurites in the presence of Retinoic Acid (RA). Cells were maintained in complete medium containing DMEM/F-12 (Gibco, Thermo Fisher, San Jose,

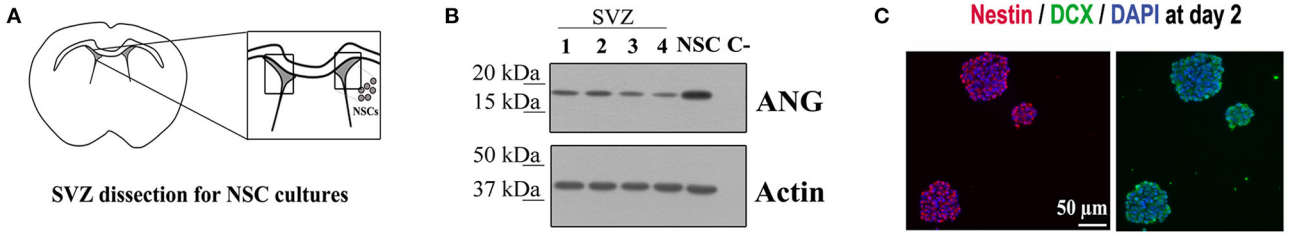

FIGURE 1 | Angiogenin expression in the SVZ niche: **(A)** schematic figure of the SVZ dissection for NSCs isolation. **(B)** Representative image of Western blot proving the presence of angiogenin in SVZ niche of different naïve mice and in primary NSCs in culture derived from the adult SVZ (see online **Supplementary Material** for the full-length Western blot film); **(C)** Neurosphere immunocytochemistry phenotyping showing expected nestin and DCX markers. ANG, angiogenin; C–, negative control (NSC culture media); DCX, doublecortin; NSCs, neural stem cells; SVZ, subventricular zone.

CA, USA), 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEA), and 1% penicillin–streptomycin (P/S). We seeded 12,500 SH-SY5Y cells onto collagen type-I-coated 24-well plates with complete media for differentiation. The medium was replaced with differentiation media after 24 h for neurite outgrowth: DMEM/F-12 (Gibco, Thermo Fisher, San Jose, CA, USA), 1% FBS, 1% NEA, and 1% P/S supplemented with ANG (100/200 ng/ml) or 10 µm RA to induce neurite differentiation. The media and treatments were changed after 2 days, and cells were imaged (three fields/well) on day 6 using an Olympus IX71 (×100 magnification), **Figure 3A**. Finally, WimNeuron automated analysis software (Wimasis Image Analysis®) was used for quantification by measuring the circuitry length and the total thin neurite length. Data are expressed as a percentage of the control condition of each independent experiment.

Western Blot

Brain dissections from the SVZ of naive C57Bl/6 mice and cell pellets from cultured NSCs were homogenized and lysed with freshly prepared ice-cold lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% BRIJ-35, 0.02% NaN_3, 1% Triton X-100, 1% phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA), and 0.5% aprotinin (Sigma-Aldrich, St. Louis, MO, USA). Homogenates were centrifuged at 12,000 rpm for 10 min at 4°C and the protein fraction in the supernatants assessed by the bicinchoninic acid assay (Thermo Scientific™, Rockford, IL, USA). A total amount of 10 µg protein was mixed with Laemmli buffer and 5% of 2-mercaptoethanol, heated for 5 min at 95°C, run into 12% polyacrylamide electrophoresis gels, and transferred into polyvinylidene fluoride (PVDF) membranes (Thermo Scientific™, Rockford, IL, USA). Then, membranes were blocked for 1 h with 10% non-fat milk (in PBS, 0.1% Tween 20, Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 4°C on a shaker with the following antibodies: anti-angiogenin (1:500, NBP2-41185, Novus, Centennial, CO, USA) or β-actin (1:5,000, A5316, Sigma-Aldrich, St. Louis, MO, USA). The membrane was then washed three times (PBS−0.1% Tween 20) and incubated with corresponding secondary antibodies at 1:2,000 for 1 h at RT with gentle agitation. Finally, membranes were washed three times (PBS−0.1%Tween 20) and briefly incubated with Pierce® ECL Western Blotting Substrate (Thermo Scientific™, Rockford, IL, USA) to visualize the chemiluminescence signal with Fujifilm FPM-100A films. Molecular weight markers were also run for reference values.

Statistical Analysis

The SPSS 20.0 package was used for all statistical analyses, and GraphPad Software was used for graph representations. The normality of continuous variables was assessed using the Shapiro–Wilk-test ($N < 30$). Normally distributed variables were analyzed using ANOVA (followed by Tukey *post-hoc*), and the Mann–Whitney *U*-test or Kruskal–Wallis-tests were used for non-normally distributed variables. For the analysis of repeated measures, the Wilcoxon test was used in non-normal distributions. Graphs represent means ± SEM or medians (interquartile range, IQR) according to the normal or non-normal distribution of the represented variable, respectively. Extreme values were excluded prior to data analyses of cell cultures using the mean ± 2SD criteria. The results with a $p < 0.05$ were considered statistically significant.

RESULTS

Angiogenin Is Expressed in the SVZ Neurogenic Niche and Increases NSCs Yields in Free-Floating Neurosphere

First, we examined for the first time the presence of ANG in this SVZ neurogenic site with the hypothesis that angiogenin could be involved in the regulation of neural precursors, which are known to respond to brain injury or physical exercise. As shown in **Figure 1B**, SVZ-naive tissues were rich in angiogenin as well as SVZ-derived NSC pools used in the present study for neurospheres formation, which, in culture, showed typical nestin and DCX markers (**Figure 1C**).

In vitro experiments with primary cell cultures of SVZ-derived mouse NSCs exposed to exogenous ANG were conducted as indicated in **Figure 2A**. Only the treatment with the highest dose of angiogenin (200 ng/ml) after the neurospheres were formed at day 3 significantly increased NSCs yields ($p < 0.001$), whereas cotreatment with neomycin (a well-known angiogenin activity inhibitor) completely abolished this proliferation ($p < 0.001$); **Figure 2B**. Indeed, no toxic evidence of neomycin was

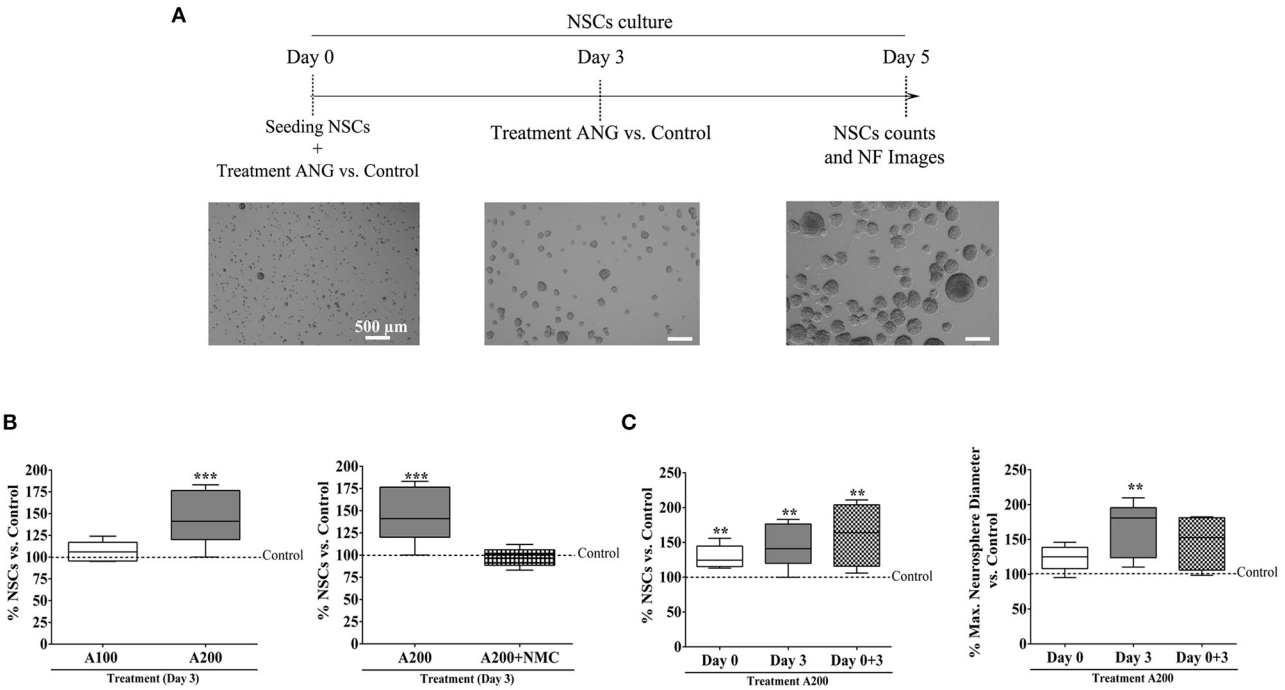

FIGURE 2 | SVZ-derived NSCs responses to angiogenin: **(A)** timeline of the experimental procedure of neurosphere cultures and representative images; **(B,C)** box plots showing quantification of the NSC-forming neurospheres after angiogenin stimulation (100 or 200 ng/ml) at different time points, the inhibition with neomycin, and the largest neurosphere diameters obtained with angiogenin treatment; $n = 4$–9; $^{**}p < 0.001$ and $^{***}p < 0.001$ vs. control. Box plots represent median (IQR) of the percentage vs. control values of each independent experiment. ANG/A, angiogenin; NSCs, neural stem cells; NMC, neomycin; NF, neurospheres.

observed on NSCs cultures ($p > 0.05$; **Supplementary Figure 1**, **Figure 1**). We also treated NSCs with the highest angiogenin dose (200 ng/ml) at the beginning of the culture prior to neurosphere formation (day 0), during neurosphere growing (day 3 after seeding), or both, and again, all treatment conditions showed a significant increase in NSCs yields vs. the control (day 0, $p = 0.002$; day 3, $p = 0.003$; and day 0–3, $p = 0.004$) with a visible increase in the neurosphere diameter; **Figure 2C**. In this regard, the maximum diameter achieved occurred when angiogenin was added to formed neurospheres on day 3 ($p < 0.01$ vs. control) (**Figure 2C**).

Finally, we investigated whether ANG could also trigger differentiation of a neuron-like cell line (SH-SY5Y cells) by its neurite outgrowth (**Figure 3A**). However, the capacity of axonal/neurite sprouting was only confirmed in the presence of retinoic acid, as expected ($p < 0.001$), but ANG did not show this mature neuron-like phenotype at any of the tested concentrations (**Figure 3B**).

Physical Exercise Increases SVZ Angiogenin After Ischemia in Areas of Active Neurogenesis

Three days after ischemia (**Figures 4A,B**), brain NAD in the SVZ was altered among the studied areas ($p = 0.0014$), showing the largest amount in the ipsilateral SVZ of the treadmill exercise group ($p = 0.0032$ vs. treadmill contralateral and $p = 0.0034$ vs. No-RHB contralateral SVZs), as seen in **Figures 4A,B**. This result was not influenced by potential differences in the ischemic lesion, since infarct volumes were similar between groups (treadmill 18.03 ± 3.2 mm^3 vs. No-RHB 16.23 ± 3.6 mm^3; $p = 0.72$, see **Supplementary Figures 2A,B**.

We also evaluated the presence of DCX+ cells in the SVZ, since neurogenesis was expected at later time points (12 days of treadmill exercise, **Figures 4A,C**). Our results show significant differences among the studied areas ($p = 0.015$) with larger DCX+ signal in the ipsilateral SVZ of treadmill-exercised mice ($p = 0.08$ vs. No-RHB ipsilateral and $p = 0.013$ vs. No-RHB contralateral, as shown in **Figure 4C**) but not in BrdU+ nuclei ($p = 0.06$, not shown).

The immunohistochemistry study showed that the ANG increase detected by ELISA in the SVZ of treadmill-exercised ischemic mice was localized in DCX+ neuroblasts emerging from the SVZ (**Figure 4D**), in the vicinity of nestin+ cells, which did not present ANG colocalizations (see magnifications in **Figure 4D**).

DISCUSSION

The present investigation focuses on describing angiogenin expression in the adult SVZ and its putative effects on neurogenic responses after stroke. Specifically, we describe that (i) for

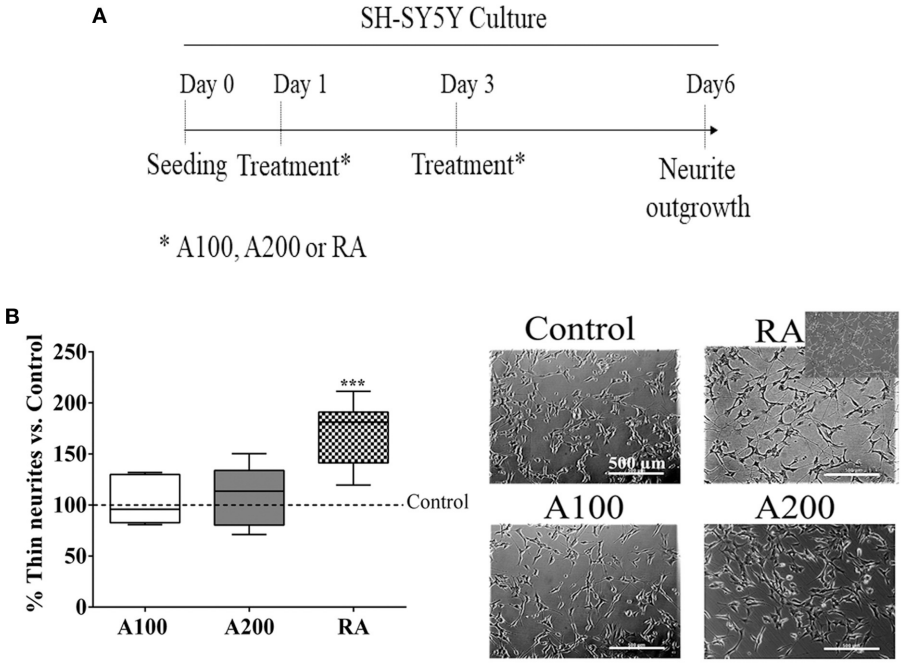

FIGURE 3 | SH-SY5Y neurite differentiation: **(A)** timeline of the experimental procedure. **(B)** Box plots and representative images showing axonal/neurite outgrowth in the presence of Retinoic Acid (RA) as expected, but not with angiogenin stimulation; $n = 5$–6. The insert in RA shows a micrograph representative of the WimNeuron analysis. Box plots represent median (IQR) of the percentage vs. control values of each independent experiment, $^{***}p < 0.001$ vs. control.

the first time, angiogenin is expressed in the adult SVZ, (ii) angiogenin increases the NSC yields in SVZ-derived neurosphere cultures, (iii) angiogenin is overexpressed after physical exercise in the SVZ of the ischemic hemisphere during neurogenesis, and (iv) SVZ angiogenin is mainly expressed in DCX+ neuroblasts. Our results position angiogenin in the neurogenic SVZ during stroke recovery, suggesting potential therapeutic interventions in neurorepair beyond the known actions on angiogenesis.

Many people survive stroke but exhibit physical and motor deficits that limit functional independence and quality of life. Current rehabilitation programs are implemented in developed countries to reduce stroke-related disabilities to ultimately compensate for the impaired functions (6–8). Several studies demonstrated that exercise improved functional recovery and activated cerebral-repair-associated processes within a plastic brain (11, 26). These data are supported by pre-clinical rehabilitation models as emerging strategies to investigate underlying mechanisms during the recovery phase of stroke and elucidate the molecular and cellular pathways activated during the rehabilitation therapies received in the clinical setting (27–30). Previous investigations in experimental models have described neurogenesis as a key mechanism regulated after stroke by showing increased neurogenesis in the SVZ and the subgranular zone (SGZ) of the hippocampus or reporting that neuroblasts from the SVZ migrated to infarct boundaries in response to the ischemic injury (31, 32). Additionally, studies in rodents under exercise conditions showed enhanced neurogenesis in the hippocampus (13, 33, 34) related to memory recovery and in the SVZ of ischemic brains (35, 36). According to these data, our post-stroke recovery treadmill moderate exercise also enhanced the DCX pools in the SVZ of the ipsilateral hemispheres. In this pos-stroke SVZ niche, we report for the first time the presence of a unique ribonuclease and potent trophic factor, angiogenin, in migrating neuroblasts in the active SVZ closely associated with other neural stem cell pools. Angiogenin is a ribonuclease protein that promotes cell proliferation and migration, and it is known to be secreted by endothelial cells (17, 20, 37). The actions of angiogenin were first described in tumor angiogenesis (38), but it also acts as a neuroprotectant in neurodegenerative diseases *in vitro* and *in vivo* (39). Furthermore, angiogenin is present during mouse embryogenesis and neuroectodermal differentiation (40), and it is also localized in axonal growth cones and neurites, where its inhibition impacts neural pathfinding (but not in embryonic cell differentiation). Among the multiple implications of angiogenin, the most important function described so far is its regulation of angiogenic-related routes in multiple experimental cell lines (17). Its role in neurogenic mechanisms is still unknown, although a close RNase (RNase A) has been recently described to induce NPC proliferation in embryonic-derived neurogenic cultures (41). In this argument line, other authors recently suggested that angiogenin, together with other proteins, participated in the prevention of neural differentiation

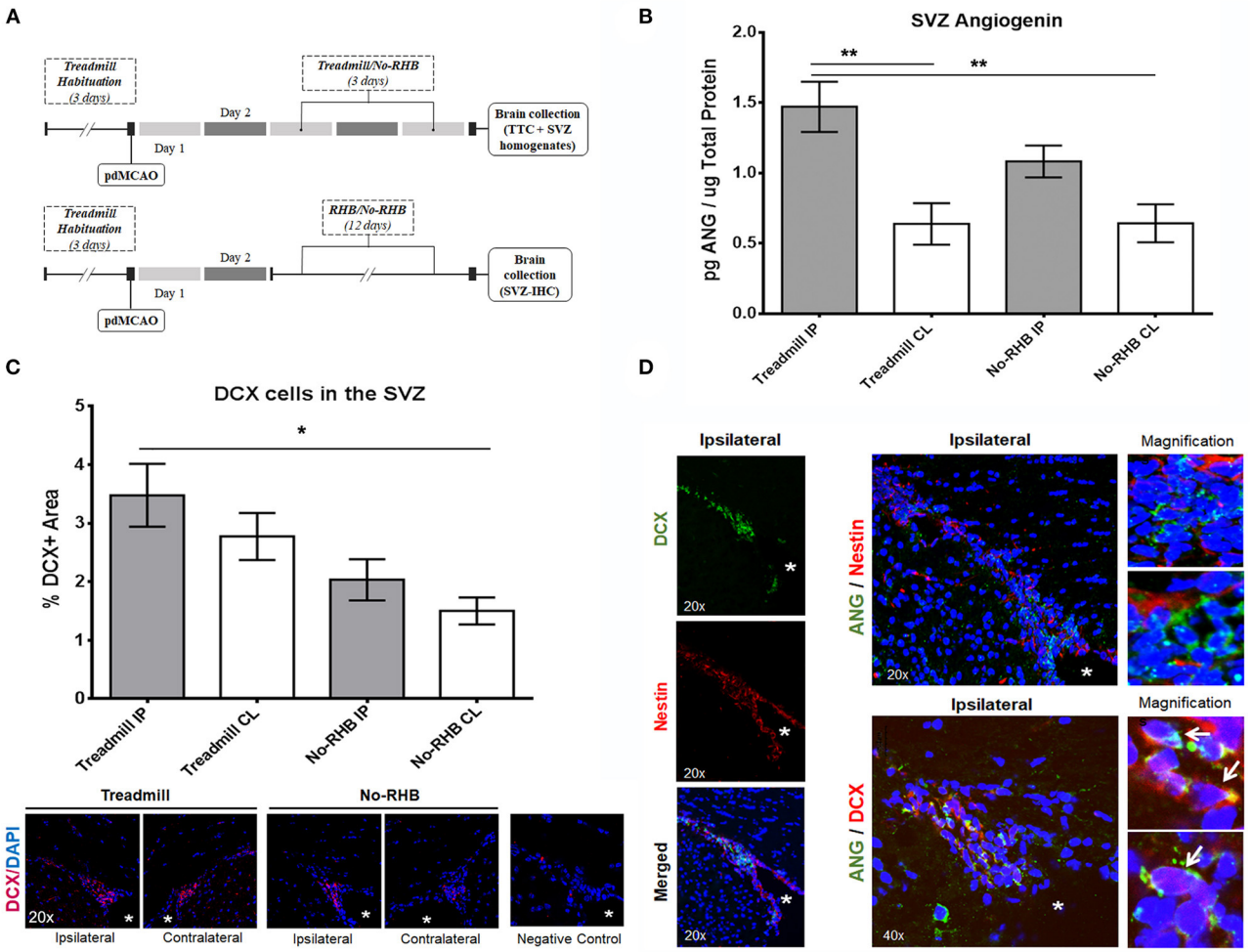

FIGURE 4 | Post-stroke SVZ neurogenesis and angiogenin expression: **(A)** timeline schemes describing the pre-clinical stroke and treadmill rehabilitation protocols. **(B)** Bar graph representing angiogenin after 3 days of treadmill rehabilitation or no rehabilitation in the SVZ area, measured by ELISA ($n = 6$/group); **(C)** bar graph representing DCX+ quantification in the immunohistochemistry study of the SVZ ($n = 5$/group) and representative immunostains of the ipsilateral hemisphere identifying the neuroblasts (DCX+). **(D)** Representative immunostains of the SVZ after stroke and treadmill exercise showing the distribution of nestin+ and DCX+ NSCs in the SVZ (left panel); notice that only neuroblasts (DCX+) had the most angiogenin expression, which is specifically shown in the insert magnifications and in white arrows. Bar graphs represent mean ± SEM, $^{*}p < 0.05$ and $^{**}p < 0.01$ as indicated by the horizontal lines. ANG, angiogenin; CL, contralateral; DAPI, 4′,6-diamidino-2-phenylindole; DCX, doublecortin; IP, ipsilateral; RHB, rehabilitation; SVZ, subventricular zone. The white * indicates the position of the lateral ventricle with contiguous dorsolateral SVZ.

of neuroepithelial stem cells (42) or demonstrated increased neurosphere formation in an embryonic carcinoma cell line after the addition of angiogenin in culture (43). Our study confirms the neurogenic actions of angiogenin protein in NSC primary cell cultures from adult SVZ niches for the first time by increasing the number of cell yields in growing neurosphere structures, which was further confirmed by adding neomycin (inhibitor of angiogenin) and suppressing the NSC responses. Importantly, we observe a dose–response effect that should be considered in any future therapeutic study design as well as the fact that the increased number of NSC yields forming neurospheres could respond to both proliferation and cell survival actions of angiogenin treatment. Angiogenin has been recently found to be present in the secretome of EPCs (20), and several authors reported that culturing NSCs from the SVZ with endothelial cells or its secretome maintained the stem-like characteristics and enhanced the proliferation of these cells (44, 45). However, the same studies demonstrated that NSCs in culture with endothelial cells under ischemic conditions migrated and differentiated to a neuroblast-like phenotype, which suggest that this mechanism serves as a repair response for neuronal replacement after injury.

SH-SY5Y cells are a subclone of a human neuroblastoma cell line and exhibit a neuroblast-like phenotype. These cells

express a marker of stem cell characteristics (46), namely, nestin, under undifferentiated conditions and differentiate into neurons in the presence of retinoic acid (47), which allows investigations on neuronal differentiation *via* the addition of drugs or molecules. In our study, we could not prove the differentiation of SH-SY5Y cells to a mature neuronal phenotype with neurite outgrowth when treated with angiogenin at the tested doses since neither circuit or neurite length were enhanced in the presence of angiogenin. These results are consistent with a previous report (41) showing that angiogenin was involved in the prevention of neural precursor maturation. Here, we should also acknowledge the existence of biphasic actions of this particular ribonuclease molecule. Initially, stress-induced responses have been extensively described in response to angiogenin actions leading to the cleavage of non-coding transfer RNA (tRNA) anticodons and producing tRNA halves (tiRNA) with cytoprotective actions involving cell survival and antiapoptotic mechanisms (48, 49); however, some recent studies point at existing cytotoxic actions of ANG (50) linked to the absence of the ribonuclease inhibitor protein, RNH1 (51).

In conclusion, the present study identifies angiogenin in the neurogenic SVZ and shows its potential actions on NSCs during neurogenesis. Additionally, in the context of stroke, ANG is overexpressed in the ipsilateral SVZ after pos-stroke exercise coexisting with the migration of SVZ-derived neuroblasts. Overall, our results support further investigations on the molecular mechanisms activated by post-stroke neurorehabilitation and the role of ANG as a therapeutic target, which should be explored *in vivo* in pre-clinical study designs of overexpression/exogenous therapeutic administration of ANG considering the potential interaction with comorbid conditions such as diabetes, age, or hyperglycemia.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the corresponding author, upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Animal Experimentation of the Vall d'Hebron Research Institute.

AUTHOR CONTRIBUTIONS

MG-S, TL, AG, MC, JM, and AR conceptualized and designed the studies. MG-S, AG, TL, CC, EM-G, and AR performed the *in vitro* and *in vivo* experiments/analyzed the data. MG-S and AR contributed to writing of the manuscript. All authors critically reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.662235/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.