Universitat Autònoma de Barcelona

MODULACIÓ DE MARCADORS DE NEUROREPARACIÓ EN TERÀPIES REHABILITADORES DESPRÉS DE L'ICTUS I EN UN MODEL PRECLÍNIC D'ISQUÈMIA CEREBRAL

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TESI DOCTORAL

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La recerca realitzada durant aquesta tesi doctoral s'ha dut a terme al Laboratori d'Investigació de Neurovascular (LIN) de l'Institut de Recerca de Vall d'Hebrón gràcies a les ajudes dels Projectes d'Investigació en Salut de l'Institut Carlos III PI13/01094 i PI16/00981, i a una beca predoctoral del VHIR atorgada a la Marina Gabriel Salazar.

Some day we will find what we are looking for.

Or maybe not,

Maybe we will find something much greater than that.
-Anonymous

RESUM

La malaltia de l'ictus té una elevada incidència, deixant a moltes de les persones afectades amb seqüeles físiques i motores permanents. Actualment, l'únic tractament disponible en la fase aguda, per intentar disminuir el dany cerebral, són les teràpies trombolítiques mitjançant fàrmacs com el t-PA (<4,5h) o mitjançant estratègies mecàniques com les teràpies endovasculars (<6h i fins a 24h en pacients seleccionats), per aquells pacients que presenten una oclusió arterial. Però malauradament, no tots els pacients poden beneficiar-se d'aquests tractaments degut als seus estrictes criteris d'inclusió. Per tant, la necessitat d'estudiar alternatives que ajudin a recuperar els dèficits neurològics causats per aquesta malaltia en fases més tardanes és essencial.

Fins a dia d'avui, la rehabilitació és l'únic tractament aprovat per ajudar a recuperar les funcions motores perdudes i millorar la qualitat de vida dels pacients. Però la resposta de cada individu a les teràpies de rehabilitació és heterogènia i es desconeix perquè alguns pacients no responen com s'esperaria a la teràpia. L'objectiu d'aquesta tesi és investigar nous biomarcadors que ajudin a conèixer l'estatus neurològic de pacients que han patit un ictus isquèmic al llarg del tractament rehabilitador i estudiar els mecanismes cerebrals subjacents que es troben activats durant les teràpies de rehabilitació mitjançant un model animal de neurorehabilitació.

L'angiogenina (ANG) i les cèl·lules endotelials progenitores (EPCs) han estat associades als processos de remodelació vascular en diferents patologies i més concretament les EPCs s'han associat com a possibles marcadors de l'estat neurològic en els pacients. És per això que són els biomarcadors candidats del nostre estudi. Els resultats reportats mostren que després d'un mes de teràpia rehabilitadora intensiva, els pacients presenten un increment dels nivells d'ANG circulants comparat amb els nivells pre-teràpia i en controls sans. En canvi, les EPCs es trobaven elevades abans de començar la teràpia com a conseqüència de l'ictus i seguien mantenint els seus nivells elevats durant els 3-6 mesos de teràpia en comparació amb els nivells en controls sans. A més, l'ANG ha estat associada a una millora funcional de l'estat dels pacients mitjançant l'índex de Barthel, Medical Research Council i el Rankin.

Per tal de complementar aquestes investigacions, es van utilitzar dos models animals de neurorehabilitació (tasca específica i exercici físic) després d'una isquèmia experimental. La rehabilitació mitjançant la tasca específica augmenta l'expressió d'*ang* en ambdós hemisferis després de 12 dies de rehabilitació, on l'exercici físic també incrementava la seva expressió en l'hemisferi contralateral. A més, ambdues estratègies activen la formació de vasos al còrtex contralateral. En canvi, l'exercici físic estimula l'expressió de neuroblasts a la zona subventricular (nínxol neurotròfic), i s'identifica per primera vegada l'expressió d'ANG en neuroblasts. Finalment en estudis *in vitro*, diferents cèl·lules mare i progenitores s'han exposat a ANG exògena i s'ha observat que l'ANG estimula les cèl·lules neurals progenitores precursores de neuroblasts augmentant la seva proliferació, però no la seva diferenciació. A més, l'ANG també estimula la funció de les EPCs *in vitro*, demostrant la implicació en l'angiogènesi, augmentant la formació d'estructures tubulars i la velocitat de migració cel·lular.

En resum, els resultats d'aquesta tesi proposen l'ANG i les EPCs com a futurs biomarcadors per avaluar la resposta dels pacients a la rehabilitació i conclouen que la rehabilitació es capaç de modular la resposta endògena potenciant els mecanismes de neuroreparació relacionats amb l'angiogènesi i la neurogènesi on l'ANG podria jugar un paper rellevant.

ABSTRACT

The high incidence of stroke leaves many people affected by physical and motor impairments. Currently, the only treatments available in the acute phase of stroke, which aims to minimize brain damage, are the thrombolitic therapies using drugs such as t-PA (<4.5h) or mechanical strategies such as endovascular therapies (<6h and within the first 24h in selected patients) for those patients who have an arterial occlusion. Unfortunately, due to the strict inclusion criteria of these treatments, only a minority of patients can benefit from these. Hence, there is a need to study alternative treatments during the chronic phases of stroke in order to improve the neurological deficits derived from this disease.
To date, rehabilitation is the only approved treatment that target to recover the motor functions and improve the quality of life of those disabled patients. However, the individual response to rehabilitation therapies is heterogeneous and it is unknown why some patients do not respond to therapy as expected. The objective of this thesis is to explore new biomarkers to deeply understand the neurological status of stroke ischemic patients during rehabilitation strategies and to study the underlying cerebral mechanisms active during rehabilitation therapies in an animal model of neurorehabilitation.

Angiogenin (ANG) and endothelial progenitor cells (EPCs) have been associated with vascular remodeling processes in different pathologies, and more specifically, EPCs have been described in patients as potential markers of neurological status. For this, they are the candidate biomarkers of our study. The results show an increase of ANG in patients after one month of intensive rehabilitation therapy compared to pre-therapy and healthy controls levels. On the other hand, the levels of EPCs increased before the therapy as a result of stroke and their levels have also been maintained during 3-6 months of therapy in comparison to healthy controls. Moreover, ANG has been associated with a functional improvement in the patient's condition in the Barthel index, Medical Research Council and Rankin scales.
In parallel to clinic investigations, we used two different animal models of rehabilitation (task-specific and physical exercise) after an experimental cerebral ischemia. Task-specific exercise increases the expression of *ang* in both hemispheres after 12 days of rehabilitation, whereas physical exercise increases its expression in the contralateral hemisphere. Interestingly, both strategies activate the formation of vessels in the contralateral cortex. Besides, physical exercise stimulates the expression of neuroblasts in the subventricular zone (neurotrophic niche) and ANG expression is identified for the first time in neuroblasts. Finally, by *in vitro* studies, different stem cells and progenitors were treated with exogenous ANG and we report a stimulation of neural stem cell precursors of neuroblasts after treatment, however not in their differentiation. Additionally, ANG stimulates the function of EPCs *in vitro*, which demonstrates its implication in angiogenesis by increasing the formation of tubular structures and the rate of cell migration.

In summary, this thesis proposes ANG and EPCs as future biomarkers to evaluate the patients' response to rehabilitation and concludes that rehabilitation is capable to modify the endogenous response by enhancing neurorepair mechanisms related to angiogenesis and neurogenesis, where ANG could play a relevant role.

INDEX

ABREVIATURES

ACM: Artèria cerebral mitja

ADP: Adenosina difosfat

AIT: Accident isquèmic transitori

AMPA: de l'anglès α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATP: Adenosina trifosfat

BBT: de l'anglès box and block test

BDNF: de l'anglès brain-derived neurotrophic factor

BHE: Barrera hematoencefàlica

BMP: de l'anglès bone morphogenetic protein

CAC: de l'anglès circulating angiogenic cells

CADASIL: de l'anglès Cerebral Autosomal-Dominant Arteriopathy with subcortical infarcts and Leukoencephalipathy

CAHAI: de l'anglès Chedocke Arm and Hand Activity Inventory

CART: de l'anglès cocaine-and amphetamine-regulated transcript

CD: de l'anglès cluster of differentiation

CIMT: de l'anglès constraint induced movement therapy

CPASS: de l'anglès Critical Periods After Stroke Study

CSE: de l'anglès cystathionine-γ-lyase

CXCR-4: de l'anglès Chemokine (C-X-C motif) receptor 4

ELA: Esclerosi lateral amiotròfica

eNOS: de l'anglès endothelial nitric oxide synthase

EPC: de l'anglès endothelial progenitor cell

EPO: eritropoietina

ESC: de l'anglès embryonic stem cells

FAC: de l'anglès functional ambulation categories

FGF: de l'anglès fibroblast growth factor

Flk-1: de l'anglès fetal liver kinase 1, receptor de VEGF

FMA: de l'anglès fugl-meyer assessment

G-CSF: de l'anglès granulocyte colony stimulating factor

GABA: de l'anglès gamma-hydroxybutyric acid

GDNF: de l'anglès glial cell line-derived neurotrophic factor

GM-CSF: de l'anglès granulocyte-macrophage colony-stimulating factor

HGF: de l'anglès hepatocyte growth factor

HIF: de l'anglès hypoxia-inducible factor

HSC: de l'anglès hematopoietic stem cell

IGF-1: de l'anglès insulin-like growth factor-1

IL: de l'anglès interleukin

iPSC: de l'anglès induced pluripotent stem cell

kDa: kilodalton

KDR: de l'anglès kinase insert domain receptor, també conegut com Flk-1, receptor de VEGF

LACI: de l'anglès lacunar infarct

MMP: de l'anglès matrix metalloproteinase

MRC: de l'anglès medical research council

mRS: de l'anglès modified rankin scale

MSC: de l'anglès mesenchymal stem cell

NHISS: de l'anglès national institute of health stroke scale

NMDA: N-metil-D-aspartat

NOS: de l'anglès nitric oxide synthase

NSC: de l'anglès neural stem cell

OCSP: de l'anglès Oxfordshire Community Stroke Project

OEC: de l'anglès outgrowth endothelial cell

OHdG: de l'anglès 8-hydroxy-2'-deoxyguanosine

OPC: de l'anglès oligodendrocyte precursor cell

PACI: de l'anglès partial anterior circulation infarct

PFT: de l'anglès pifithrin

POCI: de l'anglès posterior circulation infarct

ROS: de l'anglès reactive oxygen species

rRNA: de l'anglès ribosomal ribonucleic acid

rt-PA: de l'anglès recombinant tissue plasminogen activator

S1P: de l'anglès sphingosine-1-phosphate

Sca-1: de l'anglès stem cells antigen-1

SDF-1: de l'anglès stromal cell-derived factor 1

SGZ: de l'anglès subgranular zone

STAIR: de l'anglès Stroke Therapy Academic Industry Roundtable

STAT3: de l'anglès signal transducer and activator of transcription-3

SVZ: de l'anglès subventricular zone

TACI: de l'anglès Total Anterior Circulation Infarct

Tie-2: receptor de d'angiopoietina

TNF: de l'anglès tumor necrosis factor

TRI: Teràpia de rehabilitació intensiva

TOAST: de l'anglès Trial of Org 10172 in Acute Stroke Treatment

UEA-1: Ulex europaeus agglutinin 1

VEGF: de l'anglès vascular endothelial growth factor

VEGFR2: de l'anglès vascular endothelial growth factor receptor; també conegut com Flk-1 i KDR

vWF: de l'anglès von Willebrand factor

1.INTRODUCCIÓ

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1.1 L'ictus

1.1.1 Definició i classificacions

L'ictus o infart cerebral és un trastorn circulatori que es caracteritza per una alteració en el flux sanguini cerebral. Aquesta alteració pot ser produïda per una obstrucció, ja sigui de manera transitòria (si es restableix el flux espontàniament o terapèuticament), permanent (si no es restableix el flux) o per una hemorràgia deguda al trencament d'un o més vasos cerebrals. Com a conseqüència de l'aturada del flux sanguini poden aparèixer tot un seguit de símptomes neurològics com l'afàsia (trastorn de la parla), hemiparèsia (afectació de la capacitat motora d'un costat del cos), pèrdua de visió o trastorns del llenguatge, entre els més comuns[1]. Ara bé, el pacient presentarà uns símptomes o altres depenent de la zona cerebral afectada i aquests dèficits seran reversibles o no segons el temps d'oclusió, si es restableix o no el flux, de l'evolució clínica del pacient en la fase més aguda i de la seva resposta als tractaments rehabilitadors en cas de necessitar-los[2,3].

L'ictus representa la segona causa de mortalitat al món després de les malalties cardíaques i continua essent una de les malalties cerebrals més devastadores, causant 5 milions de morts anuals i deixant 5 milions de persones amb discapacitats motores i funcionals, segons dades obtingudes de la World Health Organization (www.who.org). A l'estat espanyol es produeixen 165 casos cada 100.000 habitants/any, sent la primera causa de mortalitat en dones i la segona en homes[4,5].

Al tractar-se d'una malaltia associada a l'edat, s'espera que incrementi la seva prevalença degut a que la població mundial, amb una esperança de vida superior als 65 anys, continua creixent especialment en els països desenvolupats[6]. Actualment, el número de supervivents després d'un ictus ha augmentat degut a la millora en l'assistència dels pacients a les unitats d'ictus i als tractaments trombolítics durant la fase aguda de la malaltia, tot i aquestes millores, un gran nombre de persones presentarà discapacitats en els propers anys[7–9].

Es coneixen un gran número de factors de risc associats a l'ictus i es poden classificar en factors modificables o no modificables. Un factor modificable és aquell que mitjançant una estratègia terapèutica es pot corregir o disminuir la seva intensitat. Alguns d'aquests factors poden estar associats a altres malalties cardiovasculars com ara, la hipertensió, la diabetis, les dislipèmies, la obesitat, síndrome metabòlic, el tabaquisme, l'alcoholisme, el sedentarisme, l'estrès i depressió, mentre que d'altres són més específics de l'ictus, com ara la fibril·lació auricular o els atacs isquèmics transitoris. En canvi, els factors no modificables són aquells que no es poden variar encara que s'intervingui sobre ells com ara, el sexe, la raça o l'edat així com el conjunt de

components genètics, ja siguin variants monogèniques com el CADASIL (Cerebral Autosomal-Dominant Arteriopathy with subcortical infarcts and Leukoencephalipathy) o malaltia de Fabry, o variants poligèniques com poden ser alteracions en els gens involucrats en la cascada de coagulació[10].

Tal i com es mostra a la **Figura 1**, els subtipus d'ictus es poden classificar en dos grups, depenent de la causa que alteri el flux sanguini cerebral: **ictus isquèmics** (quan hi ha una obstrucció d'un vas) o **ictus hemorràgics** (quan hi ha un trencament d'un vas i en conseqüència una extravasació de sang al parènquima). És important classificar els tipus d'ictus ja que això condicionarà el pronòstic i el tractament de cada pacient des de l'inici de la malaltia.

Els **ictus isquèmics** són els més comuns i representen el 85% dels infarts cerebrals. Es produeixen per una disminució del flux sanguini cerebral de forma total (isquèmia global) o parcial (isquèmia focal). Segons la durada de la isquèmia focal es defineix com accident isquèmic transitori (AIT) si es restableix el flux sense deixar lesió, o infart cerebral si no s'acaba restablint el dèficit del flux al cervell en un període curt i apareix la lesió cerebral. La isquèmia global es produeix quan hi ha una disminució del flux cerebral en tot l'encèfal de manera simultània, sent la causa més freqüent la parada cardíaca. En canvi, la isquèmia focal pot produir-se com a conseqüència d'un coàgul normalment provinent del cor (embòlic) o per plaques d'ateroma que obstrueixen la llum del vas (trombòtic)[2].

A més, els ictus isquèmics es poden classificar segons la seva localització o etiologia. La classificació segons la **localització** es basa en els criteris de la Oxfordshire Community Stroke Project (OCSP)[11] i es subdivideix en 4 categories clíniques, les sigles provenen de l'anglès.

1) TACI (*total anterior circulation infarct*): infart total en la circulació anterior cerebral.
2) PACI (*partial anterior circulation infarct*): infart parcial en la circulació cerebral anterior.
3) LACI (*lacunar infarct*) : infarts lacunars.
4) POCI (*posterior circulation infarct*): infart en la circulació posterior cerebral.

D'altra banda, en la classificació **etiològica** es fan servir els criteris TOAST (Trial of ORG 10172 in Acute Stroke Registry)[12], que s'enumeren a continuació:

1) Ictus aterotrombòtic: els pacients presenten una trombus generat per una placa ateroscleròtica (estenosi de més del 50%) o oclusió d'una gran artèria extracraneal o intracraneal. També es pot produir per la presència de plaques o per una estenosi menor del 50% associada a factors de risc cardiovasculars.

2) Ictus cardioembòlic: oclusions arterials degut a un èmbol provinent del cor.

3) Ictus lacunar o d'oclusió del petit vas: la lesió de l'infart és inferior a 1,5 cm.

4) Ictus de causa inhabitual: un cop descartades les etiologies anteriors es determina la causa menys freqüent com poden ser alteracions en la coagulació, aneurismes, infeccions, alteracions metabòliques o neoplàsies[2].
5) Ictus d'etiologia indeterminada: Després d'una exploració exhaustiva en el pacient i un cop descartades les situacions anteriors, el pacient presenta més d'una etiologia o no és possible diagnosticar el subtipus d'ictus.

Els **ictus hemorràgics** són els menys estudiats, representen el 15% del total d'ictus, són els més devastadors en cas d'afectar vasos grans i no disposen de cap tractament establert, exceptuant alguns casos d'evacuació de l'hematoma per cirurgia. Aquests, es caracteritzen per la presència de sang al parènquima o a l'interior dels ventricles laterals (hemorràgia intracerebral), o en l'espai subaracnoideu (hemorràgia subaracnoidea). La causa més freqüent és la hipertensió arterial (60% dels pacients) i altres causes associades poden ser la presència d'aneurismes o la malaltia d'angiopatia amiloide, entre d'altres[13,14].

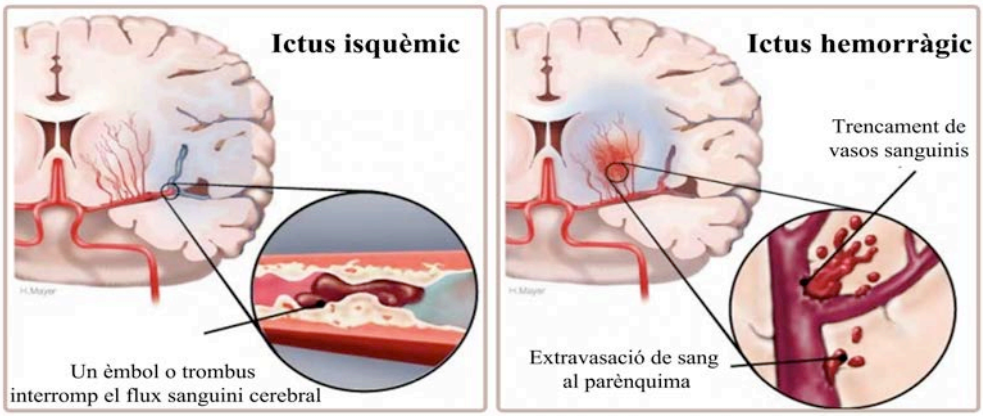


Figura 1: Classificació dels principals subtipus d'infarts cerebrals.

Finalment, per tal d'avaluar els dèficits neurològics després de l'ictus, el mètodes més utilitzats són l'escala de **National Institute of Health Stroke (NIHSS)** i **l'Escala modificada del Rankin** (mRS, de l'anglès *modified Rankin Scale*). En la NIHSS s'examina al pacient avaluant el seu grau de consciència, el moviment dels ulls, camp visual, dèficits sensorials o motors, atàxia, la parla, dèficits cognitius i l'atenció. L'escala té una puntuació de 0 a 42, de menys a més gravetat. Un increment de 4 punts significa un empitjorament, una variació de menys de 4 punts estabilitat i una disminució de 4 punts representa una millora en l'estat del pacient[15]. En la mRS es mesura el grau de dependència o discapacitat en les activitats diàries amb una puntuació de 0 a 6, on 0 representa pacients sense símptomes, 1 absència d'una discapacitat important on el pacient es capaç de realitzar les seves activitats i obligacions, 2-5 presència de discapacitat (on 5 és el grau més sever) i 6 punts la defunció[16].

1.2 Models animals d'isquèmia cerebral

Els models experimentals han estat essencials a l'hora d'entendre els mecanismes fisiopatològics que s'esdevenen després d'una isquèmia cerebral. En aquesta malaltia resulta imprescindible l'ús d'aquests models degut a la necessitat de trobar nous tractaments pel gran nombre de persones afectades per un ictus a diari, i a la limitada accessibilitat que presenta el cervell humà per investigar el que passa quan es produeix un dany cerebral, com l'alteració del flux o la mort cel·lular.

L'ús d'animals grans (gats, gossos, porcs o primats no humans) en l'estudi de l'ictus és poc utilitzat, restant més comú l'ús de rosegadors en els models experimentals degut al seu menor cost, facilitat de manipulació, monitorització i processament de teixits, així com també la seva millor acceptació ètica[17]. Cal afegir també que els rosegadors obren un gran ventall de possibilitats a l'hora d'estudiar la implicació de diferents molècules en la lesió cerebral, ja que els rosegadors poden ser modificats genèticament (transgènics) i això resulta imprescindible a l'hora que avantatjós en el estudis preclínics[18].

En general es poden dur a terme models animals d'ictus isquèmic o hemorràgic, quedant aquests últims fora del camp d'interès d'aquesta tesi. En els models experimentals isquèmics existeixen 2 tipus d'aproximacions per dur a terme una isquèmia en animals (**Figura 2**): **model d'isquèmia global** (comprèn tot el cervell) i el **model d'isquèmia focal** (comprèn una regió delimitada del cervell)[19].

En la **isquèmia global** no hi ha flux en tot el cervell, provocant així un dany neuronal ràpid en les àrees més susceptibles, com l'hipocamp. Les tècniques per dur a terme una isquèmia global completa són poc utilitzades ja que els animals presenten complicacions que poden arribar a introduir una variabilitat en els resultats, i sobretot perquè no representa la realitat clínica ja que en la majoria de casos corresponen a una isquèmia focal. A més, aquest model no es pot utilitzar per experiments a llarg termini ja que els animals necessiten una contínua monitorització i cures. Els models més utilitzats són el d'oclusió de 4 vasos o de 2 vasos. En el mètode dels 4 vasos, s'oclouen les artèries caròtides comuns i les vertebrals obtenint una reducció del flux cerebral menor del 3% al neocòrtex, estriat i a l'hipocamp. D'altra banda, el model d'oclusió dels dos vasos s'utilitza com a alternativa del model anterior. En aquest model s'oclouen les artèries caròtides comuns i s'indueix una hipotensió sistèmica, provocant així una disminució del flux sanguini en còrtex, tàlem i mesencèfal[18]. Actualment, els models d'isquèmia global no són gaire emprats en l'estudi de l'ictus.

Els models d'**isquèmia focal** reprodueixen millor els accidents cerebrovasculars humans, ja que la majoria dels ictus isquèmics són trombòtics o embòlics (80%), causant en la majoria de casos una obstrucció en l'artèria cerebral mitja (ACM). Actualment, els models més utilitzats són els que afecten només a un hemisferi cerebral per oclusió intravascular o proximal (gran vas) o per oclusió extravascular o distal (petit vas), aquesta última requereix craniotomia. La gran majoria de models es caracteritzen per l'oclusió de l'ACM, bé sigui mecànicament mitjançant un filament, un lligament, un clip o electrocauteritzant l'artèria o induint un trombe a través de la injecció de coàguls sanguinis o administrant trombina (model embòlic) o induint una vasoconstricció (endotelina-1) directament a l'ACM [17,19]. Finalment, hi ha el model fototrombòtic, en el que es produeix una lesió cortical induïda per una injecció sistèmica d'un component fotosensible (ex. Rosa de Bengala) i aquest quan s'irradia amb un làser indueix la formació de coàguls a nivell local[20].

Un factor molt important en aquests models és la durada de la isquèmia. Si la isquèmia es manté fins a l'eutanàsia de l'animal es tracta d'una oclusió permanent i si revertim l'oclusió de l'artèria després de la isquèmia, permetent així una reperfusió de la zona infartada, es defineix com a una isquèmia transitòria. La lesió produïda dependrà de molts factors com el temps d'oclusió i la localització del dany cerebral, que pot ser al còrtex, a l'estriat o a ambdós llocs. A més, hi ha d'altres factors que també determinen l'extensió de l'infart com el sexe, l'espècie i soca de l'animal, la temperatura corporal, la durada de l'anestèsia, la pressió arterial, així com l'edat dels animals[21].

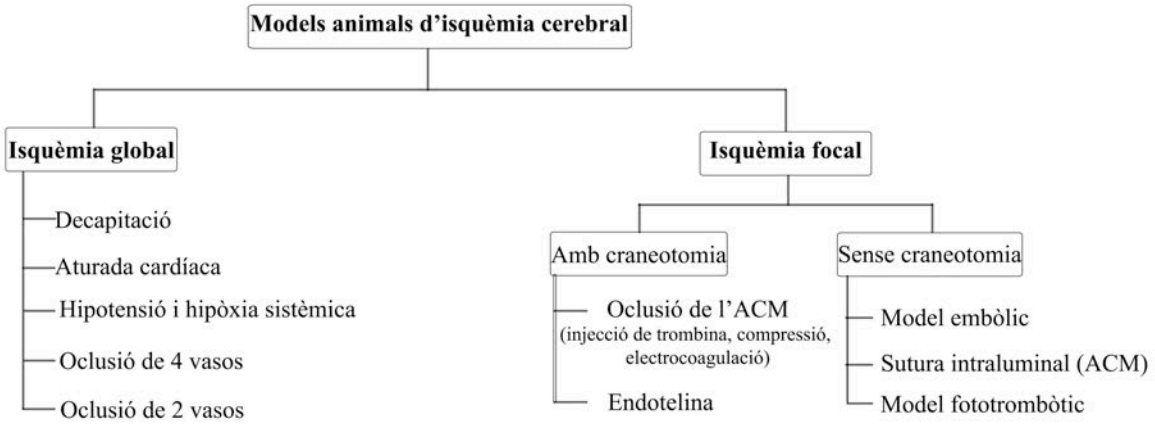


Figura 2: Principals models d'isquèmia cerebral en rosegadors (Adaptat de Kumar *et al.*, 2016)[22].

L'adequada elecció del model dependrà dels objectius de l'estudi experimental, ja que cada model té les seves característiques així com els seus avantatges i inconvenients. Per exemple, si es vol estudiar l'efecte dels fàrmacs neuroprotectors després d'una isquèmia, el model d'oclusió de l'ACM mitjançant un filament intraluminal seria adequat ja que mimetitza millor la isquèmia que presenten els pacients amb ictus més grans i extensos. Aquest model presenta una reperfusió controlable i així els fàrmacs poden arribar a la zona afectada[19,23]. Ara bé, si es vol estudiar l'efecte d'un fàrmac trombolític o adjuvant a la trombòlisi aquest model queda descartat i s'hauria d'utilitzar un model en el qual s'injectessin coàguls com el model embòlic. Per altra banda, en el cas que es vulguin estudiar els mecanismes de neuroreparació serà important escollir un model que presenti una baixa mortalitat a llarg termini i una lesió amb una zona peri-infart molt definida, aquest model podria ser el d'una oclusió distal de l'ACM mitjançant craniotomia, amb diferents modalitats per realitzar aquesta oclusió[24].

1.3 Fase aguda en l'ictus

1.3.1 La fisiopatologia de la isquèmia cerebral

La interrupció del flux cerebral després d'un ictus provoca un deficiència d'oxigen i glucosa en un àrea del parènquima cerebral. Aquesta zona afectada no és homogènia sinó que es pot classificar en dues regions segons el grau de perfusió del teixit: el nucli de l'infart o *core,* on el flux cerebral és pràcticament nul, i pot existir una àrea de penombra, definida com una zona d'hipoperfusió no infartada al voltat del *core*[25] (**Figura 3**). En l'àrea de penombra es manté, temporalment, suficient activitat elèctrica per a que els canals iònics no deixin de funcionar, és a dir les cèl·lules es troben metabòlicament actives. Així mateix, aquesta àrea és susceptible al processos de dany cerebral que esdevindran del *core* com l'exicitotoxicitat, el desequilibri iònic, l'estrès oxidatiu i una resposta

inflamatòria que contribuirà a l'expansió de l'infart[26]. Els factors més importants que contribuiran en aquesta expansió són el temps d'isquèmia, el grau de circulació col·lateral i l'estat funcional previ del parènquima i les cèl·lules en concret[27]. Per tant el concepte esdevé senzill: una oclusió fa disminuir el flux sanguini cerebral de manera que un tractament efectiu hauria de tornar a restituir aquest flux interromput i rescatar la zona de penombra. Si la situació d'hipoperfusió no es reverteix, la cascada isquèmica acaba produint un dany cerebral irreversible (**Figura 4**).

La conseqüència principal després d'una davallada del flux sanguini cerebral és un problema energètic degut a la falta d'oxigen i nutrients. La síntesi d'ATP (*adenosine triphosphate*) s'atura mentre que el cervell continua consumint energia produint una fallida metabòlica on el potencial de membrana deixa de funcionar i les membranes de les neurones i la glia es despolaritzen. En aquest moment hi ha un desequilibri iònic on s'origina un alliberament massiu de neurotransmissors i es produeix una inhibició de la recaptació d'aquests. S'allibera glutamat, un dels neurotransmissors més excitotòxics, i s'uneix als receptors N-metil-D-aspartat (NMDA) i α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) promovent un increment de l'entrada de Ca^{2+}. Aleshores, els receptors del glutamat promouen l'entrada de més ions de Na^{2+} i aigua provocant la formació d'edema, una disminució de l'espai extracel·lular i l'activació els processos catabòlics mediats per proteases, lipases i nucleases. A més, la sobrecàrrega dels nivells de Ca^{2+}, Na^{2+} i adenosine diphosphate (ADP) estimulen la producció de radicals d'oxigen mitocondrials i radicals lliures. Aquestes espècies reactives d'oxigen (ROS) produeixen un dany en els lípids, proteïnes, àcids nucleics i carbohidrats i participen en els processos d'apoptosi[28,29].

D'altre banda, el calci també activa altres vies secundàries de senyalització que condueixen a l'expressió de gens pro-inflamatoris com el factor nuclear-κB, factor d'hipòxia induible-1 (HIF-1), STAT3, així com mediadors de la inflamació, factor necròtic tumoral (TNFα) i interleucina 1β (IL-1 β). Hi ha diferents tipus cel·lulars que secreten aquestes citocines com la micròglia activada, cèl·lules endotelials, les neurones i astròcits, on aquesta producció es troba incrementada després d'un dany isquèmic. Aquestes citocines participaran en la infiltració leucocitària i augmentaran la permeabilitat de la barrera hematoencefàlica (BHE). La infiltració de leucòcits, en especial els neutròfils, contribuirà al dany cerebral produint l'alliberació de radicals lliures i l'activació de diferents proteases, augmentant així el risc d'edema i transformacions hemorràgiques cerebrals[30].

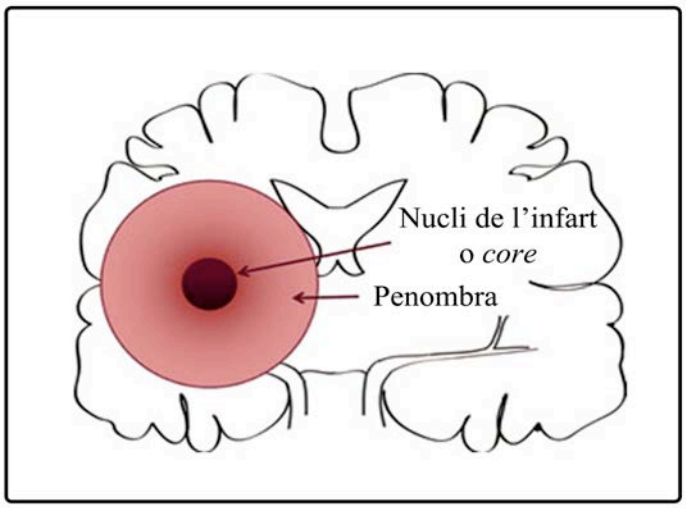


Figura 3: Representació del nucli de l'infart i l'àrea de penombra (Adaptat de Coultrap *et al.*, 2011)[31].

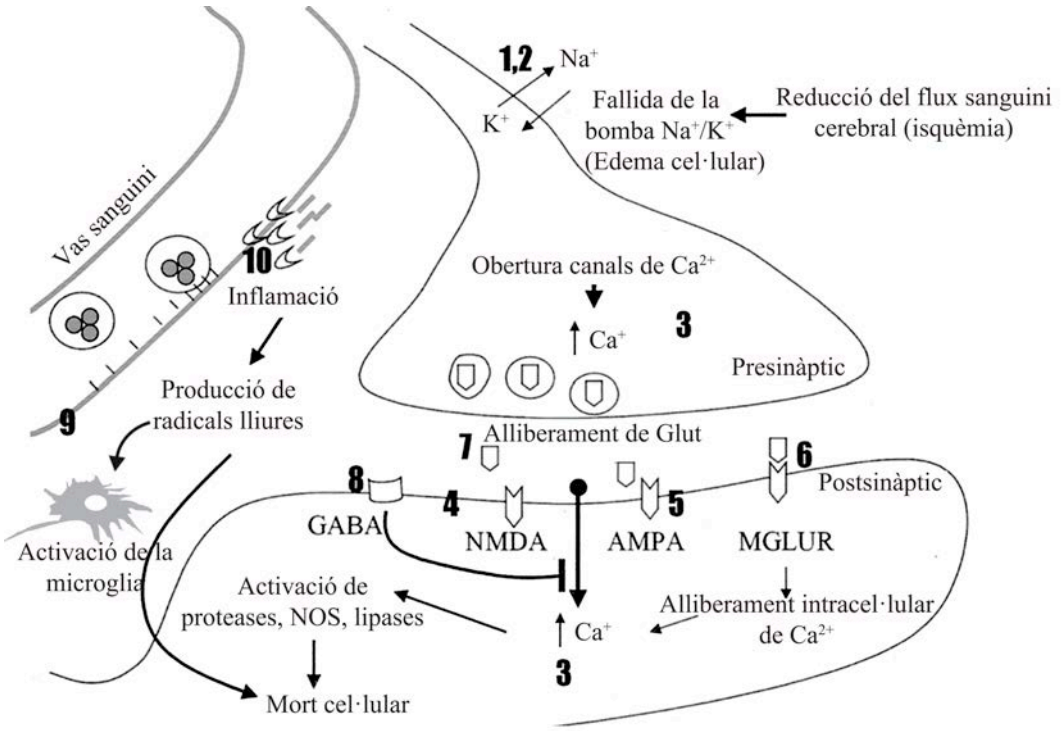


Figura 4: Resum dels mecanismes fisiopatològics després d'una isquèmia cerebral (Adaptada de Danton *et al.*, 2004)[32]. 1,2: Despolarització de membrana, desequilibri iònic. 3: Augment de Ca^{2+}. 4-6: Activació de receptors post-sinàptics que contribueixen a l'augment de Ca^{2+} intracel·lular. 7: Alliberament de glutamat. 8: Agonistes de GABA inhibeixen la despolarització de membrana i l'entrada massiva de Ca^{2+}.9: Degradació de membrana. 10: Cèl·lules inflamatòries estimulen la producció de radicals lliures contribuint al dany cel·lular.

1.3.2 Tractaments i estratègies terapèutiques

L'ictus és una malaltia que afecta a un gran nombre de persones, deixant dèficits neurològics aproximadament a un terç dels individus que pateixen aquesta malaltia i produint la mort directa a un altre terç. Així doncs, té un gran impacte social però també econòmic ja que suposa un cost rellevant dels pressupostos dels sistemes sanitaris públics anualment[33]. Per això és necessària una estratègia terapèutica més eficaç que l'actual en la fase aguda de la malaltia, com també una millora dels programes de neurorehabilitació.

Els tractaments actuals en la fase més aguda de l'ictus es basen en la monitorització propera del pacient en **unitats d'ictus especialitzades** amb controls exhaustius del ritme cardíac, els nivells de glucosa o la tensió arterial, entre d'altres. A més, pels ictus isquèmics és possible realitzar **intervencions terapèutiques de recanalització arterial** durant les primeres hores per revertir l'oclusió i minimitzar el dany posterior, revertint total o parcialment els dèficits neurològics que presenta el pacient.

Les estratègies de re-canalització poden ser **farmacològiques** com l'ús de fàrmacs trombolítics capaços de dissoldre el coàgul o mitjançant **dispositius mecànics** com les teràpies endovasculars (trombectomia), eficaces a l'hora d'extreure el coàgul.

Actualment, l'únic fàrmac trombolític aprovat per a l'ictus isquèmic és l'activador recombinant del plasminogen tissular (rt-PA), el qual transforma el plasminogen en plasmina i dissol el coàgul. Aquest fàrmac s'administra per via intravenosa durant les primeres 4,5 hores des de l'inici dels símptomes[34,35] i permet, aproximadament, un percentatge d'èxit del 50% en la re-canalització[36]. Però l'estreta finestra terapèutica en que es pot administrar aquest fàrmac i els estrictes criteris d'inclusió fa que només un 15% dels pacients pugui arribar a rebre aquest tractament[37]. A més, un 6-7% dels pacients presenta complicacions secundàries degudes al rt-PA com ara les transformacions hemorràgiques associades a un increment en la mortalitat[38,39].

Avui en dia, aquells pacients que no es puguin beneficiar dels tractaments trombolítics o en que la recanalització no hagi estat satisfactòria, tenen una alternativa, la trombectomia intravascular. Aquesta tècnica consisteix en l'extracció del coàgul mitjançant dispositius mecànics. Diversos estudis han demostrat la viabilitat i eficàcia d'aquest tractament[40,41] i recentment un estudi multicèntric ha demostrat que trombectomies realitzades fins a 8h després de l'inici dels símptomes presentaven una millora en els dèficits funcionals[42]. A més, es poden realitzar trombectomies en pacients seleccionats fins a les 24h[43]. Per tant, gràcies a aquest recent avanç terapèutic, les opcions per a tractar els malalts d'ictus tenen una finestra terapèutica més àmplia.

Tot i els tractaments de reperfusió actuals l'estratègia terapèutica més investigada en les darreres dècades, ha estat l'ús de neuroprotectors per evitar la mort neuronal (i cel·lular en general) amb més

de 1.000 publicacions en models preclínics i més de 100 assajos clínics[44]. L'objectiu dels fàrmacs neuroprotectors ha estat intentar minimitzar els danys produïts per la cascada isquèmica i prevenir la mort cel·lular, inhibint l'alliberament de calci, radicals lliures, neurotransmissors, citocines, entre d'altres. Un gran nombre de molècules neuroprotectores com les estatines, ciclosporina A, nitrones, o el natalizumab, han estat exitoses en models preclínics revelant resultats prometedors per al tractament de l'ictus (disminuint l'infart cerebral i millorant els dèficits funcionals), però malauradament han fallat en posteriors assajos clínics en pacients[45–48]. Aquest fracàs translacional ressalta la importància de millorar el disseny experimental en models animals. Amb aquest objectiu es van establir els criteris STAIR (de l'anglès, *Stroke Therapy Academic Industry Roundtable*)[49] amb diverses actualitzacions, on es detallen una sèrie de recomanacions, com escollir un model animal adequat, la dosi a administrar, temps d'administració i valorar amb més d'un test la funcionalitat motora o sensorial, però també millorar la qualitat dels estudis en termes de disseny experimental i de la forma de reportar resultats a la comunitat científica. Seguint la mateixa línia, també s'han descrit recomanacions a l'hora de dissenyar els assajos clínics de fase I/II a fase III en el context de l'ictus[50].

Amb això, avui dia encara no és coneix un tractament eficaç per disminuir el dany cerebral o reduir la mort cel·lular en termes de neuroprotecció després de l'ictus, per això cada cop es fa més imprescindible investigar teràpies associades als mecanismes de neuroreparació que s'activen en fases inicials de la malaltia però que es mantenen durant setmanes en la fase de recuperació (**Figura 5**).

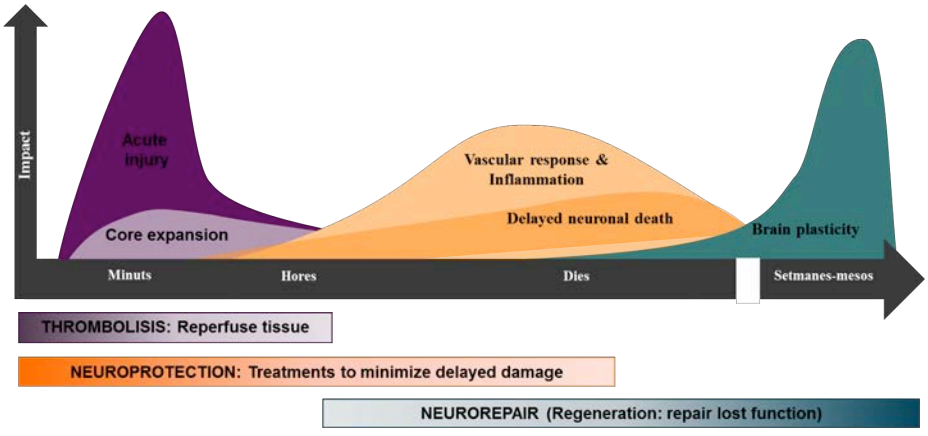


Figura 5: Cronologia dels processos de dany i reparació i de les finestres terapèutiques després d'una isquèmia cerebral (Adaptada de Zaleska *et al.*, 2009)[51].

1.4 Fase sub-aguda i crònica en l'ictus

1.4.1 Processos de neuroreparació

El parènquima cerebral està format per diferents tipus cel·lulars. Les cèl·lules dels vasos cerebrals (cèl·lules endotelials i perícits), astròcits, les neurones i els seus axons i altres cèl·lules de suport, com la micròglia i els oligodendròcits formen una xarxa complexa multifuncional dins d'una matriu extracel·lular anomenada **Unitat Neurovascular (Figura 6)**.

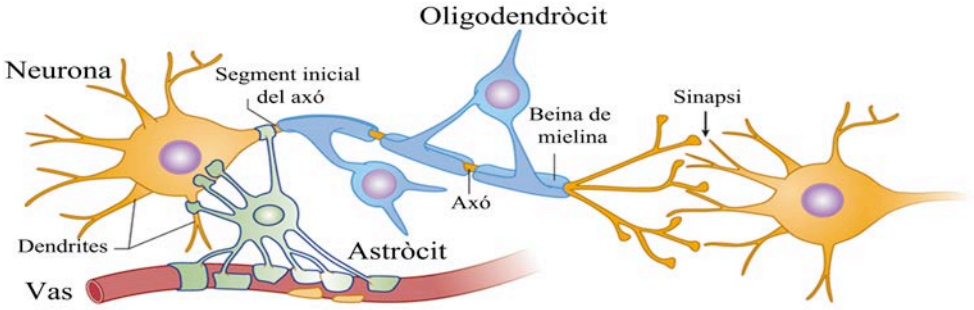


Figura 6: Esquema de la unitat neurovascular (adaptat de Bertan G. Katzung and Anthony J. Trevor: *Basic and clinical pharmacology*).

Avui en dia sabem que les cèl·lules de la unitat neurovascular es comuniquen entre sí, cèl·lula-cèl·lula o via comunicació paracrina[52,53]. I que després d'una isquèmia cerebral s'activen mecanismes que afecten al funcionament de la unitat neurovascular, però que també s'activen processos de neuroreparació, que es mantenen durant la fase sub-aguda i crònica, com mecanismes de supervivència i proliferació cel·lular, angiogènesi, neurogènesi i oligodendrogènesi, entre d'altres. Aquest processos són dinàmics, estretament relacionats entre sí i es poden activar de manera endògena o bé mitjançant diferents estratègies terapèutiques[54–56]. Així doncs, per tal de dissenyar futures teràpies efectives que recuperin la funció de la unitat neurovascular després de la fase més aguda de la malaltia, és important conèixer els mecanismes implicats en la neuroreparació i les molècules responsables del seu funcionament.

1.4.1.1 Angiogènesi

El procés de remodelació vascular té lloc tant en condicions fisiològiques com patològiques i es troba estrictament regulat. Al cervell es produeix a través de 3 mecanismes diferents: angiogènesi, vasculogènesi i creixement de vasos col·laterals. L'**angiogènesi** es defineix com la formació de nous capil·lars a partir de microvasos ja existents en el sistema vascular.

La **vasculogènesi** en canvi, es defineix com la formació de nous vasos sanguinis de nou[57]. Aquest mecanisme només es coneixia durant el desenvolupament embrionari, però la descoberta de les

cèl·lules endotelials progenitores (EPCs) en individus adults va revolucionar el camp de la remodelació vascular en l'adult, ja que les EPCs poden formar xarxes vasculars totalment funcionals en etapes posteriors al desenvolupament embrionari[58]. I per últim el creixement dels **vasos col·laterals**, que es produeix quan el diàmetre d'un vas incrementa per tal de fer arribar el flux sanguini a les zones d'alta demanda.

Després d'una isquèmia, l'angiogènesi té un rol crucial amb l'objectiu de generar nous vasos per tal d'augmentar la circulació colateral i així atenuar el dany produït. En models animals s'ha observat que minuts després de la isquèmia cerebral es produeix una sobreexpressió de gens associats als processos angiogènics juntament amb un increment de la proliferació de cèl·lules endotelials, a les 12-24h, i factors angiogènics que es mantenen elevats durant els primers 7 dies[59,60]. Nous capil·lars i vasos sanguinis apareixen 14 dies post-isquèmia[59,61]. D'altra banda, entre els 21 i 90 dies hi ha una davallada dels gens implicats en l'angiogènesi i de la densitat de vasos[59,62]. En pacients, Krupinski *et al.* van analitzar cervells post-mortem on van observar angiogènesi al voltant de l'àrea infartada, i també van descriure un augment d'aquesta 3-4 dies post-ictus. A més, l'increment de vasos sanguinis nous correlaciona amb una millora funcional i supervivència en models preclínics i en humans[54,63].

Hi ha diferents factors que regulen l'angiogènesi després d'un infart cerebral com ara, eNOS, CSE, VEGF i el seu receptor KDR, angiopoietina-1 i el seu receptor Tie-2, o l'endostatina[64–67], entre d'altres. En models animals, eNOS afavoreix la resposta angiogènica i la vasodilatació, ja que la seva inhibició suprimeix aquesta resposta[68]. VEGF es troba sobreexpressat, exercint una activació pro-angiogènica i promovent la formació vasos[60]. D'altra banda, Angiopoietina-1/Tie-2 actuen en fases més tardanes participant en la formació, manteniment i integritat dels nous vasos[69,70]. Al contrari que les molècules anteriors, l'endostatina participa en la inhibició dels processos angiogènics durant la fase aguda[71,72]. D'aquesta manera, la resposta a la isquèmia es troba regulada per l'expressió de factors promotors i inhibidors de l'angiogènesi. Juntament amb tots aquests factors, hi ha altres molècules que tenen un paper important en l'angiogènesi com l'angiogenina (que estudiem en les investigacions d'aquesta tesi).

L'angiogenina és una molècula que es troba implicada en l'angiogènesi fisio-patològica la qual interacciona amb les cèl·lules endotelials promovent la migració i proliferació d'aquestes[73]. Més endavant (apartat 1.5) s'aprofundeix en els mecanismes d'acció de l'angiogenina.

Finalment, la resposta angiogènica després d'una isquèmia es troba estretament relacionada amb l'activació dels processos neurogènics, on els capil·lars de la zona subventricular de l'hipocamp (SVZ) són permeables a factors alliberats, com FGF o VEGF, per les cèl·lules endotelials que constitueixen la unitat neurovascular. Aquesta estreta relació entre neurogènesi i angiogènesi ha

estat descrita en models experimentals d'isquèmia cerebral, on es creu que la neurogènesi és depenent de la vasculatura cerebral i la formació de nous vasos[71,74–77].

1.4.1.2 Neurogènesi

La neurogènesi és un procés que no només té lloc durant el desenvolupament embrionari sinó que continua en l'etapa adulta en els mamífers, incloent els humans. La neurogènesi es produeix en dues àrees específiques del cervell: a la SVZ i a la zona subgranular del gir dentat de l'hipocamp (SGZ)[78]. Després d'un estímul com la isquèmia pot produir-se una activació de la neurogènesi i les cèl·lules mare neurals (de l'anglès *neural stem cell* (NSCs)) comencen a proliferar[79]. Es coneix que aquest procés es troba regulat per diferents factors de creixement (FGF, IGF-1, BDNF o VEGF), mecanismes genètics intrínsecs o factors modulables com ara l'exercici físic[54,71]. Un cop aquestes cèl·lules han proliferat és necessari que migrin a les zones d'interès. En condicions no patològiques, els neuroblasts de la SVZ migren al bulb olfactori a través de l'anomenat tracte migratori rostral. Però després d'una isquèmia canvien els patrons fisiològics de migració i aquestes cèl·lules es dirigeixen, sempre en contacte amb vasos sanguinis, a la zona de l'infart on els processos angiogènics es troben actius[75,76,80]. Aquest últim mecanisme dependrà del balanç entre molècules promotores i inhibidores, de cicatrius que impedeixen la migració, i de les citocines que indueixen a la mobilització dels neuroblasts. Mitjançant l'administració d'inhibidors de l'angiogènesi, com ara l'endostatina, s'ha observat una disminució radical del número de neuroblasts a la zona peri-infart del còrtex provinents de la SVZ, evidenciant el nexe entre els processos d'angiogènesi i neurogènesi[76].

Finalment en estudis *in vitro*, les NSCs provinents dels ventricles o l'hipocamp tenen la capacitat d'autorenovar-se i diferenciar-se a neurona, astròcit o oligodendròcit[81], d'aquesta manera podem estudiar els factors que estimulen la seva proliferació i diferenciació i així conèixer millor els seus mecanismes d'acció en el parènquima cerebral.

1.4.1.3 Oligodendrogènesi

L'oligodendrogènesi es defineix com la capacitat de diferenciació de les cèl·lules precursores d'oligodendròcits (de l'anglès, *oligodendrocyte precursor cells*, OPCs) a oligodendròcits madurs mielinitzats en l'etapa adulta. Aquesta diferenciació es produeix en la substància grisa o blanca del cervell a través d'OPCs residents al parènquima cerebral o a partir d'OPCs diferenciades de les NSCs de la SVZ. Aquestes OPCs migraran al cos callós, l'estriat i al còrtex[82].

Quan es produeix una isquèmia cerebral amb un dany a la substància blanca, els oligodendròcits (cèl·lules formadores de la mielina) del sistema nerviós central esdevenen vulnerables. El dany produït en aquestes cèl·lules fa que hi hagi una alteració de la funció axonal deguda a la desmielinització. Per tal d'intentar restaurar aquest dany, hi ha una producció de nous oligodendròcits a partir d'OPCs, ja que els oligodendròcits madurs no tenen capacitat de dividir-se al cervell adult[83].

En models animals d'isquèmia cerebral s'ha observat un augment en la proliferació d'OPCs amb capacitat de madurar a oligondendròcits mielinitzats a la substància gris i blanca del peri-infart[84,85]. S'han identificat OPCs provinents de la SVZ en cervells post-mortem de pacients amb una malaltia desmielinitzant com l'esclerosi múltiple[86]. A més, hi ha estudis que postulen que els factors SDF-1α o VEGF participen en la migració d'OPCs a la zona peri-infart de la substància gris i a la substància blanca[87,88]. També es creu que els oligodendròcits secreten factors de creixement que ajuden a la supervivència i funció de les neurones confrontants[89].

1.4.1.4 Altres mecanismes de reparació

La neuroplasticitat, juntament amb la comunicació sinàptica, comprenen un altre tipus de mecanisme partícip en els processos de neuroreparació. La plasticitat sinàptica es defineix com el conjunt d'alteracions anatòmiques i funcionals en el sistema nerviós central en resposta a estímuls externs així com a lesions cerebrals. Entre els mecanismes implicats en la plasticitat s'inclouen: la regulació dels circuits cerebrals, activació de vies que es troben silents que participen en la formació de noves espines dendrítiques i creixement de les neurites formant noves connexions sinàptiques[54,90,91].

És important tenir en compte que després d'un dany cerebral es produeixen millores funcionals gràcies als processos de recuperació espontània a través de mecanismes estretament relacionats amb la formació i remodelació de noves connexions neuronals. A més, s'ha observat que després d'una isquèmia s'activen mecanismes de remodelació axonal i processos de sinaptogènesi[92,93]. Cal destacar també que en humans, aquests processos de neuroremodelació poden ser potenciats mitjançant tasques complexes com la rehabilitació, que facilita la recuperació de les funcions motrius[94].

1.4.2 Teràpies neurorehabilitadores

Com s'ha comentat anteriorment, els avenços en els tractaments durant la fase aguda i les millores en l'assistència han contribuït a augmentar la supervivència dels malalts que han patit un infart cerebral. Malgrat això, un cop superada la fase hiperaguda, segueix existint un gran nombre de persones amb dèficits funcionals i motors amb diferents graus de severitat pels quals les úniques teràpies disponibles són els programes de rehabilitació.

Com hem vist després de la isquèmia s'activen mecanismes de reparació de forma espontània però sovint no són suficients per tal de retornar a la normalitat les funcions afectades, per això és indispensable iniciar teràpies rehabilitadores.

L'objectiu principal de la rehabilitació és el d'aconseguir la màxima autonomia possible que ajudi al pacient a la inserció laboral, familiar i social. Es considera que el 50% dels supervivents a un ictus presentaran dèficits funcionals i requeriran assistència en les seves activitats diàries[95,96]. La majoria de guies de pràctica clínica recomanen iniciar la rehabilitació el més aviat possible un cop estabilitzada la situació clínica del pacient, i supervisada per un equip multidisciplinari (metges rehabilitadors, fisioterapeutes, terapeutes ocupacionals, logopedes, infermers, neuropsicòlegs i treballadors socials)[3,96–98]. Tanmateix, hi ha alguns estudis que suggereixen que s'ha d'evitar la mobilització del pacient durant les primeres 24 hores ja que, això podria afectar a la recuperació posterior del pacient[99,100].

Els programes de rehabilitació que es duen a terme en el nostre sistema sanitari són força diversos i heterogenis i estan distribuïts en diferents nivells assistencials: hospitals de dia amb o sense ingrés hospitalari, centres monogràfics, centres sociosanitaris, rehabilitació ambulatòria i domiciliària. Aquests programes varien en el temps, la intensitat i el tipus d'intervenció[98].

És important valorar per part d'un metge rehabilitador especialista les necessitats de rehabilitació de cada pacient en les primeres 24-48h de l'ictus[98]. La decisió d'incorporar al pacient a una intervenció d'alta, mitjana o baixa intensitat ve determinat per diferents factors: socio-demogràfics, gravetat del dèficit neurològic, clínics, capacitat de participar en les teràpies de rehabilitació i la presència o no del cuidador. Les característiques dels pacients per tal d'incloure'ls en cada programa són les següents[101]:

- ALTA INTENSITAT (3h al dia, multidisciplinari): Inclou aquells pacients on una actuació intensiva podria millorar el pronòstic.
- MITJANA INTENSITAT (1h al dia, multidisciplinari): Inclou pacients que independentment de l'edat i amb pronòstic favorable de recuperació, permeten seguir la rehabilitació en unitats d'estada mitjana o a domicili.

- BAIXA COMPLEXITAT (programes de manteniment): Inclou pacients de perfil de llarga estada, on la presència o no del cuidador determinarà la necessitat d'institucionalització.

Hi ha evidències que assenyalen que els pacients que inicien la rehabilitació durant la primera setmana post-ictus, tenen menys grau de discapacitat i una millor qualitat de vida[96,98,102]. A més, un tractament precoç d'alta intensitat millora la funció motora a llarg termini[102,103]. Per tant és important garantir que el pacient faci la màxima activitat terapèutica que pugui tolerar des de l'inici del tractament rehabilitador.

Per tal d'avaluar exhaustivament el grau de discapacitat (motor o funcional) del pacient hi ha diferents tests que s'utilitzen abans, durant i després de la teràpia de rehabilitació. Els seus noms provenen de l'anglès i a continuació s'enumeren els més utilitzats:

- Modified Rankin scale (mRS): Com s'ha comentat en l'apartat 1.1, és una de les escales més utilitzades on s'avalua el grau d'independència funcional del pacient[16].
- Granger modified Barthel Index: S'avalua la independència del pacient en les activitats de la vida diàries amb una puntuació de 0 a 100 (0: dependent total i 100 independent)[104].
- Fugl-Meyer assessment (FMA): avaluació del dèficit motor, sensorial, equilibri, rang de moviment i dolor articular. Puntuació de 0 a 66, on 0 indica màxima severitat[105].
- Functional ambulation categories (FAC): mesura únicament la marxa del pacient de 0 a 5, on la màxima puntuació indica normalitat[106].
- Chedoke Arm and Hand Activity Inventory (CAHAI): avalua la independència a l'hora de realitzar tasques quotidianes amb ambdues mans. Puntuació de 13 a 91, on la màxima puntuació indica independència[107].
- 10-meter walk test: temps que triga el pacient a fer 10 metres. Així es pot observar la seva mobilitat funcional, la marxa i l'equilibri[108].
- Medical Research Council (MRC): Avalua la força muscular de les extremitats superiors i inferiors. La seva puntuació va de 0 a 5, on la puntuació màxima indica estat normal[109].
- Box and Block Test (BBT): mesura la capacitat funcional, on es mesura el número de cubs que trasllada el pacient d'una caixa a una altre en un minut[110].

Conjuntament amb aquesta bateria de tests, també és mesura el grau d'espasticitat degut a que els pacients d'ictus poden arribar a tenir una lesió en les motoneurones. Aquesta mesura es porta a terme mitjançant l'escala d'Asworth, on es mesura el to muscular amb una puntuació de 0 a 5. Una puntuació de 0 indica to muscular normal[111].

1.4.3 Biomarcadors de la rehabilitació

La resposta de cada pacient a la rehabilitació és heterogènia, per això en els últims anys, s'està donant importància a l'estudi de biomarcadors que permetin conèixer tant l'estat del pacient com el seu potencial de recuperació i pronòstic. Amb l'ajuda d'aquests marcadors podríem entendre les diferències en la resposta de la rehabilitació entre pacients i poder oferir un tractament clínic més personalitzat segons les necessitats individuals.

Els biomarcadors són molècules indicadores de l'estat de la malaltia i són una eina indispensable en la pràctica clínica actual, que reflecteix els mecanismes cel·lulars i moleculars subjacents que són difícils de mesurar directament. Un bon biomarcador per a la recuperació després d'un infart cerebral seria aquell que reflexa el que està passant al cervell i que correlaciona amb l'estat del pacient. Diferents estudis han estat publicats amb evidències prometedores en l'ús de molècules predictores o mètodes d'imatge durant la rehabilitació. Tot i això actualment no existeix cap biomarcador aprovat per la malaltia de l'ictus.

Els diferents tipus de biomarcadors estudiats fins el dia d'avui en fases de rehabilitació post-ictus són escassos i es poden classificar en:

- Fluids biològics (sang, orina, saliva...): nivells de 8-hydroxy-2'-deoxiguanosine (8-OHdG) en orina, les MMPs (concretament, MMP3), marcadors inflamatoris i d'estrès oxidatiu han estat descrits com a possibles predictors de pronòstic durant i després de la rehabilitació[112–114].
- Neuroimatge: Mesures del tracte corticospinal per avaluar la integritat de la substància blanca han estat identificats com a possibles biomarcadors d'imatge en ressonància magnètica associats a la recuperació funcional, entre d'altres[115,116].
- Neurofisiològics: potencials motors evocats en resposta a l'estimulació magnètica transcranial, també poden predir el pronòstic a nivell motor. Altres tècniques com magnetoencefalografia o electroencefalografia s'han fet servir per mesurar oscil·lacions neuronals en neurotransmissors (GABA, glutamat) al sistema nerviós central[117].

Finalment, la possibilitat de combinar més d'un d'aquests marcadors pot augmentar les possibilitats a l'hora de conèixer la resposta a la teràpia rehabilitadora després d'un infart cerebral i oferir informació que permeti modular i personalitzar encara més les teràpies rehabilitadores actuals.

1.4.4 Tractaments experimentals amb teràpies avançades en neuroreparació

A banda dels tractaments no farmacològics com la rehabilitació, existeixen alternatives per tal d'activar o afavorir els processos endògens de neuroreparació. Algunes d'aquestes alternatives són la teràpia cel·lular i l'administració de factors tròfics o fàrmacs que potenciïn la neuroplasticitat. La diferència principal entre la teràpia cel·lular o factors tròfics i l'administració de fàrmacs és que els primers interaccionen amb el parènquima cerebral depenent del microambient mentre que els fàrmacs interaccionen segons les seves característiques farmacològiques[118].

Les cèl·lules mare han demostrat tenir gran potencial per reparar el dany cerebral i recuperar la funció neurològica afectada. Aquestes cèl·lules provenen de diferents orígens i poden actuar modulant l'angiogènesi i la neurogènesi, tant en humans com en models animals[56,119]. A la **Taula 1** es resumeixen els resultats obtinguts a partir de l'administració de diferents tipus de cèl·lules mare/progenitores després de la isquèmia en models animals i en assajos clínics. Les cèl·lules més utilitzades fins al moment han estat les cèl·lules mare mesenquimals (MSCs, de l'anglès *mesenchymal stem cells*) i derivades de NSCs (de l'anglès *neural stem cells*) degut als prometedors resultats obtinguts en models preclínics, i actualment es troben en diversos assajos clínics https://clinicaltrials.gov. Tanmateix, malgrat els resultats satisfactoris de la teràpia cel·lular en models animals, el principal problema de seguretat en l'administració cel·lular en els pacients és la resposta immunogènica de l'organisme o la possible aparició de tumors degut a no tenir un fenotip diferenciat, com és el cas de les cèl·lules mare pluripotents induïdes (iPSCs). Per tal d'evitar la resposta immunogènica s'han realitzat assajos clínics amb trasplantament de cèl·lules autòlogues, però el número de cèl·lules obtingudes per trasplantar pot ser limitat[120,121]. Una alternativa, és l'administració dels factors o components (ex. exosomes) alliberats per les pròpies cèl·lules, ja que s'ha vist que mecanismes com l'angiogènesi s'activen per mitjà d'accions paracrines mitjançant el secretoma, o directament administrant exosomes aïllats de diferents tipus cel·lulars[122,123]. En aquest sentit els efectes de l'administració de factors després de la isquèmia ha estat molt investigat en models animals, sent el VEGF el factor més estudiat degut a la seva implicació directa en la formació de nous vasos. En canvi, no s'han observat resultats satisfactoris en els assajos clínics (**Taula 2**).

Finalment, l'ús de tractaments d'origen químic o biològic (**Taula 3**), representen la tercera estratègia coneguda per potenciar els processos neuroreparadors. L'últim estudi publicat en el que s'administra àcid retinoic (d'origen biològic) a rates isquèmiques deixa la porta oberta a nous tractaments per a restablir l'afectació després d'un procés isquèmic[124].

Taula 1: Resum de les teràpies cel·lulars post-isquèmia cerebral.

Tipus cel·lular	Models animals	Assajos clínics
Cèl·lules mare embrionaries (ESCs)	ESCs condicionades a fenotip de neurona i ESCs no condicionades incrementen la recuperació funcional motora i sensorial[125,126].	No hi ha estudis.
Cèl·lules mare neurals (NSCs)	Milloren el control motor i redueixen el volum d'infart[127].	La seguretat ha estat demostrada i els pacients presenten una lleu millora al cap del temps, no hi ha resultats concloents [128].
Cèl·lules mare mesenquimals (MSCs)	Redueixen el volum d'infart, els processos apoptòtics, el dany per estrès oxidatiu, la neuroinflamació i faciliten la recuperació motora incrementant la neuroplasticitat[129–133].	La seguretat ha estat demostrada i el tractament amb MSC fa que els pacients presentin millores en la recuperació neurològica i en el flux sanguini cerebral [134–137].
Cèl·lules mare hematopoiètiques (HSCs)	Redueixen el volum d'infart[138].	Resultats no disponibles (assaig clínic número: NCT01518231 (AHSCTIS)).
Cèl·lules mare pluripotents induïdes (iPSCs)	Millores a nivell motor i poden arribar a restaurant la funció sensorial[139,140].	No hi ha estudis.
Cèl·lules progenitores endotelials (EPCs)	Redueixen el volum d'infart, milloren el dèficit neurològic i participen en els processos de neuroreparació vascular[141–143].	Resultats no disponibles (assaig clínic número: NCT02605707 i NCT01468064 (AMETIS).

Taula 2: Resum dels factors administrats post-isquèmia (Adaptat i actualitzat de Gutiérrez-Fernández *et al.*,2012 i Wu *et al.*, 2017[56,119]).

Factor	Models animals	Assajos clínics
VEGF	Redueix la mort neuronal, augmenta l'angiogènesi i la permeabilitat vascular, redueix el volum d'infart i millora la recuperació funcional [70,144].	Hi ha una correlació entre els nivells en sang de VEGF i la millora clínica però no hi ha estudis clínics per a l'administració de VEGF[145]
HGF	Protegeix el cervell del dany isquèmic, participa en procesos anti-apoptòtics, angiogènics i neurogènics i disminueix la formació de la cicatriu glial[146,147].	No hi ha estudis, ja que en models animals s'ha observat que participa en processos de tumorogènesi-angiogènesi[147].
bFGF	Promou la neurogènesi i estimula la proliferació a la zona subventricular i al gir dentat[148,149] .	Arriba a fase III i es va aturar l'assaig ja que no es va demostrar un efecte beneficiós als pacients[150].
SDF-1	Redueix el volum d'infart, augmenta l'expressió de proteïnes anti-apoptòtiques i millora la funció motora [151].	No hi ha estudis.
BMP7	Millora la recuperació funcional a través de la proliferació de precursors neurals[152].	No hi ha estudis.
BDNF	Activa la migració de neuroblasts i augmenta la neurogènesi a l'hipocamp [153].	No hi ha estudis.
GDNF	Augmenta la proliferació cel·lular en SVZ i provoca un reclutament de neuroblasts a l'estriat[154].	No hi ha estudis.
G-CSF	Promou la formació de nous vasos sanguinis, presenta activitat antiinflamatòria, antiexcitotòxica. També és neuroprotectora, augmenta la supervivència i té efectes en el pronòstic funcional[155,156].	Es va demostrar la seguretat però no l'eficàcia. El grup tractat amb G-CSF presentava una tendència en la reducció del creixement de l'infart [157,158].
EPO	Redueix el volum d'infart i millora els dèficits neuronals[159].	En un primer estudi pilot es va demostrar la seguretat i l'eficàcia, on millorava el pronòstic neurològic i disminuïa el volum d'infart. Malauradament, en un estudi en Fase II/III es van trobar resultats negatius i els pacients que rebien EPO presentaven més hemorràgies cerebrals i augmentava la mortalitat [160].
EPO+G-CSF	Augmenta l'angiogènesi i la plasticitat tissular promovent la recuperació funcional[161].	No hi ha estudis.

Taula 3: Altres tractaments neuroreparadors.

Altres tractaments	Models animals	Assajos clínics
PFT-α (inhibidor de p53)	Incrementa la supervivència de les cèl·lules neurals progenitores i millora la funció motora[162].	No hi ha estudis.
CART	Augmenta la proliferació i migració de les cèl·lules progenitores neurals a la zona subventricular[163,164].	No hi ha estudis.
S-nitrosoglutathione	Augmenta l'angiogenesi[165].	No hi ha estudis.
9-cis-retinoic acid	Augmenta la recuperació de la funció motora i la proliferació cel·lular a la zona subventricular[124].	No hi ha estudis.

1.4.5 Models animals de rehabilitació en la isquèmia cerebral

Els models animals de rehabilitació són importants per entendre millor els mecanismes implicats durant les teràpies de rehabilitació i així conèixer els processos de reparació que es produeixen durant aquesta fase de la malaltia. Aquests models han de mimetitzar els programes de rehabilitació en pacients i poder ser el més translacionals possibles.

Actualment, hi ha diferents models animals de rehabilitació després d'una isquèmia com l'ús de models forçant l'exercici de l'extremitat afectada, models locomotors, l'ús d'ambients enriquits/estimulants o mitjançant l'exercici de tasques específiques (**Taula 4** i **Figura 7**).

La primera estratègia és el model on es força l'extremitat afectada, de l'anglès ***constraint induced movement therapy*** (CIMT). Aquesta tècnica també es fa servir en la pràctica clínica i consisteix en encoratjar als pacients, mitjançant programes intensius diaris, a utilitzar l'extremitat superior afectada i en cap cas fer ús de l'extremitat superior no afectada[166]. En el model de rosegadors, també se'ls força a fer servir l'extremitat afectada amb diferents estratègies: Devow *et al.* fan servir una armilla al voltant del cos de la rata[167], mentre que d'altres grups utilitzen una escaiola[168]. Un dels avantatges d'aquest model és que el temps en que l'animal exercita l'extremitat afectada és controlable (posant i traient l'armilla/escaiola) i es pot forçar a rehabilitar l'extremitat d'interès. Per altra banda, els animals poden estar subjectes a un elevat nivell d'estrès i això es pot veure reflectit en els resultats. L'ús d'aquest model en rates isquèmiques, s'ha demostrat que fa augmentar la neurogènesi en la SVZ i el gir dentat, a més de millorar els dèficits neurològics[169].

En segon lloc, la **locomoció**, on les 4 extremitats de l'animal s'exerciten, ja sigui de manera forçada o voluntària. L'exercici acostuma a durar entre 10 i 60 min, i la distància recorreguda pot ser d'entre 1 i 7 km, durant dies o setmanes[170]. Aquest tipus de rehabilitació pot induir-se de manera forçada, mitjançant la cinta de córrer[171] o rodes d'exercici[172], o voluntària, mitjançant rodes d'exercici d'accés lliure dins de la gàbia[55] o amb pilotes d'activitat[173]. Com ja s'ha comentat, una rehabilitació de manera forçada pot provocar alts nivells d'estrès, però al contrari que l'activitat voluntària podem

controlar la distància i el temps per cada animal i així evitar una alta variabilitat en l'estudi experimental. Els estudis en animals mitjançant l'exercici físic mostren una reducció del volum d'infart i l'apoptosi, un augment de la neurogènesi i angiogènesi a més a més d'una millora dels dèficits neurològics[171,174,175].

En tercer lloc, es pot utilitzar un **ambient enriquit.** En aquest, es fan servir diferents objectes que s'introdueixen a la gàbia com escales, figures amb diferents formes o joguines. En aquest cas es tracta d'un exercici que es realitza de manera voluntària en el que es promou una estimulació sensorial, cognitiva i social, augmentant la recuperació després de la isquèmia[170] .

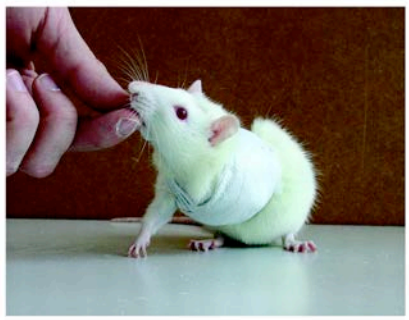
I finalment, el model realitzant una **tasca específica,** on l'animal és forçat a realitzar una tasca concreta amb les extremitats superiors i que requereix un aprenentatge previ, ja que en aquest model no s'utilitzen maniobres d'immobilització.

Hi ha diferents aproximacions per a realitzar exercicis de tasca específica, com el model d'aconseguir *pellets*[176] o trossos de pasta disposats sobre d'una matriu de metacrilat (anomenat *pasta matrix*)[177] en la part oposada a l'extremitat afectada, d'aquesta manera si l'animal vol aconseguir la recompensa ha de fer ús de l'extremitat afectada. En models animals d'isquèmia que porten a terme aquest exercici, els animals milloren les seves funcions motores i presenten un increment de factors neurotròfics[178,179]. A més, aquest model presenta l'avantatge de poder rehabilitar tant una extremitat com l'altre i així estudiar tant els mecanismes de recuperació en l'hemisferi afectat com els mecanismes de compensació relacionats amb l'extremitat no afectada. Cal tenir en compte que aquest és un mètode complex que requereix una restricció de la ingesta per tal d'augmentar la motivació durant l'exercici de tasca específica.

D'altra banda, s'han desenvolupat mètodes robòtics, en els que no es necessita una restricció de la ingesta i la dosi de rehabilitació es troba estrictament controlada. Aquests aparells tracten d'imitar sistemes utilitzats en la pràctica clínica per en rehabilitar les extremitats superiors afectades[180,181].

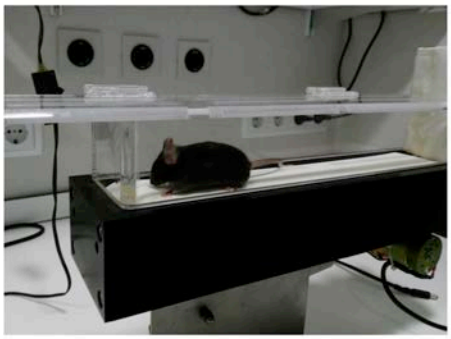
Taula 4: Models animals de rehabilitació després d'una isquèmia en rosegadors (Adaptació de Livingston-Thomas and Tasker; 2013)[170].

Estratègies de rehabilitació	Exemples de models de rehabilitació
Ús forçat de l'extremitat afectada	-*Constraint induced movement therapy* (CIMT).
Locomoció (voluntària/forçada)	-Cinta de córrer (*Treadmill*). -Rodes de locomoció. -Pilotes d'activitat .
Ús d'ambient enriquit o estimulant	-Introducció d'objectes nous i plataformes per escalar dins de la gàbia.
Exercici mitjançant una tasca específica	-Model *pasta matrix*/Agafar *pellets*. -Rehabilitació basada en sistemes robotitzats.



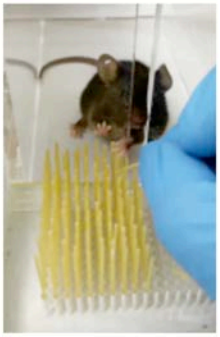
Constraint induced movement therapy

Ambient enriquit o estimulant



Cinta de córrer (*treadmill*)

Tasca d'aconseguir *pellets*



Pasta matrix

Figura 7: Estratègies pre-clíniques de rehabilitació.

1.5 L'Angiogenina

1.5.1 Definició de l'angiogenina

L'angiogenina és una proteïna que pertany a la família de les ribonucleases. Té un pes de 14kDa i va ser purificada per primera vegada de sobrenedants d'una línia cel·lular de càncer de còlon (HT-29) convertint-se en la primera proteïna derivada d'una línia tumoral humana amb activitat angiogènica *in vivo*[182].

En humans, l'angiogenina es localitza al cromosoma 14 i només presenta un gen funcional (*ANG)*, en canvi en ratolins s'han descrit 5 gens, on l'*angiogenina-1* és l'únic que presenta activitat angiogènica i té la mateixa estructura que l'humà[183]. L'expressió d'angiogenina en humans s'ha detectat en diferents òrgans; cor, melsa, pulmó, fetge, colon, pròstata, cervell, retina, melanòcits, entre d'altres[183].

Els processos cel·lulars i moleculars en els que participa l'angiogenina són diversos; actuant tant a l'espai extracel·lular com al citoplasma i al nucli (**Figura 8**)[183]. Extracel·larment l'angiogenina pot unir-se a un receptor transmembrana de 170 kDa[184] i desencadenar diferents respostes, activant cascades de senyalització secundàries i vies de supervivència cel·lular[185]. A més, en l'espai extracel·lular, s'uneix a l'actina que es troba en la superfície de les cèl·lules endotelials estimulant el t-PA que transforma el plasminogen en plasmina. L'activació de la plasmina produeix la degradació de la laminina i la fibronectina de la membrana basal[186].

Una vegada ha translocat al nucli pot iniciar la degradació o la transcripció de l'rRNA estimulant el creixement cel·lular[187,188].

El seu paper citoplasmàtic és poc conegut però es coneix que participa en processos de migració cel·lular[189]. Així mateix a banda de la seva activitat a nivell cel·lular regulant la proliferació, migració, invasió i adhesió necessàries per als processos angiogènics, l'angiogenina podria tenir altres funcions com mantenir la homeòstasis dels vasos sanguinis o actuar com antimicrobiòtic[73].

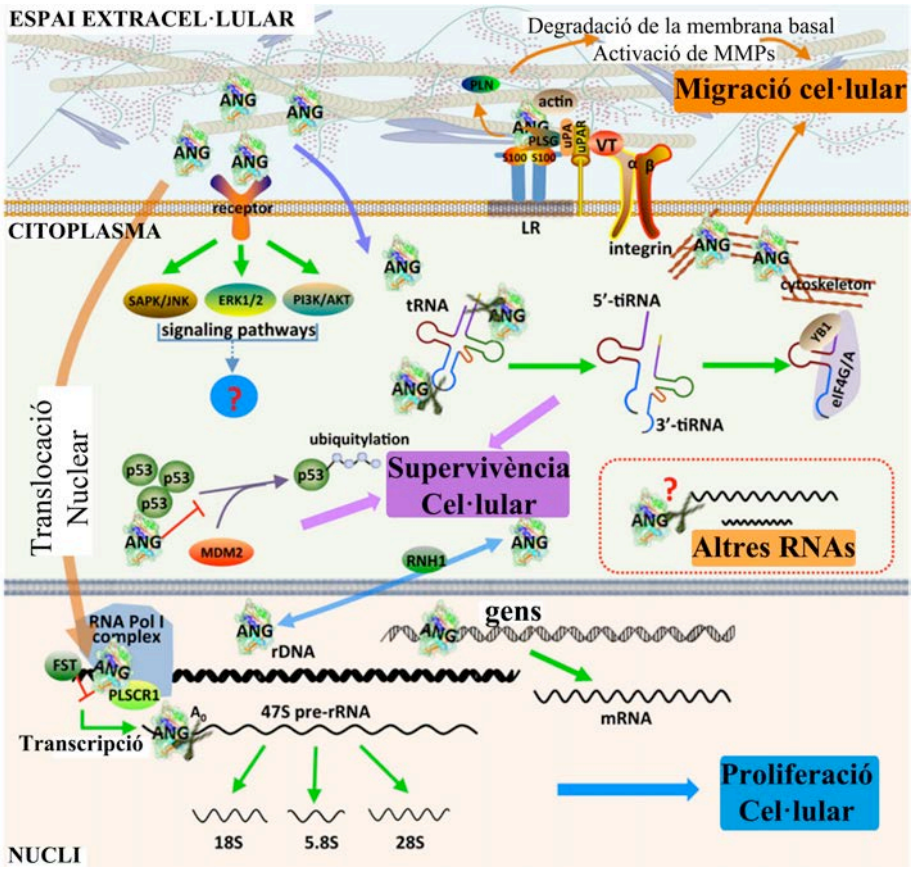


Figura 8: Representació esquemàtica dels mecanismes d'acció de l'Angiogenina

(Adaptat de Sheng J and Xu Z; 2016)[183].

1.5.2 L'angiogenina com a molècula pro-angiogènica

Com s'ha descrit anteriorment, el procés angiogènic requereix que les cèl·lules endotelials presents en els vasos es divideixin i migrin a través de la membrana basal. Aquesta proliferació i migració, es troba guiada per diferents molècules com l'angiogenina i diferents proteases que modifiquen la matriu extracel·lular durant el procés. La seva funció pro-angiogènica ha estat descrita en diferents processos, ja siguin fisiològics o patològics.

Per exemple, durant l'embaràs, en humans, s'activen mecanismes angiogènics i de remodelació vascular en teixits normals[190]. És en les primeres setmanes de gestació on l'angiogenina es troba incrementada i la seva expressió ha estat descrita en diferents cèl·lules de la placenta[191], el que suggereix que té un paper important durant el desenvolupament placentari.

Un altre mecanisme fisiològic en el que es postula que està implicada és en l'homeòstasi vascular. Aquesta funció se li atribueix al fet que els seus nivells en plasma es troben fisiològicament elevats, a una concentració entre 200-400 ng/ml, en individus sans i a la seva funció en la neovascularització quan es produeix un dany en la paret vascular, facilitant ràpidament el creixement dels vasos i la reparació tissular[192,193].

D'altra banda, aquesta molècula té una implicació directa amb els processos neoplàsics i de metàstasi amb capacitat d'activar rutes de supervivència cel·lular. L'angiogenina és una proteïna de resposta a situacions d'estrès cel·lular i sota condicions d'hipòxia la seva expressió es troba incrementada en diferents línies cel·lulars[194,195]. A més, els mastòcits que envolten els tumors també tenen la capacitat de secretar-la[196]. Estudis en models preclínics han descrit tractaments que bloquegen l'expressió d'angiogenina, podent arribar a inhibir la progressió tumoral[197].

Finalment, l'angiogenina juga un paper crucial per a que altres factors angiogènics exerceixin la seva funció, com bFGF i VEGF. Nivells baixos d'angiogenina, redueixen la capacitat per induir creixement tumoral i l'angiogènesis, encara que els nivells de bFGF i VEGF es trobin elevats[198].

1.5.3 L'angiogenina en l'ictus

La majoria de coneixements que tenim de l'angiogenina provenen d'estudis en el camp de la oncologia, mentre que el paper de l'angiogenina després d'una isquèmia cerebral ha estat molt poc estudiat. Hu *et al.* van demostrar un increment en els nivells d'angiogenina en la fase aguda de l'ictus isquèmic (fins a 7 dies post-ictus) mostrant que aquests nivells es correlacionaven positivament amb el volum d'infart. Paral·lelament a aquestes investigacions, els mateixos autors van validar els seus resultats en un model isquèmic en rata, mostrant un increment dels nivells proteics d'angiogenina al cervell en els primers 7 dies post-isquèmia, on l'expressió d'angiogenina es localitzava en les neurones[199,200].

L'altre estudi rellevant s'ha realitzat en un model embòlic en rates diabètiques on els autors demostren que la inhibició de l'angiogenina després d'una isquèmia redueix el volum d'infart, el trencament de la BHE i millora el dèficit funcional dels animals[201].

1.5.4 Altres funcions de l'angiogenina

Durant la última dècada, l'angiogenina també ha estat investigada en malalties neurodegeneratives com l'esclerosi lateral amiotròfica (ELA) o el Parkinson. En aquestes malalties s'han trobat diferents mutacions familiars i esporàdiques en el gen de l'angiogenina humà, que fan que aquest perdi la seva funció[202–204]. En models *in vitro*, aquesta molècula exerceix un efecte protector sobre cultius de motoneurones de ratolí davant un estímul excitotòxic o hipòxic. En la mateixa línia, l'administració d'angiogenina en un model animal d'ELA incrementa la supervivència i la funció motora en els rosegadors[205].

També s'ha estudiat el paper de l'angiogenina en el sistema immune. Aquesta molècula forma part de les llàgrimes protegint la superfície ocular i actuant com un agent antimicrobià[206]. També es troba en la mucosa de l'intestí prim, més concretament en les anomenades cèl·lules de Paneth on es creu que participa en la defensa d'invasors a l'intestí[207]. A més de la seva funció antimicrobiòtica,

s'evidencia que té capacitat antivírica, suprimint la replicació d'algunes soques de virus[208]. Finalment, es creu que juga un paper en la resposta antiinflamatòria degut al seu augment en plasma després d'una resposta als processos d'inflamació[209].

1.6 Les cèl·lules endotelials progenitores (EPCs)

1.6.1 Definició de les EPCs

Les EPCs deriven del moll de l'os i tenen un origen hematopoètic amb capacitat de proliferació, migració i diferenciació durant l'etapa adulta. En particular, aquestes cèl·lules tenen la capacitat de diferenciar-se en una cèl·lula endotelial madura i així poder formar nous vasos sanguinis. La identificació d'aquestes cèl·lules es remunta a 1997 quan Asahara et. al van observar que cèl·lules mononuclears ($CD34^+$ i $Flk1^+$) aïllades de sang perifèrica humana tenien capacitat de formar nous capil·lars *in vitro* i *in vivo*[58]. Aquesta descoberta va fer canviar el concepte sobre la formació de vasos sanguinis després d'un dany tissular en l'individu adult, ja que anteriorment es creia que la vasculogènesi (capacitat de formar vasos sanguinis *de novo*) es trobava restringida a la fase embrionària, i que en la fase adulta només es produïen processos d'angiogènesi (formació de vasos sanguinis a partir d'altres ja existents) i d'arteriogènesi[210].

Actualment, no hi ha consens sobre quins marcadors específics són expressats exclusivament per les EPCs, i si aquests existeixen. Segons el descobriment inicial, les EPCs van ser definides com a cèl·lules positives per CD34, marcador que també es troba en cèl·lules mare hematopoètiques, i VEGFR2, marcador endotelial[58]. Més tard, es va observar que el CD34 es trobava expressat en les cèl·lules endotelials madures, això va fer que altres grups d'investigació proposessin el CD133 en combinació amb aquests dos, per tal d'augmentar l'especificitat de les EPCs a l'hora d'aïllar-les. El marcador CD133 s'expressa en les cèl·lules hematopoètiques més immadures amb capacitat de diferenciar-se a cèl·lules endotelials *in vitro*[211], però no s'ha demostrat que desaparegui amb la maduració a fenotip endotelial.

També s'han estudiat altres marcadors com CXCR4 , CD105 (endoglina) o CD45 per a mostres humanes i c-kit, Sca-1 i CD34 en combinació amb Flk-1 (receptor de VEGF; KDR humans) en ratolins[212]. El marcador CD45 es pot utilitzar en combinació amb altres, ja que aquest marcador l'expressen les cèl·lules hematopoètiques i no les cèl·lules endotelials madures. La població cel·lular $CD34^+$ $CD45^-$ forma capil·lars *in vitro* [213], per tant, l'ús de CD34 esdevé indispensable a l'hora de caracteritzar les EPCs humanes. Malgrat tots aquests estudis, la identificació d'una combinació única de marcadors específics per les EPCs que permeti discriminar-les de la resta de cèl·lules hematopoiètiques encara és desconeguda. Degut a la falta d'aquest marcador específic, Yoder proposà 5 criteris que ha de complir una cèl·lula per ser anomenada EPC[214]: 1) una cèl·lula circulant

amb capacitat clonogènica, 2) amb diferenciació restringida a llinatge endotelial, 3) capacitat de formar capil·lars *in vitro*, 4) capacitat de formar vasos sanguinis funcionals *in vivo* (implantació als teixits) i 5) capacitat per formar i remodelar la capa íntima d'arteries, venes i estructures capil·lars[214]. Per altra banda, les EPCs de sang perifèrica humana o de cordó umbilical, es poden expandir en cultiu, *ex-vivo,* tractats prèviament amb fibronectina (o d'altres components de la làmina basal com el col·lagen). D'aquesta manera es poden diferenciar en dos tipus d'EPCs segons la seva forma, característiques i marcadors de superfície. En aquests cultius trobem les early-EPCs, anomenades més tard *circulating angiogenic cells* (CACs) o les late-outgrowth EPCs (OECs), anomenades més tard *endothelial colony forming cells* (ECFCs). Les early-EPCs apareixen als 4-7 dies de cultiu i tenen morfologia fusiforme, de l'anglès *spindle-shape*. Aquests cultius són molt heterogenis i també es poden observar la presència de cèl·lules amb morfologia monocítica més rodones. Les early-EPCs expressen CD34 i KDR i presenten activitat pro-angiogènica mitjançant la secreció de citocines *in vitro*, però *in vivo* els hi manca la capacitat de formar nous vasos. En canvi les OECs, presenten morfologia llamborda de l'anglès c*obblestone,* en aquests cas, els cultius són més homogenis, formant monocapes clonogèniques (**Figura 9**). Aquest tipus d'EPCs poden formar estructures en forma de vas *in vitro* i *in vivo*[215,216]. A més, expressen marcadors com CD31, CD105, CD144, CD146, VWF, KDR i UEA-1 (ulex europaeus agglutinin-I). Però no expressen CD45 o marcadors de monòcits[212,217]. Finalment, la caracterització d'aquestes cèl·lules mitjançant els seus marcadors de superfície segueix sent un repte en el camp de les EPCs. La seva completa caracterització ens permetria acabar d'esclarir la seva identitat.

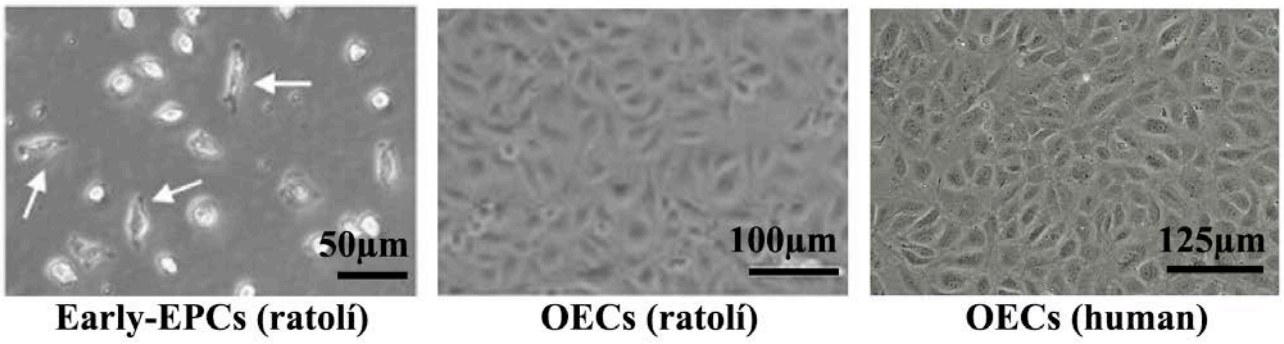


Figura 9: Tipus d'EPCs[218].

1.6.2 Les EPCs en l'ictus

1.6.2.1 Reclutament i mobilització de les EPCs

Com s'ha descrit anteriorment les EPCs s'originen al moll de l'os i és des d'aquí d'on poden ser alliberades mitjançant diferents estímuls. La isquèmia, tractaments amb citocines o fàrmacs[219–221] poden desencadenar l'alliberament de les EPCs, que migraran i participaran en el procés de neovascularització incorporant-se als teixits o intervindran via accions paracrines[222]. La mobilització de les EPCs a la circulació pot estar influenciada per diferents factors com són els nivells de VEGF, SDF-1 (de l'anglès *stromal cell-derived factor-1*), angiopoietina-1, GM-CSF (de l'anglès *granulocyte/macrophage-colony stimulating factor),* G-CFS (de l'anglès *granulocyte-colony stimulating factor)*, eritropoietina, però també per fàrmacs com les estatines o amb l'exercici físic[55,222–224].

En el cas de l'ictus, sota una situació d'hipòxia hi ha un augment en l'expressió de SDF-1, VEGF, S1P (de l'anglès *sphingosine-1-phosphate*) , HIF-1α, entre d'altres. L'increment de HIF-1α fa augmentar els nivells de VEGF, factor implicat directament en els processos d'angiogènesi i vasculogènesi[222]. D'altres autors han mostrat com l'administració de VEGF en models animals fa augmentar el número d'EPCs circulants[225] mentre que SDF-1 s'unirà al seu receptor CXCR4 afavorint la mobilització de les EPCs[226,227]. Un cop aquestes cèl·lules migren als teixits mobilitzades pel gradient de citocines participen en la formació de nous vasos sanguinis[228]. Els diferents marcadors que expressen les cèl·lules des que s'alliberen del moll de l'os fins que es diferencien a cèl·lules endotelials madures és encara desconegut. Es creu que la pèrdua del marcador CD133 podria reflectir la transformació d'EPC a cèl·lula endotelial madura, ja que el CD133 no es troba expressat en les cèl·lules endotelials com en cèl·lules de cordó umbilical[229].

1.6.2.2 EPCs com a biomarcadors

A banda de les implicacions de les EPCs en els processos d'angio-vasculogènesi, aquestes cèl·lules han estat investigades com a possible biomarcador de l'ictus per diversos autors, cap d'ells ha reportat fins ara els efectes de la rehabilitació sobre elles. Hi ha diferents estudis en pacients, tal i com es mostra a la **Taula 5**, on les EPCs han estat relacionades amb la severitat de l'ictus, volum d'infart i evolució neurològica dels pacients. Però existeixen controvèrsies entre aquestes investigacions, la majoria d'elles degudes a la manca d'un mètode únic i consensuat per identificar i quantificar les EPCs. Tsai *et al.*, postulen que hi ha nivells més elevats d'EPCs en individus sans que en pacients, però moltes d'altres investigacions demostren el contrari, on la isquèmia cerebral afavoreix l'augment de les EPCs circulants en fase aguda. A més, Navarro-Sobrino *et al.*, associen aquest increment de les EPCs a les 24 hores amb la severitat de l'infart, però un cop superada la fase més aguda, els mateixos autors, en consonància amb altres estudis publicats, han establert una

associació dels nivells d'EPCs amb una disminució del volum d'infart i amb una millora neurològica i funcional als 21 dies, 3 mesos i a l'any posteriors a l'accident cerebrovascular.

Taula 5: Resum dels estudis de les EPCs com a biomarcadors en l'ictus.

Tipus d'ictus	Marcadors EPCs/Metodologia	Resultats	Referència
Isquèmic	CD34, CD133; citometria.	Increment de $CD34^+$ al dia 7 vs. 6 h post-ictus, correlació positiva de $CD34^+$ amb el flux cerebral.	Taguchi *et al.*, 2004[230]
Isquèmic	EPCs-CFU; cultiu cel·lular.	Un augment de les EPCs en la fase aguda correlaciona amb una millora funcional als 3 mesos (mRS: *modified rankin scale*) i amb una disminució del volum d'infart.	Sobrino *et al.*, 2007[231]
Isquèmic	CD31/CD34, CD62E/CD34, CD34/KDR; citometria.	$CD34^+KDR^+$ correlacionen amb la millora neurològica (NIHSS) a les 3 setmanes.	Yip *et al.*, 2008[232]
Isquèmic	CD34/CD133/KDR; citometria.	Nivells elevats d'EPCs correlacionen negativament amb volum d'infart i disminució del creixement de la lesió.	Bogoslovsky *et al.*, 2010[233]
Isquèmic	CD133+/CD34+/KDR+/CD45+; citometria i cultiu.	Increment d'EPCs en ictus isquèmics. controls. I els nivells s'associaven amb la severitat de l'infart (NIHSS).	Navarro-Sobrino *et al.*, 2010 [219]
Isquèmic i hemorràgic	*Early*-EPCs: $CD34^+/KDR^+/CD133^+$; *Late*-EPCs: $KDR^+/CD34^+$; Citometria.	Número d'*early* i *late* EPCs elevat en pacients vs. controls.	Paczkowska *et al.*, 2013[234]
Isquèmic	Poblacions: $CD133^+/CD34^+$ i $KDR^+/CD34^+$; citometria.	Els nivells de les dues poblacions eren més baixos en pacients vs. controls.	Tsai *et al.*, 2014[235]
Isquèmic	$CD34^+/KDR^+/CD133^+$; citometria.	Els nivells d'EPCs al dia 7 es troben elevats vs. temps basal post-ictus. Nivells elevats d'EPCs s'associen a una millora en l'estat funcional (mRS).	Martí-Fábregas *et al.*, 2015[236]
Hemorràgic	$CD133^+/CD34^+/KDR^+$; citometria.	Els pacients que mostraven una millora neurològica a l'any, presentaven nivells elevats d'EPCs al dia 7 post-ictus. Correlació negativa d'EPCs amb el creixement del volum d'infart.	Pías-Peleteiro *et al.*, 2016[237]

2.OBJECTIUS

2. OBJECTIUS

1. Implementar un model experimental de rehabilitació post-isquèmia en ratolí.

2. Determinar la modulació de factors relacionats amb l'angiogènesi com l'angiogenina o les EPCs durant la rehabilitació en pacients amb un ictus i determinar el seu valor com a biomarcadors de la millora funcional.

3. Traslladar els resultats observats en pacients al model preclínic de rehabilitació post-isquèmia.
4. Avaluar els mecanismes de neuroreparació *in vivo* que es produeixen després de la isquèmia cerebral en dos models experimentals de rehabilitació diferents: exercici amb una tasca específica o per exercici físic.

5. Estudiar l'efecte tròfic de l'angiogenina *in vitro* en els diferents tipus cel·lulars associats amb els processos d'angiogènesi, neurogènesi i oligodendrogènesi.

3. MÈTODES I RESULTATS

Article 1

Importance of Angiogenin and Endothelial Progenitor Cells after Rehabilitation both in Ischemic Stroke Patients and in a Mouse Model of Cerebral Ischemia

Frontiers in Neurology (under review). Manuscript Nº: 373534

Importance of Angiogenin and Endothelial Progenitor Cells after Rehabilitation both in Ischemic Stroke Patients and in a Mouse Model of Cerebral Ischemia.

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ABSTRACT

Background: Rehabilitation therapy is the only available treatment for stroke survivors presenting neurological deficits; however, the underlying molecules and mechanisms associated with functional/motor improvement during rehabilitation are poorly understood.

Objective: Our aim is to study the modulation of angiogenin and endothelial progenitor cells (EPCs) as repair-associated factors in a cohort of stroke patients and mouse models of rehabilitation after cerebral ischemia.

Methods: The clinical study included 18 ischemic strokes admitted to an intensive rehabilitation therapy (IRT) unit, 18 non-ischemic controls and brain samples from 3 deceased patients. Angiogenin and EPCs were measured in blood obtained before and up to 6 months after IRT together with an extensive evaluation of the motor/functional status. In parallel, 70 mice underwent middle cerebral artery occlusion, and the pasta matrix reaching-task or treadmill exercises were used as rehabilitation models. Angiogenin RNA expression was measured after 2 or 12 days of treatment together with cell counts from EPCs cultures.

Results: Brain angiogenin was identified in both human and mouse tissue, whereas serum levels increased after 1 month of IRT in association with motor/functional improvement. EPC populations were increased after stroke and remained elevated during follow-up after IRT. The mouse model of rehabilitation by the task-specific pasta matrix exercise increased the number of EPCs at 2 days and increased angiogenin expression after 12 days of rehabilitation.

Conclusions: Angiogenin and EPCs are modulated by rehabilitation after cerebral ischemia, suggesting that both angiogenin and EPCs could serve as biomarkers of improvement during rehabilitation or future therapeutic targets.

Key words: stroke, rehabilitation, angiogenesis, angiogenin, endothelial progenitor cell, biomarker

INTRODUCTION

Stroke is one of the leading causes of death and long-term disability worldwide and leads to 5 million people becoming permanently disabled annually (1–3). Even with the new advances in diagnosis and therapeutic options that are available in the acute phase of a stroke (4), the only approved treatment in subacute and chronic phases is neurorehabilitation to reduce stroke-related disability, thereby leading to an improved quality of life and independence in daily living activities (5).

Rehabilitation after stroke needs an inter-disciplinary care-team including physiotherapists, occupational therapists, language and speech therapists, working under the direct supervision of a physiatrist, who might be assisted in medical decisions by the use of biomarkers monitoring the neurorepair process (6). The use of clinical measures, physiological parameters or neuroimaging biomarkers to predict long-term motor recovery in the context of rehabilitation has been studied in recent years (7,8), but minor contributions have been achieved for molecular biomarkers. It has been described that the improvements in neurological function during rehabilitation respond to repair or compensatory mechanisms that induce plasticity changes involving multiple molecular pathways (9). By identifying these pathways and bio-molecules, we predict that personalization of rehabilitation treatments may be possible. In this regard, some studies have positively shown associations between the levels of oxidative stress markers, neurotransmitters and proteases in biological fluids and motor function in stroke patients undergoing rehabilitation programs (10–13).

In the present study, we would like to focus on angiogenesis and vascular remodeling, which are mechanisms activated early after stroke that are tightly coupled to neurogenesis and oligodendrogenesis (14) and that can be modulated by physical exercise (15–17). More specifically, angiogenin and endothelial progenitor cells (EPCs) as molecular and cellular responders to rehabilitation therapy after stroke, previously associated with new vessel formation (18–20). Our hypothesis is that angiogenin and EPCs are modulated during rehabilitation after cerebral ischemia serving as biomarkers of functional/motor outcome related to their participation in plasticity mechanisms during neurorepair. To test this hypothesis, our study combines a cohort of stroke subjects under intensive rehabilitation therapy (IRT) in which angiogenin and circulating EPCs levels are analyzed. Additionally, the neurological status is monitored in patients using a battery of tests during a 6-month period. Further, two rehabilitation models in mice after cerebral ischemia (task-specific based exercise or physical exercise) are used, where brain and circulating angiogenin determinations together with EPCs cultures are analyzed.

MATERIAL AND METHODS

Study cohorts and rehabilitation intervention

Stroke and control cohorts are part of the SMARTS (Studying Markers of Angiogenesis during Rehabilitation Therapy after Stroke) study (13). In the current investigation, ischemic stroke patients were prospectively admitted in a neurorehabilitation from February 2014 to May 2015. Inclusion criteria were as follows: first-ever ischemic stroke, age < 70 years, somatosensory or ataxic hemiparesis, time until start of IRT< 3 weeks after stroke, stable medical condition, and endurance to participate for a minimum of 3 hours/day in a therapy program. Exclusion criteria were malignant infarctions, sensory or global aphasias, cognitive

deficits (Mini-Mental State Examination score <23), terminal illness, inflammatory or infectious diseases, or previous deficits of the upper/lower limb. A total of 71 patients with stroke received IRT in our hospital; however, only 18 met the inclusion criteria of our study. The excluded patients presented with hemorrhagic stroke (n=24), malignant infarction (n=13), global aphasia (n=10), human immunodeficiency virus infection (n=1), or carotid tumor (n=1), time to IRT >3 weeks (n=1) or denied the informed consent (n=3). The included patients followed a comprehensive intensive rehabilitation program, including physiotherapy, occupational therapy, speech therapy, and/or neuropsychology, if required, for a minimum of 3 hours/day and 5 days/week as recommended by the clinical practice guidelines for the management of stroke in our health care system (21).

The protocol started with a physiatrist assessing patient's deficits within the first 48 hours after stroke and designing an initial rehabilitation program. Passive mobilizations performed in bed started within the first 24 hours after stroke if there were no medical conditions that contraindicate it, and at 48 hours patients started a moderate-intensity rehabilitation program. Those patients who presented severe or moderate deficits in ≥2 functional areas and who met the inclusion criteria for an intensive rehabilitation program were transferred to the neurorehabilitation unit for inpatient IRT, whereas those patients who were able to walk with some support/assistance were released to their household and started the daily IRT at the day hospital of the neurorehabilitation unit. IRT continued until completion of a minimum of 75% of the proposed objectives or when functional stability was achieved. If there were further objectives, the patient continued a high- to moderate-intensity outpatient rehabilitation program.

In parallel, 18 non-ischemic healthy individuals without any known neurological, malignant, infectious, or inflammatory diseases volunteered to participate as the non-stroke cohort; a portion of the individuals were subjects with hypertension studied in the Investigating Silent Strokes in Hypertensives Study (22) or patient relatives (spouse) who volunteered.

The study protocol was approved by the local clinical research ethics committee (Num. 317/2013), and all patients/healthy volunteers signed the corresponding informed consent in accordance with the Declaration of Helsinki.

Clinical study protocol

Of the 18 included stroke patients, 1 patient was excluded after having a recurrent stroke, whereas 2 other patients chose to withdraw from the study during the follow-up visits (Figure 1). Data related to subject demographics, risk factors, medications, comorbidities, exercise and clinical stroke characteristics were obtained from both cohorts by researchers blinded to the angiogenin and EPC determinations. Specific visits were performed by an experienced physiatrist at baseline prior to starting the IRT and at 1, 3 and 6 months after starting the therapy program. During these visits, motor function and functional status were assessed as described below, and peripheral blood was extracted in serum-separating tubes and centrifuged at 1500 × g for 15 minutes to obtain serum, which was stored at -80ºC in EDTA tubes for EPC counts as described below.

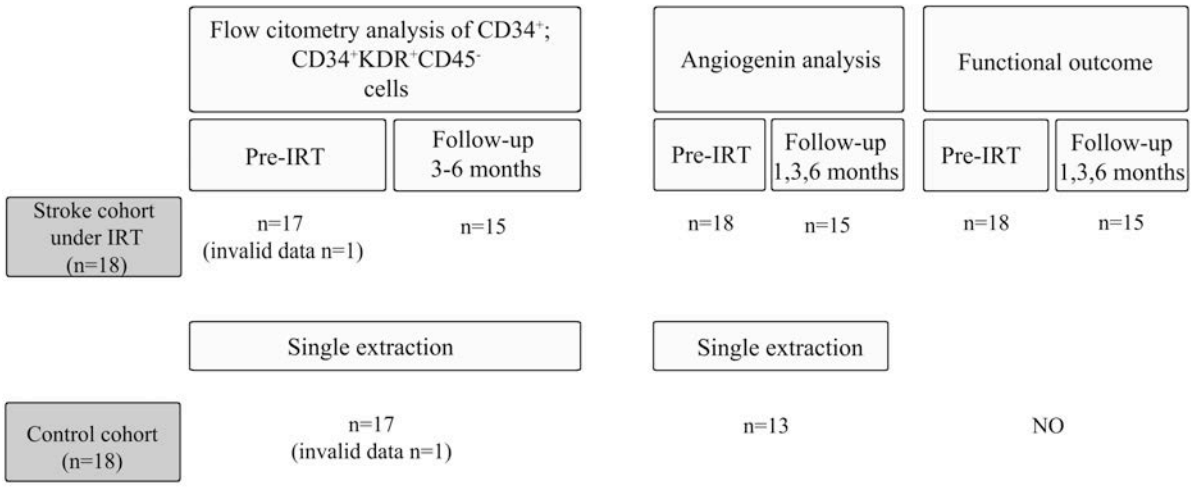

Figure 1: Study overview for the control and the stroke cohort.

Infarct volume evaluation and functional follow-up

Infarct volume was evaluated in follow-up CT (computed tomography) scans performed 24-48 hours after stroke by an experienced neurologist, blinded to clinical information, angiogenin levels and cell populations of interest. Infarct volume was measured according to the formula AxBxC/2, where A and B represent the largest perpendicular diameters through the hypodense area on CT scan, and C represents the thickness of the infarct (23).

Baseline and follow-up visits included different functional/motor tests: the modified Rankin scale (scores 0-6), the Granger modified Barthel Index (BI) (scores 0-100) (24) and dependence categories (25), the Fugl-Meyer Assessment (FMA) score for the upper extremity (scores 0-66), the Functional Ambulation Categories (FAC) (scores 0-5), the Chedoke Arm and Hand Activity Inventory (scores 13-91) (26), the 10-meters walk test, and the Medical Research Council (MRC) scale (scores 0-5) of the disabled hemisphere (upper and lower extremities at the proximal/distal level).

Improvement classifications were obtained after comparing the scores during follow-up visits versus baseline scores: Rankin improvement was defined as a decrease of ≥1 points. For the FMA, improvement was defined as an increase ≥10 points, described previously as the minimal clinical important difference (27). For the Chedoke Arm and Hand Activity Inventory, an improvement was defined as an increase of ≥ 7 points (28). For the 10-m walk test, the walking velocity was calculated, and improvement was considered if walking velocity increased by >0.3m/s (29). The FAC was categorized into 3 categories: cannot walk (score 0), dependent walk (scores 1-3), and independent walk (scores 4 and 5). Improvement was defined as a shift to an upper category (30). For the MRC scale, our analysis differentiated between normal (score 5) or impaired (scores 0-4) muscle strength.

Animal habituation

A total of 74 C57BL/6 mice (males between 6-12 weeks) purchased from Janvier laboratories (Saint Berthevin, France) were

used in this study. The mice were housed in groups of 3 to 5 animals in a temperature/humidity-controlled room and maintained on a 12-hour light-dark cycle. All animals were given water and food *ad libitum,* but 7 days before habituation and throughout the entire experimental procedure, food was restricted to 2-2.5 g/animal/day of chow pellets in order to increase the motivation for the specific task, while weight was controlled during the entire procedure. Experimental procedures were approved by the Ethics Committee of Animal Experimentation of the Vall d'Hebron Research Institute (protocol number 21.16) and were conducted in accordance with Spanish legislation and the Directives of the European Union.

All animals were habituated and trained on the pasta matrix reaching task with a protocol adapted from Kerr and colleagues (31) or habituated to the treadmill apparatus. In brief, all 74 mice were offered with 4 pieces of uncooked pasta per animal (1.6 cm each, Capellini pasta DeCecco) and placed in their housing-cages during 7 consecutive days. To avoid neophofic responses mice were simultaneously habituated to the Methacrylate chamber (20x15x8.5cm with two apertures) with small pasta pieces on the floor and the filled pasta matrix structure in front of one of the apertures for three consecutive days (10min/day); see Figure 2A.

The habituation protocol for the treadmill was conducted after the pasta matrix training described below. Briefly, from 3:00 pm to 6:00 pm, all mice were placed in a treadmill apparatus 10 min/day for 3 consecutive days, to avoid posterior stress responses from a new environment during rehabilitation.

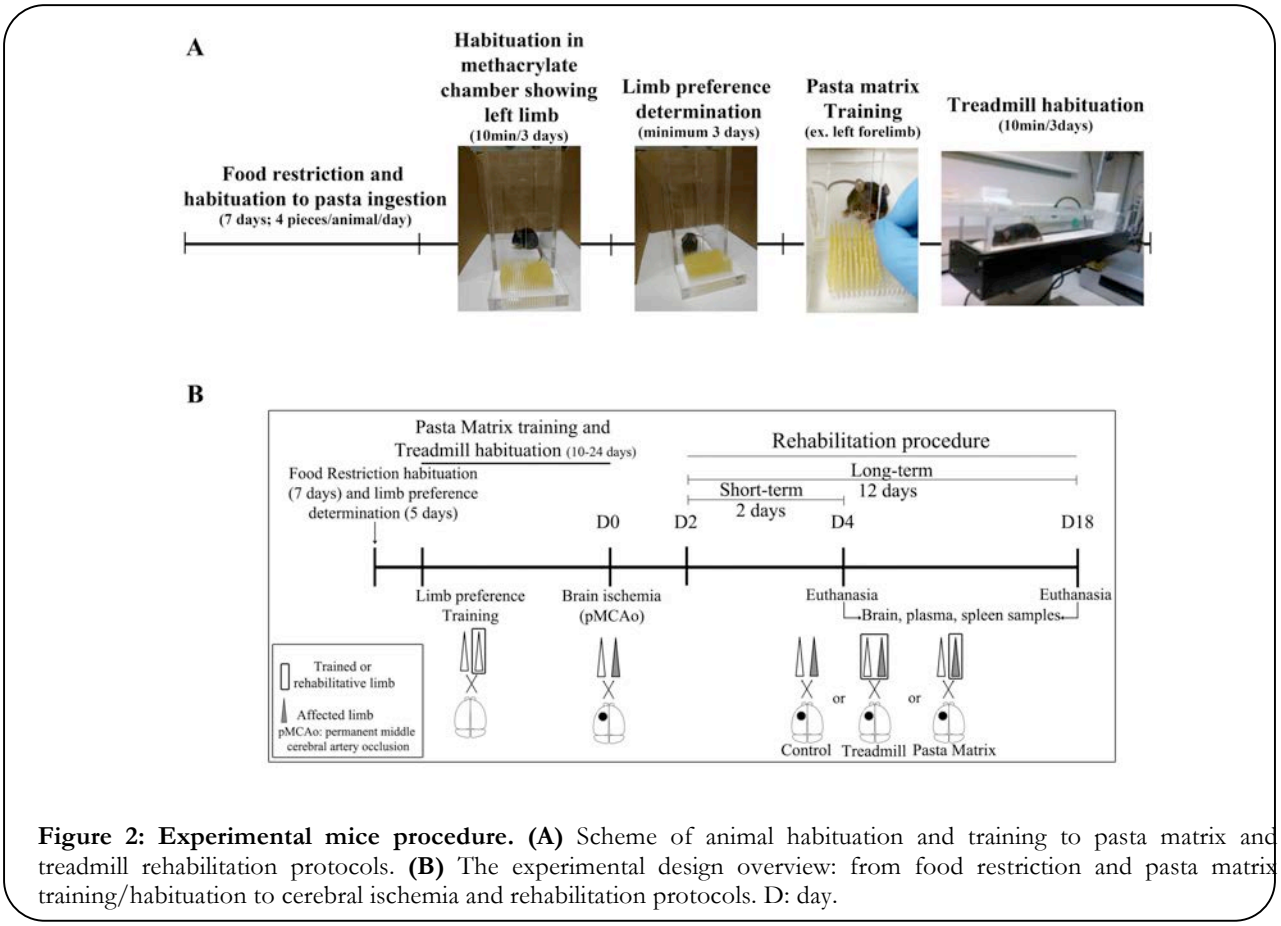

Figure 2: Experimental mice procedure. (A) Scheme of animal habituation and training to pasta matrix and treadmill rehabilitation protocols. **(B)** The experimental design overview: from food restriction and pasta matrix training/habituation to cerebral ischemia and rehabilitation protocols. D: day.

Forelimb laterality and pasta-matrix training

After the pasta matrix habituation, limb preference was established by testing the mice for a minimum of 3 days between 9:00 am to 1:00 pm. Mice were placed in the testing chamber with a matrix full of pasta in the front, and they were encouraged to reach pasta pieces from the aperture for 10 minutes or a maximum 10 attempts. The number of attempts with the right or the left forelimb was recorded and the limb preference was determined by the 70% of limb dominance. After individual laterality preference was established, mice were trained for their preferred forelimb by filling only half of the matrix with pasta (contralateral of the preferred limb; Figure 2A). All animals performed at least 10 days of training (five days/week) consisting on 100 attempts or 15 minutes in the testing chamber. The training was finished the day each mice was able to break a minimum of 9 pieces of pasta consistently for a minimum of 3 consecutive days.

Permanent focal cerebral ischemia model

Permanent cerebral ischemia was performed on 70 mice as previously described (32) by the permanent distal occlusion of the middle cerebral artery (pMCAo), depending on the animal limb preference, while 4 other mice received sham surgery.

Animals were anesthetized with isofluorane (Abbot Laboratories, Spain) for a maximum period of 30 minutes via face-mask (4% for induction and 1-2% for maintenance in Medicinal Air, 79%N_2/21%O_2) and body temperature (36.5º-37ºC) was controlled by a probe thermometer with mice laying on a heating pad. Mice eyes were protected from corneal damages during surgery using an ophthalmic lubricating ointment (Lipolac™, Angelini Farmaceutica, Spain). A small craniotomy, between retro-orbital and ear areas, was performed in order to expose the distal part of the MCA as described. MCA was compressed using a micromanipulator and indirectly electrocauterized by heating the 30-G needle compressing the MCA. Cerebral blood flow (CBF) was monitored using a Laser-Doppler flowmetry (Moor Instruments, UK) with a probe inserted distally to the cauterization site, only animals with a reduction in CBF to below 80% were included. Buprenorphine (0.05-0.1mg/Kg) was administered subcutaneously after the surgery, the temporal muscle was placed to the original position, skin sutured with a silk suture and mice were allowed to spontaneously recover from anesthesia. All sham animals were operated with the same surgical procedure with the exception of the pMCA occlusion (pMCAo). After 4 or 18 days of ischemia (corresponding to 2 or 12 days of rehabilitation) or after 4 days of surgery in the sham group, animals were euthanized following specific procedures described below.

Pre-clinical rehabilitation models

Rehabilitation treatments began 48 hours after pMCAo for all animals. Mice were randomly distributed into 3 different groups: No-Rehabilitation (No-RHB); Pasta Matrix (PM), where the reaching-task consisted of performing 100 attempts with the affected limb inside the chamber; and Treadmill, where mice received 30 min of exercise by increasing the speed every 10 minutes (10 cm/s, 15 cm/s and 20 cm/s). All mice were weighed every session, which consisted of 2 (short-term) or 12 (long-term) days of rehabilitation (Figure 2B). Finally, after every session, each animal received 4 small pieces of pasta (1.6 cm/piece) in their home-cages in order to balance the pasta ingestion between groups. After completing the rehabilitation treatments, mice were euthanized under deep anesthesia as described below to obtain brain, spleen and plasma samples for further analysis.

Grip strength test

The maximum forelimb forced was assessed with grip test as previously described (33). For the test, the mice were placed in a

horizontal plane and when pulled backwards from the tail the animal exerts force in the grid. Scores of each animal were determined by averaging 6 trials. This test was performed before and after 24h, 4 days and 18 days of ischemia (corresponding 2 or 12 days of rehabilitation).

Infarct volume assessment in animals

Animals were euthanized after 4 days of pMCAo (2 days after RHB) by transcardial perfusion of cold saline with deep anesthesia as described. Brains were removed and cut into 1mm-thick coronal sections and stained with 2,5% of 2,3,4-triphenyl-2H-tetrazolium chloride (TTC; Sigma, MO, USA) for 10 minutes at room temperature (RT) when TTC solution was replaced by cold saline and images acquired for infarct quantification. A treatment-blinded researcher measured the infarct volume by the ImageJ free software as described previously (32). Finally, the results were corrected for brain edema taking into account the following equation: infarct volume corrected=infarct volume/edema (ischemic area/contralateral area), and expressed in cubic millimeters (mm^3).

Glucose level monitoring

Glucose levels were monitored in a sub-group of animals before and after food restriction together with pMCAo (24 h and 4 days). Blood was obtained by making a small incision on the tail vein of mice, placing one droplet of blood on a glucose test strip and using a glucometer (GLUCOCARD™ G+).

Angiogenin measurements by ELISA

For human samples, serum levels of angiogenin were measured by ELISA kit (R&D systems, MN, USA). Samples from strokes were obtained before IRT (n=18), after 1 month (n=15), 3 months (n=15) and 6 months (n=15) of rehabilitation intervention, while 13 samples were tested for the non-ischemic control cohort (Figure1). Briefly, 200µl of diluted serum samples (1:200) were loaded to the sample wells to test angiogenin levels by ELISA following the manufacturer's instructions.

For mouse samples, venous blood was obtained from cardiac puncture under deep anesthesia and collected in EDTA tubes at day 2 or 12 post-rehabilitation and after sham surgery (Figure 2B). After centrifugation (3000 g, 10 min at 4ºC), plasma was separated and stored at -80ºC until analysis. After blood collection mice were transcardially perfused with cold saline, brains were collected and cortex was isolated from the ipsilateral and contralateral hemispheres and immediately frozen at -80ºC until use. Briefly, 100 µl of the diluted plasma (1:20) were loaded to the sample wells to quantify angiogenin levels by a sandwich ELISA kit (LSBio, WA, USA) according to manufacturer's instructions.

All samples were tested by duplicate and only values with a coefficient of variation <20% were accepted for the statistical analysis. For those molecules in which the inter-assay CV was higher than 20%, values were standardized prior to statistical analysis by calculating the Z-score value by substracting the mean and dividing by the standard deviation (SD) of each plate and adding three units to avoid results below zero in any sample.

Flow cytometry for EPC cell counts in stroke and control cohorts

Fresh blood samples were collected in EDTA tubes from stroke patients before IRT, during the follow-up after 3-6 months of IRT and in control subjects as described.

Blood (4ml) was diluted with 8 ml of PBS containing 2% of fetal bovine serum (Gibco, MD, USA) and the diluted blood was carefully placed over 5ml of Ficoll-Paque Plus™ (GE healthcare, Sweeden). Cells were centrifuged for 30 min (400 g) at room temperature (RT). Mononuclear cell (MNC) layer was isolated and centrifuged for 5 min at RT (400 g) to discard the supernatant. Then, cell pellets were resuspended in 3 ml of PBA

buffer (1% bovine serum albumin, 0.1% sodium azide in PBS) and 1 ml of blocking reagent was added (AB Human Serum, Invitrogen, CA, USA) and incubated for 10 minutes. Afterwards, 3 ml of the total mixture were used to incubate with primary antibodies and 1 ml was also incubated with corresponding fluorescent isotype control antibodies in order to control the non-specific antibody binding. Primary antibodies and isotypes were incubated for 45 minutes at RT (Antibodies: 0.3 μg/ml CD34-BV421, 0.3μg/ml CD45-APC and 0.75 μg/ml CD309-KDR-PE. Isotypes: 0.3 μg/ml IgG1-BV421, 0.75 μg/ml IgG1-PE (BD Pharmingen, CA, USA) and 0.3 μg/ml IGG1-APC (eBioscience, CA, USA)). Tubes were centrifuged for 5 min at RT (350 g), discarding the supernatant, the cell pellet was resuspended in PBA buffer (0.8 ml for the sample and 0.5 ml for isotype) and transferred into 5 ml poliestirene plastic tubes. Before being analyzed, the samples were filtered through 30 □m Cell Tricks filters (Partec, Münster, Germany). Finally, 10^6 events per sample and 1 to 2 x10^4 events for isotypes were analyzed using the BD LSRFortessa™ cell cytometer (BD Pharmingen, CA, USA). $CD34^+$ and $CD34^+KDR^+CD45^-$ populations were analyzed using FCS Express™ version 3, Research Edition (DeNovo Software, CA, USA). Extreme values represented in box-plots were excluded from the analyses.

EPCs cultures from mouse spleens

Mouse spleens were used to obtain early EPCs as previously described (34). Spleens were obtained after 2 or 12 days of RHB treatment and 2 spleens from the same group were pooled for cultures. Spleens were mechanically minced, placed at 37ºC for 10 minutes in a 1mM EDTA solution and run through a 40-μm nylon membrane to obtain a cell suspension. Mononuclear cells (MNCs) were obtained by density gradient centrifugation (400g, 20 minutes) with Ficoll-Paque Plus (GE healthcare, Sweeden), shortly washed with red blood cells lysis solution (150 mmol/L NH_4Cl, 10 mmol/L $NaHCO_3$ and 0.1 mmol/L EDTA in distilled water) and gently washed with complete endothelial growth medium-2 (EGM-2; Clonetics®, CA, USA), which is composed of endothelial basal medium (EBM) containing 20% fetal bovine serum (FBS), human epidermal growth factor (hEGF), vascular endothelial growth factor (VEGF), human basic fibroblast growth factor (hFGF-B), insulin like growth factor 1 (R3-IGF-1), GA-1000 (gentamicin and amphoterecin-B), heparin, hydrocortisone and ascorbic acid. Isolated MNCs were finally resuspended in EGM-2 and 10^7 MNCs were seeded on fibronectin-coated 12-well cell culture plates and incubated in 5% CO_2 at 37ºC. Under daily observation, first media change was performed 3 days after plating and, thereafter, media was changed every 2 days. At day 5, images from 4 representatives fields/well were taken at 100X magnification (IX71, Olympus). Before data analysis, extreme values were excluded by mean ± 2SD criteria.

Immunohistochemistry (IHC)

IHC was performed in human and mouse brain slices for angiogenin, NeuN antibodies and lectin blood-vessel marker. For human tissue, post-mortem brain samples from stroke patients (see Supplementary Table 1 for details) were collected from infarct tissue and healthy contralateral hemispheres by an experienced neuropathologist and supported by computed tomography images. For animal samples, mice under 12 days of rehabilitative treatment received daily intraperitoneal injections of 5-Bromo-2□-deoxyuridine (BrdU, 50mg/kg in saline, B9285, Sigma-Aldrich, MO, USA) (35), which started after 48 hours of pMCAo. Later Dylight 594-labeled tomato lectin (80 □g/mouse, DL-1177, Vector Laboratories, USA) was injected intravenously (retro-orbitally) 10 minutes before euthanasia. Afterwards, animals were euthanized by transcardial perfusion with cold paraformaldehyde (PFA, 4%) under deep anesthesia. Both human and animal brains were fixed using 4% paraformaldehyde overnight or 2 hours respectively, changed to 30% sucrose until the brains sank, embedded in OCT and kept frozen at -80ºC until use.

Brain sections were obtained by cutting twelve-µm-thickness slices in a cryostat. Human and mouse slices were tempered for 30 min and human sections were fixed with cold acetone for 15 min. Tissue slices were washed 3 times: 5 min at RT with 0.1% PBS-Tween, 5 min with 0.3%-PBS-Triton X-100 and 5 min with 0.1% PBS-Tween. Samples were blocked by using 0.1% PBS-Tween containing 1% of BSA (Sigma-Aldrich, MO, USA) and 5% of goat serum (Merck Millipore, MA, USA) for 1 hour. Then, slices were incubated overnight with the following primary antibodies: 1:100 Angiogenin (NBP2-41185; Novus, Littleton CO), 1:100 NeuN (MAB377X; Merck Millipore, Billerica, MA), and for human vessel analysis 1:100 lectin glycoprotein (RL-1062; Vector labs. CA, USA) was used. Before the secondary antibody incubation, sections were washed 3 times for 5 min at RT with 0.1% PBS-Tween. As secondary antibodies, Alexa fluor 488 goat anti-rabbit IgG (1:1000, mouse; 1:500, human) and Alexa fluor 568 goat anti-mouse IgG (1:500) (Invitrogen, CA, USA) were incubated for 1 hour at room temperature. Later, 3 washes for 5 min with 0.1% PBS-Tween at RT were performed. Additionally, human samples were incubated 10 min at RT with Sudan Black B staining to reduce the brain tissue autofluorescence and washed with 0.1% PBS-Tween 3 times for 5 min after the incubation. Human and mice sections were then mounted in Vectashield™ with DAPI (H-1200, Vector Laboratories, USA) to stain the cell nuclei and analyzed with Olympus BX61 microscope (Olympus, Japan). Finally, two slices of infarct and healthy contralateral areas from three stroke patients were double-stained for NeuN/angiogenin and lectin/angiogenin and the total angiogenin positive area was calculated using ImageJ free software.

For mice sections analysis, 2 slices from 6 animals were imaged at 100X magnification. Two images from each section were captured from the infarct boundary area and contralateral cortex. Total area of angiogenin positive staining was calculated in mice sections using ImageJ free software to compare ischemic side *vs.* contralateral side. Image analysis was performed by a blinded investigator.

Western Blot

Western blot analysis for angiogenin was performed in human and mouse ischemic and non-ischemic brain cortex homogenates. Human stroke brain homogenates (see Supplementary Table 1 for details), mice ischemic brain homogenates (core of infarct, 24 hours post-pMCAo) and corresponding contralateral tissues were homogenized 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, Switzerland) and 0.5% aprotinin (Sigma-Aldrich, USA). Lysates were collected from the supernatant after centrifugation at 15300g for 12 min at 4°C and Pierce™ Comassie (Bradford) assay was used to determine the total protein content (Thermo Scientific™, IL, USA). A total amount of 40µg for human samples and 10µg for mouse samples was diluted in Laemmli Buffer (5%, 2-mercaptoethanol), incubated 5 minutes at 95ºC and run into 12% polyacrylamide gel electrophoresis, transferred into PVDF membranes (Thermo Scientific™, IL, USA), blocked for 1 hour with 10% non-fat milk (PBS, 0.1% Tween 20 (Sigma-Aldrich, MO, USA)) and incubated overnight at 4ºC on a shaker with anti-angiogenin (1:200, NBP2-41185, Novus, CO, USA) or β-actin (1:5000, A5316, Sigma-Aldrich, MO, USA). The membrane was then washed 3 times (PBS-0.1% Tween) and incubated with secondary antibodies (GE Healthcare, UK) anti-rabbit-horseradish peroxidase from donkey or anti-mouse horseradish peroxidase from sheep (1:2000, respectively) for 1 hour at RT with

gentle agitation. Finally, membranes were washed 3 times (PBS-0.1%Tween) and incubated with Pierce® ECL Western Blotting Substrate (Thermo Scientific™, IL, USA) and signal visualized with Fujifilm FPM-100A films. Scanned western blots were quantified using the ImageJ free software. Results are expressed in arbitrary units and angiogenin band-intensities were corrected by actin band-intensities. Molecular weight markers were also run for reference values.

Quantitative reverse transcriptase PCR (qRT-PCR) for angiogenin expression

Mice tissues from 2 and 12 days of rehabilitation were used for angiogenin RNA isolation with the PARIS ™ kit (Invitrogen, CA, USA), following the manufacturer's procedure. The quality and quantity of RNA was measured using Nanodrop Spectrophotometer and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT-PCR reaction was performed using a mixture of 5 μL of TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 μL of TaqMan® Gene Expression Assay (Gapdh: Mm99999915_g1, angiogenin (Ang): Mm01316661_m1; Applied Biosystems, Foster City, CA, USA), 3.5 μL of RNase-free water and 1 μL of cDNA sample. A sample calibrator was used to compare samples between different assay plates. 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).

Statistical analysis

The SPSS 20.0 package was used for statistical analyses. Descriptive statistics were used to define demographic data and risk factors, stroke characteristics, functional scores, angiogenin and cell analysis results. Chi-square tests were conducted for categorical variables. The normality of continuous variables was assessed by the Shapiro-Wilk test ($N<30$) and Kolmogorov-Smirnov ($N\geq30$). Normally distributed variables were analyzed by the ANOVA and LSD post-hoc or t-test, while Mann-Whitney U-test or Kruskal Wallis test were used for non-normally distributed variables. For the analysis of repeated measures, paired t-tests or repeated measures ANOVA followed by the Bonferroni post hoc test were performed for normally distributed variables, while the Friedman followed by Wilcoxon tests were used for non-normal distributions. Bar graphs represent mean±SEM or IQR according to normal or non-normal distribution. For correlation analysis, Pearson (normal distribution) or Spearman (non-normal distribution) tests were used. Results with a p value less than 0.05 were considered statistically significant. Statistical trends ($p<0.1$) are also reported in the results section.

RESULTS

Characteristics of the study cohorts

The baseline characteristics of the stroke rehabilitation cohort and control subjects are described in Table 1. In summary, non-stroke controls were significantly older (64 *vs.* 54.06 years, p=0.002), had higher alcohol intake (p=0.034), had a higher body mass index (p=0.019) and were more often on antihypertensive drugs (p=0.04) than stroke patients.

Table 2 shows the clinical features associated with the ischemic stroke. In summary, the median National Institutes of Health Stroke Scale (NIHSS) score on admission was 8 (5-10), and after 3-4 days, it improved to 5 (4-9) with 38.9% of patients showing a neurological improvement (≥4 points). Strokes were mainly located in the carotid territory (77.8%), presenting large infarcts (TACI, 55.6%), with more than half affecting the left hemisphere (55.6%). Blood samples were taken at 11.5 days after the

ischemic event, and the mean number of days for patients to enroll in IRT was 12.4 days.

Angiogenin, $CD34^+$ and $CD34^+/KDR^+/CD45^-$ cells increase after stroke related to IRT

More brain angiogenin content was found during the acute phase of stroke in infarct tissue compared to the contralateral hemisphere (mean fold increase of 6.5; Figure 3A), although differences were not signifficant. Immunohistochemistry experiments revealed that this expression of brain angiogenin was mainly localized in neurons ($NeuN^+$, Figure 3B) and in vessel-like structures ($lectin^+$, Figure 3C), with higher expression in infarct tissue (Figure 3D).

Table 1: Baseline Characteristics of the Control and Stroke Cohorts.

	Control cohort (n=18)	Stroke cohort (n=18)	P-value
Age	64±7.7	54±9.6	0.002*
Sex (male)	50 (9)	72.2 (13)	0.171
Risk factors			
Alcohol	50 (9)	16.7 (3)	0.034*
Tobacco	16.7 (3)	33.3 (6)	0.443
Hypertension	83.3 (15)	66.7 (12)	0.443
Dyslipidemia	66.7 (12)	44.4 (8)	0.18
Diabetes mellitus	22.2 (4)	16.7 (3)	1
Atrial fibrillation	0 (0)	11.1 (2)	0.486
Obesity	66.7 (12)	38.9 (7)	0.095
Body mass index (kg/m^2)	26±3.3	23.2±3.5	0.019*
Comorbidities			
Osteoarticular disorders	22.2 (4)	11.1 (2)	0.658
Ischemic cardiopathy	0 (0)	5.6 (1)	1
Psychiatric disorders	22.2 (4)	22.2 (4)	1
Previous exercise			
Physical activity	77.8 (14)	47.1 (8)	0.06
Physical activity (h)	7 (5-10)	0 (0-7)	0.116
Previous medication			
Antiplatelets	27.8 (5)	16.7 (3)	0.674
Anticoagulants	0 (0)	5.6 (1)	1
Statins	38.9 (7)	27.8 (5)	1
Antihypertensives	77.8 (14)	44.4 (8)	0.040*
Antidiabetic	16.7 (3)	11.1 (2)	0.639

Variables are expressed as percentage, mean±SD, median (IQR) and the number of cases as (n); h: hours. *p-value<0.05.

In the context of post-stroke rehabilitation, circulating angiogenin levels were significantly increased after 1 month of IRT compared to controls (p=0.012) and compared to pre-rehabilitation values (p=0.036), decreasing after 3 months (p=0.001) and 6 months (p=0.002) of IRT as shown in Figure 3E. Regarding EPCs populations, we observed that $CD34^+$ and $CD34^+/KDR^+/CD45^-$ cells increased after stroke compared to controls (p<0.001 and p=0.005, respectively) and were elevated during the follow-up (3-6 months) compared to controls (p<0.001 and p=0.028, respectively; Figure 3F and 3G) despite a decrease in $CD34^+/KDR^+/CD45^-$ cells compared to pre-IRT levels (p=0.004; Figure 3G). No association was observed between angiogenin and EPCs at baseline, although serum angiogenin at 1 and 3 months associated negatively with $CD34^+$ cells during the follow up (R=-0.636, p=0.011; R=-0.679, p=0.005; respectively).

Table 2: Clinical characteristics of the Stroke Cohort.

	Stroke cohort (n=18)
NIHSS score at admission	8 (5-10)
NHISS motor score at admission	4 (2-7)
NHISS score after 3-4 d	5 (4-9)
NHISS motor score after 3-4 d	2.5 (2-5)
Early neurological outcomes	
Improvement	38.9 (7)
Stability	50 (9)
Worsening	11.1 (2)
Etiology	
Lacunar	11.1 (2)
Cardioembolic	33.3 (6)
Atherothrombotic	11.1 (2)
Others	16.7 (3)
Unknown	27.8 (5)
Location, territory	
Vertebrobasilar	22.2 (4)
Carotid	77.8 (14)
Stroke laterality	
Right	44.4 (8)
Left	55.6 (10)
OCSP classification	
TACI	55.6 (10)
LACI	16.7 (3)
PACI	11.1 (2)
POCI	16.7 (3)
Other	
Thrombolytic therapy	35.3 (6)
Hemorrhagic transformation	17.6 (3)
Time stroke-baseline sample before IRT (d)	11.5±5.7
Time stroke-evaluation before IRT (d)	12.4±5

Variables are expressed as percentage, mean±SD or median (interquartile range). Early neurological outcomes were defined as improvement (decrease ≥4 points in the NHISS admission score), worsening (increase ≥4 in the NHISS admission score), or stability (as any other change in the NHISS admission score).

Abbreviations: OCSP, Oxfordshire Community Stroke Project; TACI, total anterior cerebral infarct; LACI, lacunar cerebral infarct; PACI, partial anterior cerebral infarct; POCI, posterior cerebral infarct; IRT: intensive rehabilitation therapy; d:days.

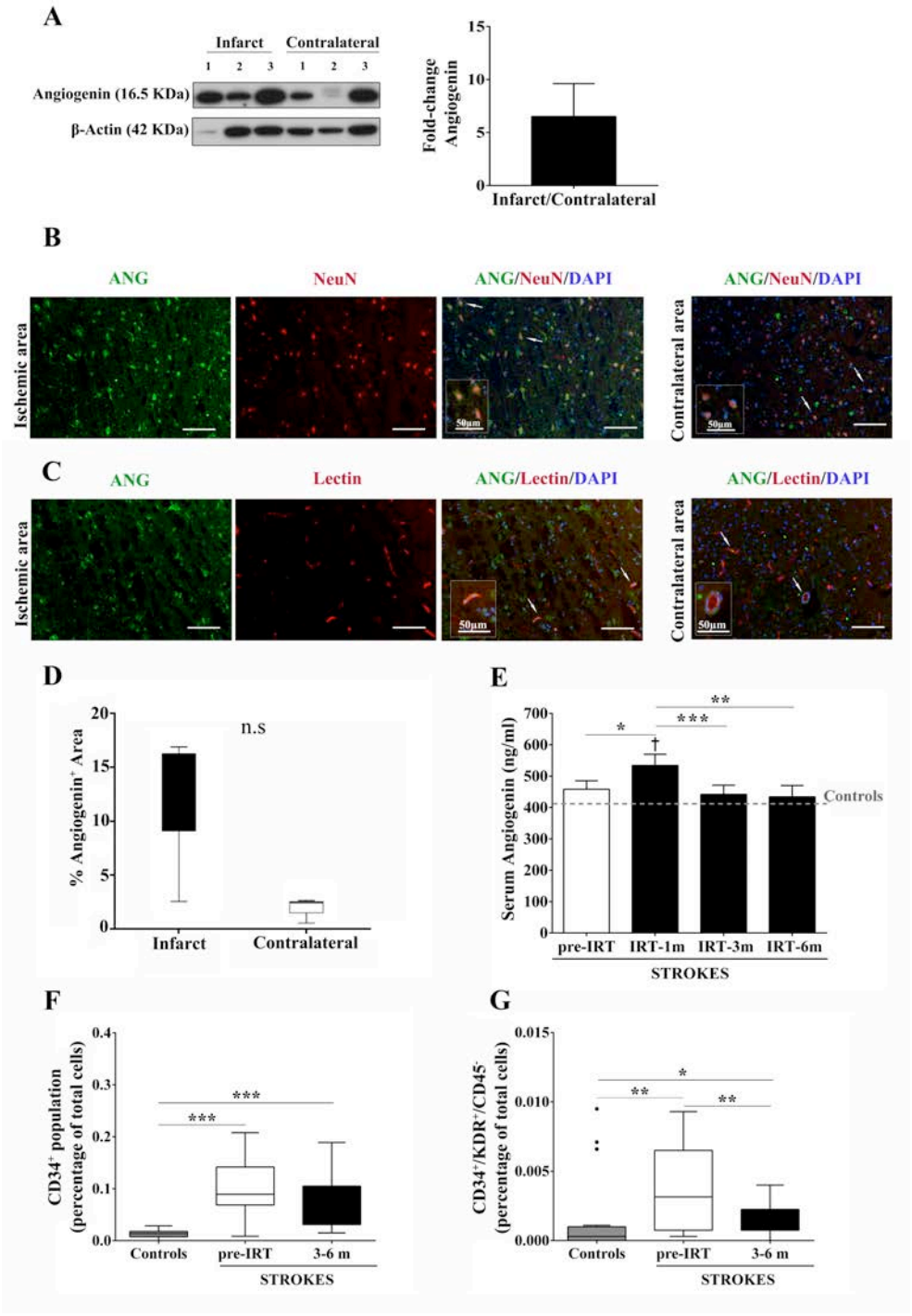

Figure 3: Angiogenin and EPCs modulation after human stroke and rehabilitation. (A) Infarct tissue and contralateral brain homogenates (≤4 days) were analyzed by western blot for angiogenin (n=3). The densitometry results (arbitrary units) were corrected by the actin load and bar graph showing the angiogenin fold-change of the ipsilateral vs. contralateral signal. **(B** and **C**) Representative immunofluorescence images of ischemic and contralateral areas, respectively, of stroke patients showing angiogenin$^+$/NeuN$^+$ cortical neurons and angiogenin$^+$/lectin$^+$ vessels**. (D)** Box plots representing the percentage of the fluorescent Angiogenin$^+$ area in infarcted and non-infarcted brain samples from stroke patients (n=3) **(E)** Bar graphs showing the temporal profile of serum angiogenin levels during IRT. *p<0.05; **<0.01 and ***p<0.001; †p<0.05 vs. Controls (Controls n=13; Strokes in IRT n=15-18). **(F)** and **(G)** Box plots representing cell populations with angiogenic potential such as CD34$^+$ and CD34$^+$/KDR$^+$/CD45$^-$ cell populations; *p<0.05, **<0.01. ***p<0.001; Controls n=17 and Strokes in IRT n=15-17. Bar graphs are represented as the mean±SEM, and box plots are represented as the median (IQR). N.S: Non-significant; IRT: intensive rehabilitation therapy; m: month.

High angiogenin during IRT is associated with better neurological outcome

Neither baseline angiogenin nor EPC populations were associated with the baseline NIHSS score or infarct volume (p>0.05; data not shown). Additionally, the increased angiogenin levels observed at 1 month were not associated with baseline infarct volume (p>0.05; Figure 4A). Regarding improvement, a strong correlation was found between angiogenin levels and Barthel Index scores at 1 month (R=0.618, p=0.014), and patients with higher Barthel Index scores (mild and complete independence) presented the highest angiogenin levels (p=0.048 and p=0.015, vs. severe dependence); see Figure 4B and 4C. Similarly, patients presenting the highest angiogenin levels presented a normal MRC score at 6 months (p=0.043; Figure 4D) and functional outcomes assessed by the Rankin scale also showed a trend towards better improvement at 1 month (p=0.07; Figure 4E), showing the association between high angiogenin levels and better neurological improvements.

Regarding circulating EPC populations, we observed a trend only towards higher $CD34^+$ levels during IRT follow-up and normal MRC scores for superior-proximal and distal extremities at 3 months (p=0.086, respectively, data not shown), whereas the highest baseline levels of $CD34^+/KDR^+/CD45^-$ cells were observed in those patients presenting a Rankin improvement at 1 month (p=0.09; Figure 4F).

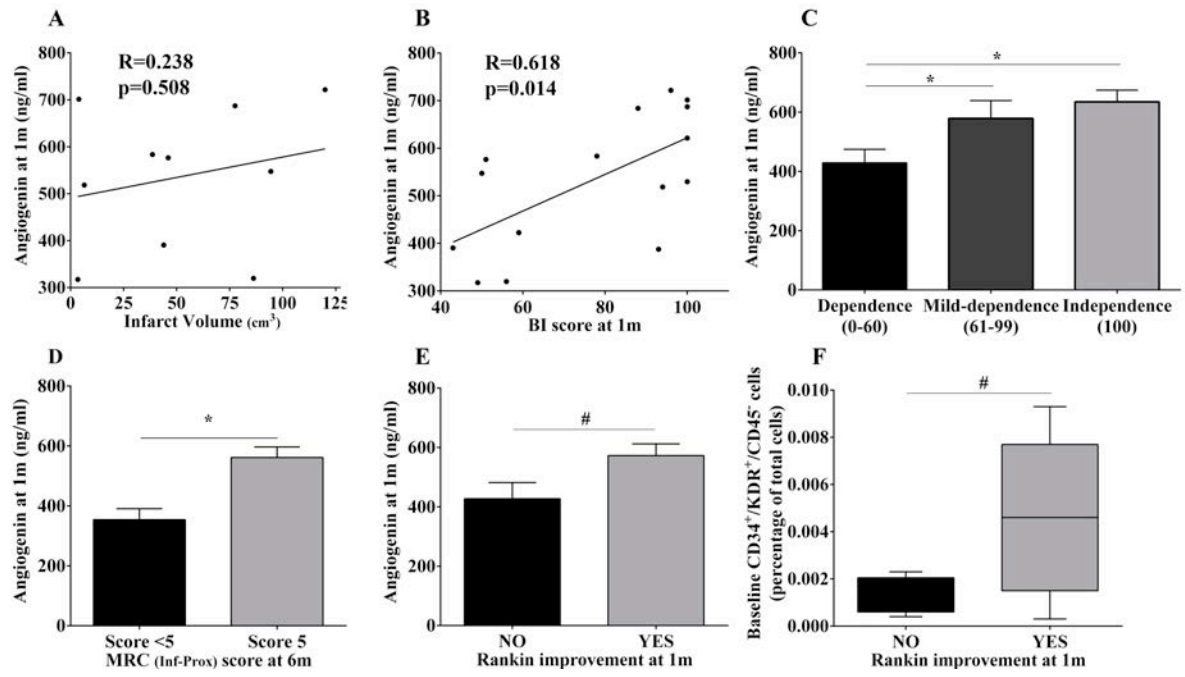

Figure 4: Association of molecular and cellular markers with infarct volume and motor/functional scores in stroke patients. (A) and **(B)** Scatter plot showing the correlations of angiogenin levels at 1 month with infarct volume and the BI score, respectively. **(C), (D)** and **(E)** Bar graphs showing angiogenin levels at 1 month with BI score categories, MRC scores and Rankin improvement at different time points;*p<0.05, #p<0.1). **(F)** Bar graphs representing baseline $CD34^+/KDR^+/CD45^-$ cells according to the Rankin improvement at 1 month. Bar graphs are represented as the mean±SEM, and box plots are represented as the median (IQR). Abbreviations: BI: Barthel index; m: month; MRC: Medical Research Council, Inf-Prox: Inferior-proximal.

Characteristics of the mouse rehabilitation models after cerebral ischemia

Our cohort of mice presented a preference for the left forelimb (61.3%) *vs.* right forelimb (38.7%) (Figure 5A) and learned the pasta matrix-reaching task as expected between day 10 and 24 of training (Figure 5B). As a consequence of food restriction, the weights did not increase, although no significant weight loss was observed and no significant differences were observed between rehabilitation groups (Figure 5C).

Importantly, at the beginning of rehabilitation (2 days), infarct volumes were similar among groups ($p>0.05$; Figure 5D). Food restriction decreased the levels of blood glucose as expected, but no correlations with infarct size were observed ($p>0.05$; Figure 5E and 5F).

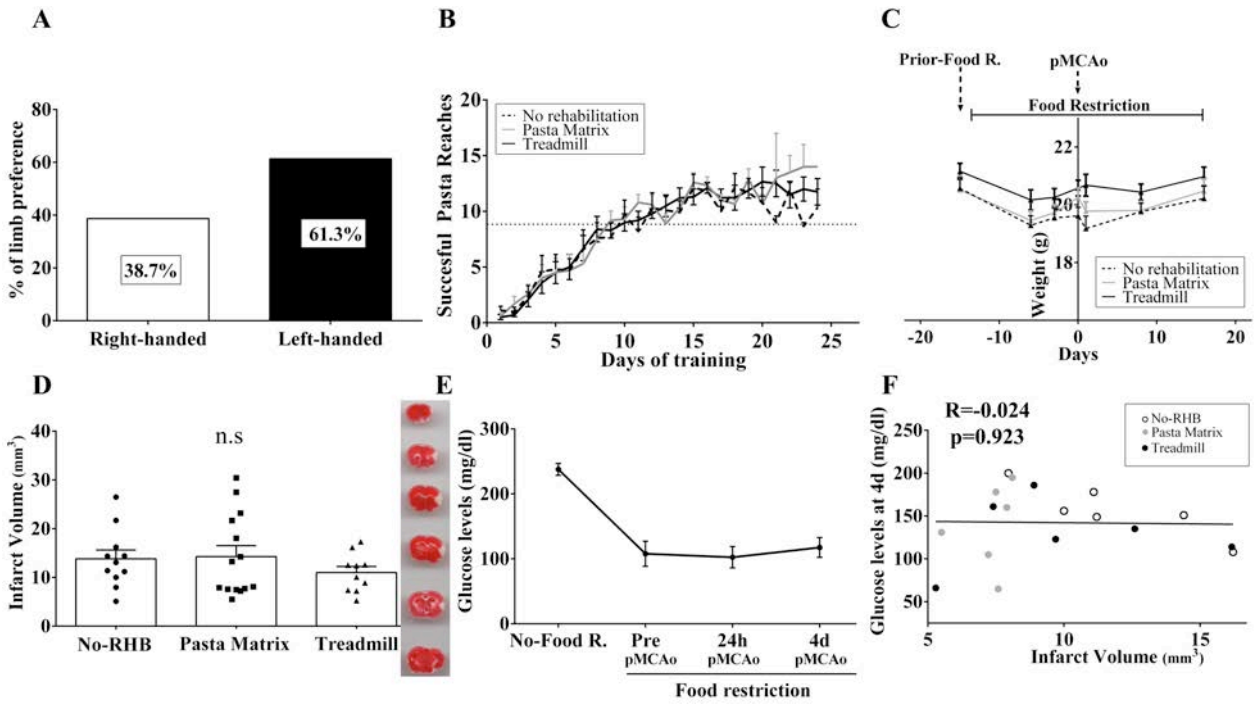

Figure 5: Mouse rehabilitation model. (A) Graph showing limb laterality preference in our cohort of mice (n=74) **(B)** Graph representing the temporal profile of pasta matrix reaching-task training in n=10 animals/group. **(C)** Body-weight graph during the entire procedure in n=10 animals/group. **(D)** Bar graphs showing the mean infarct volume during the short-term phase of rehabilitation with no significant differences between groups (n=10-14); **(E)** The glucose monitoring graph during acute cerebral ischemia. **(F)** Scatterplot showing no association between infarct volume and glucose levels during the short-term phase of rehabilitation (4 days pMCAo, n=6/group). Data are represented as percentage, mean±SEM or single values in the scatter plot. Abbreviations: N.S: Non-significant; RHB: rehabilitation; pMCAo: permanent middle cerebral artery occlusion; R: restriction; d: days.

Angiogenin and endothelial progenitor cells are modulated in the rehabilitation models after cerebral ischemia.

Angiogenin was expressed in brain tissue homogenates 24 hours after ischemia before rehabilitation, presenting a non-significant 3.5-fold increase in the ipsilateral *vs.* the contralateral hemisphere (Figure 6A).

RNA expression of angiogenin increased early after rehabilitation in the ipsilateral hemisphere in all groups ($p<0.05$), but angiogenin did not correlate with the infarct volume (data not shown). Importantly, angiogenin remained elevated in the ipsilateral hemisphere only in mice performing the pasta-matrix-reaching task for 12 days compared to the contralateral hemisphere ($p=0.043$) and compared to the ipsilateral of non-RHB mice ($p=0.016$). The contralateral hemispheres from the pasta matrix and treadmill groups also showed higher levels of angiogenin than the contralateral of non-RHB mice ($p=0.028$ and $p=0.016$, respectively); see Figure 6B. We observed a decrease in ipsilateral angiogenin over time only in non-RHB mice ($p=0.045$), whereas ipsilateral angiogenin from the pasta matrix and treadmill groups remained elevated over time, and contralateral angiogenin increased in both rehabilitation groups ($p=0.014$ and $p=0.007$, respectively).

We validated the RNA findings in the pasta matrix reaching-task model at the protein level by IHC, confirming that angiogenin was increased in the ipsilateral vs. the contralateral hemisphere after 12 days of rehabilitation ($p=0.003$), with a mean fold increase of 4.6, as shown in Figure 6C. Strong angiogenin expression was identified in neurons of the ipsilateral cortex (see details in Figure 6C).

The analysis of blood angiogenin in the mouse rehabilitation models showed a decrease at 12 days after rehabilitation only in the control group ($p=0.029$ *vs.* short-term rehabilitation), while the pasta matrix and treadmill presented an increase at the beginning of rehabilitation that remained elevated at the end of the treatment; however, these differences were not significant (Figure 6D). Forelimb force was decreased 24 hours after ischemia ($p<0.001$; data not shown); however, there were no differences between groups after rehabilitation.

Finally, spleen-derived endothelial progenitor cells from the pasta matrix reaching-task rehabilitation group showed an increase in the number of spindle-shaped cells compared to the non-rehabilitation in the short-term ($p=0.025$). In the long-term, the number of cells decreased compared to that in the non-rehabilitation group ($p=0.025$); see figure 6E.

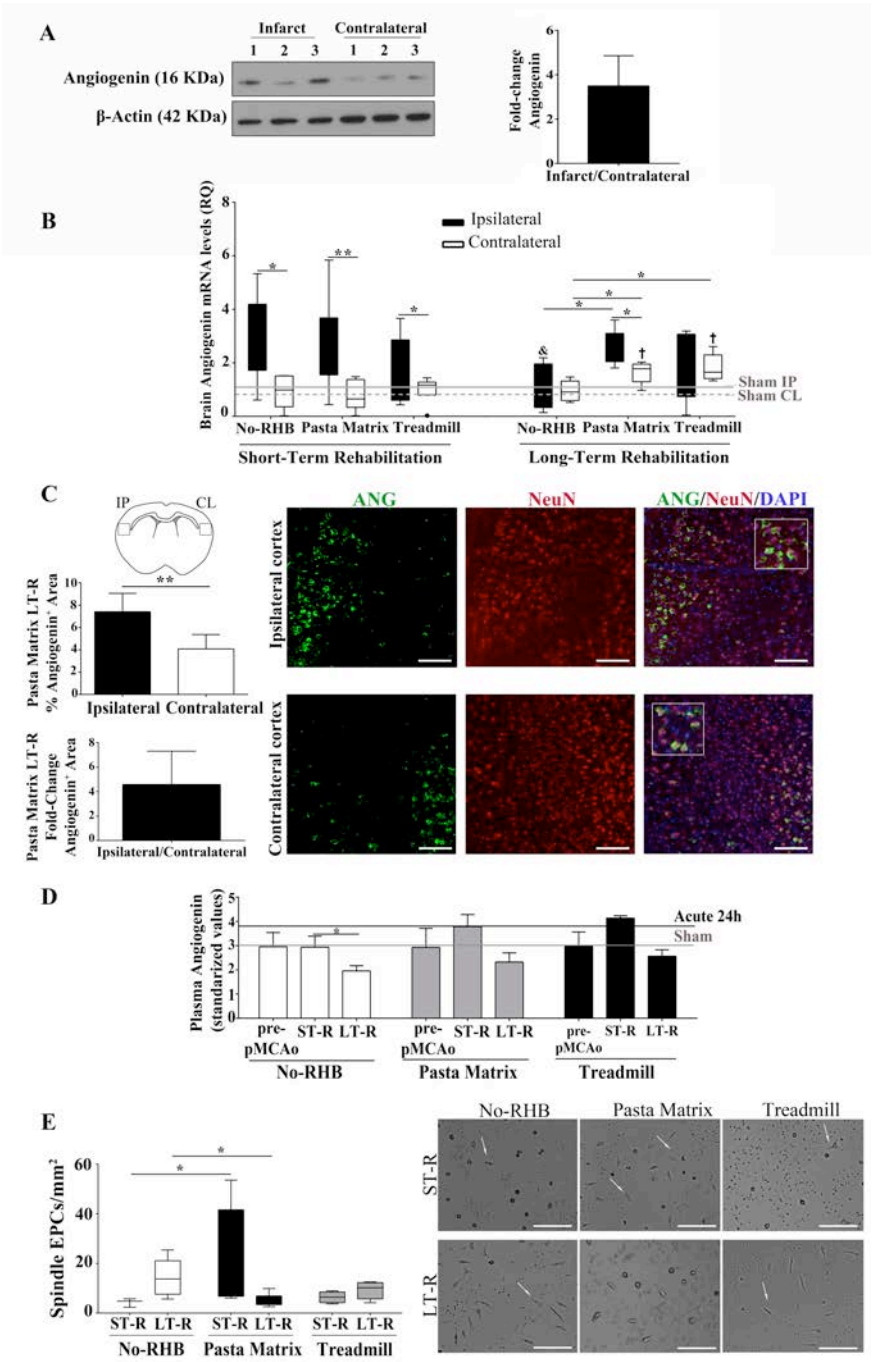

Figure 6: Angiogenin and EPCs modulation after mouse cerebral ischemia and rehabilitation. (A) Infarct and contralateral mouse brain cortical homogenates were analyzed by western blot to quantify angiogenin protein (n=3). The densitometry results (arbitrary units) were corrected by the actin load and are represented as fold-change of the ipsilateral vs. contralateral signal. **(B)** Bar graphs representing the angiogenin RNA expression in the ischemic and contralateral cortex in the short-term (n= 6-9) and long-term rehabilitation groups (n=5), **p<0.01, *p<0.05 as indicated by horizontal lines. Non-RHB ipsilateral short-term vs. long-term; &p<0.05. Pasta Matrix and Treadmill contralateral short-term vs. long-term; †p<0.05. **(C)** Bar graphs showing the percentage of the Angiogenin+ area in the pasta matrix long-term rehabilitation group (n=6) together with images of representative brains: scale bar represents 100 µm. *p<0.05. **(D)** Graph showing the plasma angiogenin temporal profile of No-RHB, Pasta matrix and Treadmill groups (n=3-4/group); *p<0.05. **(E)** Box plots representing the cell density of EPCs from the 3 different RHB groups (n=3-5 short-term; n=6-7 long-term) and representative images of the primary cultures; scale bar represents 250 µm. Data are represented as the mean±SEM or as box plots indicating the median (IQR). IP: ipsilateral; CL: contralateral;ST-R: short-term rehabilitation; LT-R: long-term rehabilitation; No-RHB: No-Rehabilitation; EPC: endothelial progenitor cells.

DISCUSSION

Currently, the only approved treatment for recovery after the acute phase of stroke is rehabilitation with the aim of maximizing independence and improving physical disabilities. In this study, we have examined the role of angiogenin and EPCs during IRT in ischemic stroke patients and in a neurorehabilitation animal model after cerebral ischemia. Our results describe for the first time an increase of angiogenin and EPCs levels during rehabilitation therapy after stroke. We also identify an association between serum angiogenin levels and functional and motor improvement of outcome measurements. These observations are supported by in vivo experiments in a neurorehabilitation animal model where RNA expression and primary endothelial progenitor cells are increased after rehabilitation interventions. These data suggest that angiogenin and EPCs are modulated during rehabilitation therapies and might act as biomarkers to monitor patient status or to potentially serve as therapeutic targets.

Despite the enormous therapeutic advances with reperfusion therapies in combination with well-coordinated teams in stroke units, one-third of stroke patients still present functional deficits (36,37). With this scenario, the only therapeutic opportunities are neurorehabilitation programs that are evidence-based multidisciplinary interventions to restore the impaired function and improve patient independence and quality of life (5,38). However, the patient response to rehabilitation is heterogeneous and might depend on individual endogenous neurorepair mechanisms (39), thus limiting recovery during rehabilitation. In this regard, preclinical and clinical studies should aim to understand the ongoing mechanisms during rehabilitation and to identify new biomarkers to monitor patient baseline conditions, monitor the neurorepair process and adjust personal rehabilitation programs to maximize the optimal recovery.

Angiogenin is a member of the ribonuclease superfamily and is expressed in many cell types. Angiogenin acts as a potent angiogenic factor that can trigger a wide range of biological processes, such as proliferation, cell migration, invasion and formation of tubular structures (18). However, uncontrolled activity of angiogenin is implicated in pathological processes. A high expression of angiogenin has been described in different types of cancer (40), and mutations in the angiogenin gene have been characterized in amyotrophic lateral sclerosis (41) and Parkinson's disease (42). Angiogenin has also been studied in cardiovascular diseases showing an increase in blood in chronic heart failure or in acute coronary syndrome in association with severity or prognosis (43,44). In the context of stroke, Huang and colleagues reported that higher serum angiogenin levels within the first week after stroke are associated with larger infarct size (45). This up-regulation of angiogenin in the acute phase of cardiovascular diseases has also been observed in our study by identifying angiogenin in neurons or vessels of the brain within the first 4 days in infarcted areas. In this regard, several authors have used cell culture experiments to reveal that angiogenin is expressed in motoneurons and is protective under hypoxic conditions (41). But angiogenin also mediates tumor angiogenesis in cancer (46).

In the present study, we have described the temporal profile of angiogenin from the subacute phase to the most chronic phases after ischemic stroke during rehabilitation. In patients, before starting IRT, angiogenin levels were not different from those of the controls. This result is in accordance with a previous publication showing a decrease in angiogenin levels at day 14 after an initial acute increase after 48 h (45). In our study, angiogenin levels measured before IRT were not correlated with infarct size, probably because those measures were performed after the most acute phase of the disease. One of the most interesting findings in the present study is the increase in blood angiogenin at 1

month after starting IRT, followed by a decrease at 3 and 6 months. More importantly, this increase is clearly associated with an improvement in Barthel Index, MRC and Rankin scores, confirming the potential use of angiogenin as a biomarker to monitor rehabilitation.

In parallel, the pasta matrix and treadmill exercise for rodents were used to support our findings in stroke patients. The pasta matrix task has recently been included as a rehabilitative approach in mice by Kerr et al. in 2014 (31) as an experimental task-oriented test to study the brain mechanisms targeting only the affected or the contralateral forelimb. The pasta-matrix approach requires a food-restricted diet, which was established for all animals in the study and caused an expected hypoglycemic status. Importantly, the achieved glucose levels during ischemia and rehabilitation were not different between groups, and there was no association with infarct size. The treadmill task has been widely used to study exercise-related mechanisms after ischemia in rodents (47,48), with several authors reporting neuroprotective effects when the activity is performed before ischemia or within 24 h of ischemia (47–49). As observed in human stroke samples, angiogenin was found in the mouse brain after cerebral ischemia and was increased in the ipsilateral cortex and more specifically in neurons. Another study conducted in a rat stroke model previously described elevated brain angiogenin levels during the first week with a clear nuclear expression in neurons (50).

We have shown an increase in ipsilateral angiogenin RNA expression in all groups at the beginning of rehabilitation with no differences between groups; importantly, higher RNA expression was found after 12 days of rehabilitation in the task-oriented group compared to the non-rehabilitation group. Additionally, the contralateral brains of both rehabilitation groups exhibited increased angiogenin levels compared to the non-rehabilitation group. These observations link brain angiogenin with rehabilitation tasks, being different in task-oriented exercises for the impaired forelimb than in general-running exercise where both extremities are used. Unfortunately, we could not demonstrate a functional improvement on the grip strength meter test in the mice with overexpressed brain angiogenin that received rehabilitation. We attribute this result to the spontaneous recovery observed in mice (33) and the need to evaluate the forelimb force separately between affected and non-affected sides.

Finally, EPCs have been investigated as key elements of neovascularization and for their use as biomarkers after stroke during rehabilitation. These cells have the capability to form new vessels in the adult, can mobilize to specific areas in response to ischemia and differentiate to endothelial cells (19,34,51). Different studies in stroke patients have shown an increase in circulating EPCs in the acute and subacute phases, and several studies have reported a peak in EPCs after 7 days of ischemic stroke associated with better outcome (52) followed by a decrease in the EPCs counts at 3 months (53). In our study, we report that the initial stroke-driven increase in EPCs is maintained during rehabilitation follow-up after several months. Finally, our pre-clinical experiments have revealed an early increase in spleen-derived EPCs in culture early after pasta-matrix rehabilitation but not in long-term. According to previous studies, we were expecting to also observe an increase in the mice undergoing daily exercise (15). Some differences could be explained by the fact that we have established a model of forced vs. voluntary exercise (15) or that our food restriction conditions could influence the spleen as described by others (54).

In conclusion, we have described for the first time the modulation of angiogenin and EPCs in stroke patients under IRT and in a mouse model of post-stroke rehabilitation task-oriented exercise. Our findings suggest that these molecules may be novel biomarkers to monitor and predict stroke recovery.

It is important to remark that the main limitation of our study is that we have not included a control cohort of stroke patients of similar characteristics who did not receive IRT to truly elucidate the role of rehabilitation in the modulation of angiogenin and EPCs. Ethical principles demand that all patients admitted to our hospital who are candidates for receiving IRT must be enrolled in the program. Future multicentric studies including other rehabilitation programs and enlarging the number of studied patients should be designed in the future. As an approach to this non-rehabilitation condition after stroke, we have presented a pre-clinical model of stroke with or without rehabilitation intervention.

Author Contributions

M.G.-S and A.R conceived and designed the experiments; M.G.-S, A.R, X.B, N.G and S.R organized the database and contributed to data collection; M.G-S and D.G performed the statistical analysis; M.G.-S, A.M, G.C, A.F and A.B participated in data acquisition. M.G.-S and A.R wrote the first draft of the manuscript. J.M contributed to review the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Statement

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.

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SUPPLEMENTARY MATERIAL

Importance of Angiogenin and Endothelial Progenitor Cells after Rehabilitation both in Ischemic Stroke Patients and in a Mouse Model of Cerebral Ischemia.

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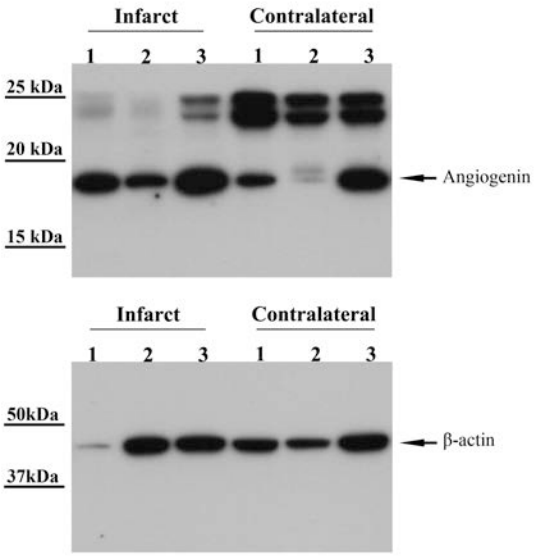
Supplementary Table 1: Demographic and clinical data from postmortem samples of ischemic stroke patients.

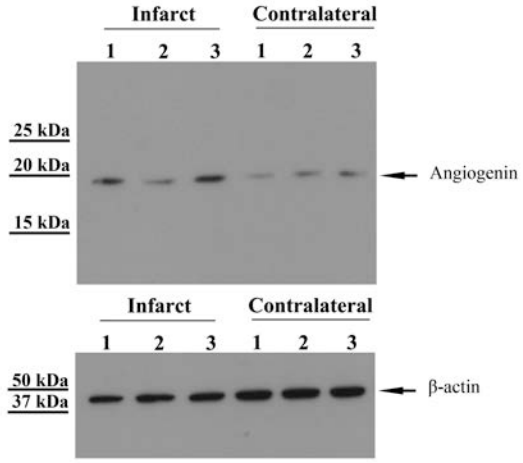
Sex	Age	T-O-D (h)	PMI (h)	IHC	WB
Female	83	100	14.5		X
Male	84	40	8	X	X
Female	73	44	4	X	
Male	75	19	5	X	X

Abbreviations: T-O-D: time from onset of stroke symptoms to death; h:hours; PMI: postmortem interval (from death to brain samples collection) ;IHC: immunohistochemistry; WB: western blot.

Supplementary Figure 1: Full image of western blot shown in Fig.3A.



Supplementary Figure 2: Full image of western blot shown in Fig.6A.



Article 2

Physical exercise enhances sub-ventricular zone neurogenesis and cortical angiogenesis while angiogenin stimulates primary neural stem cells and endothelial progenitor cells function.

Manuscript in preparation

Physical exercise enhances sub-ventricular zone neurogenesis and cortical angiogenesis while angiogenin stimulates primary neural stem cells and endothelial progenitor cells function.

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ABSTRACT

Background: Stroke-related disabilities compromise quality of life, and rehabilitation is the only effective treatment to compensate for these motor and functional impairments. However, little is known about the brain repair mechanisms that are activated during rehabilitation. Preclinical models of rehabilitation and investigations of the progenitor cells involved in neurorepair would help elucidate these mechanisms and identify new therapeutic targets.

Objective: The present study examines the modulation of brain recovery-associated routes in a rehabilitation animal model after cerebral ischemia and the effect of angiogenin in primary cultures of different progenitor cells (neural stem cells, NSCs; oligodendrocyte precursor cells, OPCs; and endothelial progenitor cells, EPCs) related to neurorepair processes.

Methods: Male mice underwent permanent occlusion of the middle cerebral artery. 48h later, mice were randomly assigned to non-rehabilitation, task-specific and physical-exercise groups and performed 12 daily-sessions of rehabilitation. Neurogenesis was assessed in the SVZ, and angiogenesis was measured in the peri-infarct cortex. White matter remodeling was measured as the final thickness of corpus callosum sections. Different primary cell cultures were established for NSCs, OPCs, EPCs, and neuron-like cells (SH-SY5Y) which were treated with exogenous angiogenin. NSC proliferation, neurosphere formation, OPC proliferation and differentiation, and endothelial tubulogenesis and migration were measured.

Results: Physical exercise increased DCX^+ cells in the SVZ of the ischemic hemispheres and vessel density in the contralateral hemispheres. Notably, SVZ-neurogenic sites with NSCs ($nestin^+$) were rich in angiogenin expression, especially in neuroblast cells. Treatment with exogenous angiogenin *in vitro* increased NSC neurosphere formation and proliferation and SH-SH5Y proliferation (but not differentiation into a neuron-like phenotype) and improved the angiogenic response of EPCs in tubulogenesis and migration assays.

Conclusions: Our findings demonstrate that post-stroke rehabilitation modulates neurorepair responses involving neural stem cells and angiogenesis, and angiogenin might play a central role, which suggests targeting this factor in future pre-clinical studies.

Keywords: stroke, rehabilitation, neurogenesis, neural stem cells, angiogenin, angiogenesis.

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 therapies are the only available treatments during the acute phase of ischemic stroke to prevent death and minimize functional and motor incapacities[3,4]. However, the narrow time window limits these strategies, and only a small number of patients benefit from these therapies. Although these vessel-recanalization strategies are effective, a large percentage of stroke survivors suffer unilateral motor disabilities and neurological deficits[5]. 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 independency for daily activities[6,7]. In spite of the proven benefits of multidisciplinary rehabilitation programs, these do not guarantee complete recovery for all patients despite their wide therapeutic window, and individuals exhibit variable responses to similar treatments[8]. Therefore, the biological responses responsible for the individual functional improvements were investigated to identify brain plasticity mechanisms and targets to modulate the natural evolution of brain repair by rehabilitation [9–11].

Neurorehabilitation animal models are indispensable for investigating repair-associated responses after stroke[12,13]. A few pre-clinical studies associated rehabilitation with restorative brain plasticity, including mechanisms of neuro-angiogenesis[14–18]. However, knowledge of the molecules modulated by rehabilitation and potentially associated with brain plasticity is incomplete. The present study used two different rehabilitation animal models: treadmill (as a physical exercise) and pasta matrix (as a task-specific performance). Both tasks are included in multidisciplinary rehabilitation programs for stroke patients. Therefore, these models were used to examine neurogenesis, remyelination and angiogenesis via modulation of progenitor/precursor cell function. To further explore the implications of rehabilitation at the molecular level we have focused on the angiogenin protein and its activity in *in vitro* cultures of different brain progenitor/precursor cells. Angiogenin is a protein that promotes cell proliferation and migration and it is secreted by endothelial cells[19–21]. The actions of angiogenin were described in tumor angiogenesis[22], and it acts as a neuroprotectant in neurodegenerative diseases *in vitro* and *in vivo*[23,24]. Our previous study demonstrated its potential role as a blood biomarker during rehabilitation of motor and functional improvements, and its overexpression in the ischemic brain following cerebral ischemia was related to specific rehabilitation tasks (see article 1).

Our hypothesis is that angiogenin might modulate rehabilitation-enhanced neurorepair responses via progenitor/precursor cell function.

METHODS

Brain Tissue Samples

We used tissue slides from a previous protocol in which cerebral ischemia was induced in mice via permanent electrocauterization of the distal branch of the middle cerebral artery to investigate cerebral neurogenesis and angiogenesis after ischemia. Mice were randomized to the following groups: non-rehabilitation group, physical-exercise rehabilitation or task-specific

rehabilitation (see article 1); n=9-11/per group.

C57BL/6 mice (8-12 weeks old) were used to obtain the SVZ neurogenic-sites for NSC cultures and western blot analysis. NSCs were obtained as previously described[25] and stored in liquid nitrogen until used.

Mouse habituation and training protocol

The Ethics Committee of Animal Experimentation of the Vall d'Hebron Research Institute approved all experimental animal procedures (protocol number 21.16), which were performed in accordance with the Spanish legislation and the Directives of the European Union. Briefly, C57BL/6 mice (males 6-12 weeks old) were purchased from Janvier Laboratories (Saint Berthevin, France). Mice were housed in a temperature/humidity-controlled room and maintained on a 12-hour light-dark cycle. All animals were given water and food *ad libitum*. All mice were food-restricted for 7 days prior to the habituation procedure, as described (see article 1). Mice were habituated and trained on the Pasta Matrix Reaching task using a protocol adapted from Kerr and colleagues[26] and the treadmill apparatus (see article 1). Notably, individual limb preference was established by testing the mice for a minimum of 3 days in the pasta matrix reaching task, and the number of attempts with the right or left forelimb was recorded. Limb preference was determined as 70% of limb dominance. Finally, mice were trained for their preferred forelimb by filling only half of the matrix with pasta (contralateral to the preferred limb). Our group previously fully described this protocol (see article 1).

Permanent focal cerebral ischemia model

Mice underwent into permanent cerebral ischemia as described previously[27]. The distal occlusion of the right or left middle cerebral artery (pMCAo) was dependent on animal limb preference. Body temperature control and cortical cerebral flood flow were monitored as described previously (see article 1). Briefly, animals were anesthetized with isofluorane (Abbot Laboratories, Spain) for a maximum of 30 minutes via face-mask (4% for induction and 1-2% for maintenance in Medicinal Air, 79%N_2/21%O_2), and mice eyes were protected from corneal damage using an ophthalmic lubricating ointment (Lipolac™, Angelini Farmaceutica, Spain). A small craniotomy was performed between the retro-orbital 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 via heating of the needle and compressing the MCA. Cerebral blood flow (CBF) was monitored using a laser-Doppler flowmetry (Moor Instruments, UK) as described previously (see article 1), and only animals with a reduction in CBF below 80% were included. Buprenorphine (0.05-0.1 mg/kg) was administered subcutaneously after surgery, and the skin was sutured. Mice were allowed to spontaneous recovery from anesthesia. Mice were euthanized after 18 days of ischemia (corresponding with 12 days of rehabilitation) following the specific procedures described below.

Pre-clinical rehabilitation models

Rehabilitation treatments began 48 hours after pMCAo and consisted of 12 days of exercise as described previously (Article 1). Mice were randomly distributed into 3 different groups: No-Rehabilitation (No-RHB), Pasta Matrix (PM) or Treadmill.

Article 1 fully describes the rehabilitation protocol.

Immunohistochemistry (IHC)

All animals received daily intraperitoneal injections of 5-Bromo-2'-deoxyuridine (BrdU, 50 mg/kg in saline, B9285, Sigma-Aldrich, MO, USA) beginning 48 h after pMCAo to label proliferating cells. Animals 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 10 minutes prior to euthanasia, to label functional blood vessels. Afterwards, animals were euthanized via transcardial perfusion with cold paraformaldehyde (4% paraformaldehyde, PFA) under deep anesthesia. Whole brains were obtained and post-fixed with 4% PFA for 2 hours followed by 30% sucrose for cryoprotection. Brains were embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Fisher Scientific, US) and frozen at -80ºC until use (see article 1). Slices (12-μm thick) slices were cut in a cryostat and placed at room temperature for 30 min. Slices were washed 3 times: 5 min in 0.1% PBS-Tween, 5 min in 0.3%-PBS-Triton X-100 and 5 min in 0.1% PBS-Tween. Slices were 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 diving cells. Sections were blocked using 0.1% PBS-Tween containing 1% BSA (Sigma-Aldrich, MO, USA) and 5% goat serum (Merck Millipore, MA, USA) for 1 hour (mouse anti-doublecortin (DCX) was blocked with 1% BSA, 5% of goat serum and 0.5% Triton X-100). Then, slices were incubated with the following primary antibodies overnight (O/N) or for 3 hours at RT: 1:400 rabbit anti-DCX (ab18723, Abcam, UK), 1:100 mouse anti-DCX (sc-271390, Santa Cruz, USA), 1:100 rat anti-BrdU (ab6326, Abcam, UK), 1:100 rabbit anti-angiogenin (NBP2-41185, Novus, USA), 1:100 mouse anti-nestin (556309; BD Biosciences, USA), and 1:1000 rabbit anti-myelin basic protein (MBP; ab40390, Abcam, UK). Sections were washed 3 times for 5 min at RT with 0.1% PBS-Tween prior to secondary antibody incubation. Alexa fluor 488 goat anti-rabbit IgG (1:500 or 1:1000), Alexa fluor goat anti-rat IgG (1:500), Alexa fluor 647 goat anti-rabbit IgG (1:500) and Alexa fluor 633 goat anti-mouse IgG (1:500) from Invitrogen (CA, USA) were used as secondary antibodies. Slices were incubated with secondary antibodies for 1 hour at room temperature and washed with 0.1% PBS-Tween as described above. Finally, sections were mounted in Vectashield™ with DAPI (H-1200, Vector Laboratories, USA) to counterstain cell nuclei and visualized using an Olympus BX61 microscope (Olympus, Japan) or a confocal laser scanning biological microscope for the Alexa fluor 633 and 647 dyes (FV1000, Olympus, Japan).

Two brain slices were imaged at 100X, 200X and/or 400X magnifications for image analyses. Two images from each section were captured from the entire dorsolateral part of the subventricular zone (for DCX, Angiogenin, Nestin and BrdU labeling) or the peri-infarct boundary areas and corresponding contralateral sides (for lectin measurements). The total area of DCX- or lectin-positive fluorescence and double-positive cells (DCX+/BrdU+) was calculated using ImageJ software. For MBP analysis, we examined the corpus callosum in two different areas (lateral and medial), where two images were captured and the thickness was measured using ImageJ software. An investigator who was blinded to the treatment group analyzed all images.

Cell culture protocols

NSCs

Neural stem cells (NSCs) were obtained from the sub-ventricular zone (SVZ) as previously described[25]. Frozen NSCs were thaw at 37ºC and cultured in a mixture of 1:1 DMEM and F12 (Gibco, Thermo Fisher, USA) supplemented with 0.25% of P/S, 8 μg/ml of Heparin (H-3149; Sigma-Aldrich, USA), 0.02 μg/ml of hFGF-B (PHG0024; Thermo Fisher, USA), 0.02 μg/ml of hEGF (PHG0314; Thermo Fisher, USA), 2% of B27 (12587010; Thermo Fisher, CA, USA) and 1% of L-glutamine (25030149;Thermo Fisher, CA, USA). After 2 days in culture, NSCs started forming 3D proliferating structures known as neurospheres[25].

Endothelial progenitor cells

Frozen outgrowth endothelial cells (OECs) with an endothelial-like phenotype were obtained from wild-type FVB mouse spleen cultures[28] and used for *in vitro* angiogenesis. Note that this strain is a different mouse strain than the *in vivo* cerebral ischemia model because EPC cultures from C57BL/6 mice do not form OEC colonies in our hands. OECs were grown in endothelial growth medium-2 (EGM-2; Clonetics®, CA, USA), which is composed of endothelial basal medium (EBM) containing 10% FBS, human epidermal growth factor (hEGF), vascular endothelial growth factor (VEGF), human basic fibroblast growth factor (hFGF-B), insulin like growth factor 1 (R3-IGF-1), GA-1000 (gentamicin and amphoterecin-B), heparin, hydrocortisone and ascorbic acid.

SH-SY5Y culture and differentiation

The human neuroblastoma cell line SH-SY5Y was purchased from ATCC (ATCC® CRL-2266) and maintained in complete medium containing DMEM/F-12 (Gibco, Thermo Fisher, CA, USA), 10% fetal bovine serum (FBS), 1% non-essential aminoacids (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: DMEM/F-12 (Gibco, Thermo Fisher, CA, USA), 1% fetal bovine serum (FBS), 1% non-essential aminoacids (NEA) and 1% penicillin-streptomycin (P/S) supplemented with 10 μm all-trans-retinoic acid (RA).

Oligodendrocyte Precursor Cell (OPC) differentiation

NSCs were differentiated to OPCs. Briefly, 60,000 NSCs were seeded in 24-well plates coated with poly-L-lysine (Sigma-Aldrich, USA) followed by the addition of oligodendrocyte differentiation media: 1:1 mixture of DMEM/F-12 (Gibco, Thermo Fisher, USA) supplemented with 0.25% P/S, 0.02 μg/ml hFGF-B (PHG0024;Thermo Fisher, USA), 2% B27 (12587010; Thermo Fisher, CA, USA), 0.015 μg/ml PDGF-AA (PHG0035;Thermo Fisher, USA) and 1% L-glutamine (25030149;Thermo Fisher, CA, USA). Differentiation continued for 6 days with media changes every 2-3 days.

Experimental cell culture protocols

MTT assay for cell proliferation

This assay was used to assess cell proliferation in SH-SY5Y cells and EPCs. This assay shows the capacity of cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) into purple-colored formazan, which is a product of mitochondrial integrity in live cells. More formazan indicates more proliferating cells. Briefly, the proliferation of

SH-SY5Y cells was tested on day 5 of differentiation after treatment with angiogenin, RA or both agents. SH-SY5Y cells were washed 2 times with 1X PBS and MTT reagent was added at 0.5 mg/ml diluted in DMEM/F-12 (basal medium). Control wells were treated with basal medium. EPCs were seeded at a density of 30,000 cells in 24-well plates coated with fibronectin (10μg/ml) in complete medium. Cells were washed with 1X PBS 24 h later and treated with EBM (basal medium) and angiogenin (200 ng/ml) or EBM (as control). MTT (0.5 mg/ml in EBM) was added 24 h after the treatment, and cells were incubated for 90 min. Cells were lysed following the addition of DMSO, and absorbance was measured at 590 nm in a Synergy Mx BioTek reader (Bio-Tek Instruments, VT, USA). Each sample was measured in duplicate to obtain a mean value. The results are expressed as a percentage of the control group absorbance.

NSC counts and neurosphere growth

Cell suspension cultures of NSCs forming neurospheres were assessed using the Trypan blue exclusion method and a hemocytometer to quantify NSC proliferation after angiogenin treatment. 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 days with 100 or 200 ng/ml of angiogenin. We also treated these 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 100X 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 1500 rpm for 5 min. The cell pellet was gently resuspended in 300 μl of NSC media to obtain a single cell suspension, which was quantified as follows: 10μl of trypan blue was mixed with 10 μl of the cell suspension, and 10 μl of the mixed solution was pipetted into one side of the Neubauer hemocytometer. The number of cells with clear cytoplasm was counted per quadrant, and the following formula was used: total nº of cells/nº of fields*dilution factor*10^4 cells/ml. Data are expressed as a percentage of the control condition.

Cell proliferation assay (Counting Kit-8 (CCK-8))

The CCK-8 (Dojindo Molecular Technologies, Inc) assay was used to measure the cell viability of OPCs using the WST-8 reduction by dehydrogenases. NSCs were treated with 200 ng/ml angiogenin during the 6 days of OPC in differentiation media. OPCs were incubated with a 10% CCK-8 solution for 2 hours at 37ºC on day 6. The absorbance of the culture medium was measured at 450 nm using the Synergy Mx BioTek reader (Bio-Tek Instruments, VT, USA). The results are expressed as a percentage of the control treatment (differentiation media) absorbance.

Neurite outgrowth of SH-SY5Y cells

SH-SY5Y cells exhibit a neuron-like phenotype with outgrowth neurites in the presence of retinoic acid, as detailed above. Briefly, 12,500 cells were seeded in collagen-coated 24-well plates and treated in differentiation and non-differentiation media with angiogenin (100 and 200 ng/ml) to test proliferation and neurite outgrowth stimulation. The media and treatments were changed after 2 days of differentiation and/or treatment with angiogenin, and cells were imaged (3 images/well) on day 5 using an Olympus IX71 at 100X magnification.

Finally, WimNeuron automated analysis software (Wimasis Image Analysis®) was used for quantification. Neurite outgrowth was evaluated by measuring the circuitry length and the total thin neurite length. Data are expressed as a percentage of the control condition.

Western Blot

Mice brain dissections from the SVZ of naïve mice (n=4), and cultured NSCs and OPCs (n=4) were homogenized 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, USA) and 0.5% aprotinin (Sigma-Aldrich, USA). Brain homogenates obtained in a previous study (see article 1) from peri-infarct and contralateral tissue of ischemic mice under rehabilitation were also analyzed. Cell and tissue lysates were centrifuged at 12000rpm for 10 min at 4°C and the protein fraction contained in the supernatants was assessed by the bicinchoninic acid (BCA) (Thermo Scientific™, IL, USA). A total amount of 10μg for mouse tissue and cell lysates were mixed with Laemmli Buffer and 5% of 2-mercaptoethanol, heated 5 minutes at 95ºC, run into 12% polyacrylamide electrophoresis gels, transferred into PVDF membranes for angiogenin (Thermo Scientific™, IL, USA) or into nitrocellulose membranes for MBP, Nestin and PDGFRα detection. 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-angiogenin (1:500, NBP2-41185, Novus, CO, USA), MBP (1:1000 for brain homogenates and 1:250 for OPC homogenates; Thermo Fisher, USA), nestin (1:1000; BD Biosciences, USA), PDGFRα (1:200; R&D Systems, USA), β-actin (1:5000, A5316, Sigma-Aldrich, MO, USA) and GAPDH (1:2000, Thermo Fisher, USA). The membrane was then washed 3 times (PBS-0.1% Tween 20) and incubated with secondary antibodies at 1:2000 (donkey anti-rabbit-horseradish peroxidase, sheep anti-mouse horseradish peroxidase from GE Healthcare, UK) or at 1:4000 (rabbit anti-goat horseradish peroxidase from GE Healthcare, UK), for 1 hour at RT with gentle agitation. Finally, membranes were washed 3 times (PBS-0.1%Tween 20) and briefly incubated with Pierce® ECL Western Blotting Substrate (Thermo Scientific™, IL, USA) to visualize the chemiluminescence signal with Fujifilm FPM-100A films. Scanned films were quantified using the ImageJ free software and corrected by the corresponding actin/gapdh band-intensities. Molecular weight markers were also run for reference values.

Immunocytochemistry

Differentiated OPCs treated with or without angiogenin (200ng/ml) were immunostained for nestin, PDGFRα, MBP and A2B5 on day 6. The endothelial-like phenotype of EPCs was confirmed using the von Willebrand Factor (vWF), KDR and CD133 markers. Briefly, cells growing on coverslips were fixed with 4% PFA for 10 min, followed by 3 washes with 1X PBS and blocking with 3% BSA (Sigma-Aldrich, MO, USA) for 1 h (PDGFRα was blocked with 3% BSA and 0.5% Triton X-100). The following primary antibodies were incubated overnight: mouse anti-nestin (1:200, BD Biosciences, USA); mouse anti-MBP (1:200, Thermo Fisher, USA); goat anti-PDGFRα (1:100, R&D Systems, USA); mouse anti-A2B5 (1:200, Merck Millipore, USA); rabbit anti-vWF (1:100, Sigma-Aldrich, USA); mouse anti-

KDR (1:50, Sigma-Aldrich, USA); and rabbit anti-CD133 (1:50, Santa Cruz, USA). Cells were washed 3 times, and the following secondary antibodies were added to cells: Alexa Fluor 488 goat anti-mouse IgG; Alexa Fluor 488 donkey anti-goat IgG; and Alexa Fluor 568 goat anti-mouse IgG. Finally, cells were mounted in Fluoroshield™ with DAPI to stain cell nuclei, and 4 pictures per coverslip were acquired using the Olympus BX61 microscope (Olympus, Japan) at 200X magnification. Mean fluorescent intensity (MFI) was measured in OPC cultures using ImageJ software and data are expressed as a percentage of the control condition.

Wound healing assay

OECs ($6x10^5$ cells/ml) were seeded in complete medium in culture inserts composed of 3 independent wells with two wound areas in between (Ibidi, Germany). The culture insert was removed from the plate after 24 h to leave two clear wound areas. The cells were washed 2 times with 1X PBS and treated with basal medium (EBM, control) or EBM plus 200 ng/ml angiogenin (ab151351; Abcam, UK). Three images were obtained per wound area (corresponding to time 0h) using the Olympus IX71 microscope at 40X magnification and after 8 h, 24 h and 48 h of treatment. Finally, the percentage of the covered area and migration speed were calculated using WimScratch automated software from Wimasis Image Analysis®. The results are expressed as a percentage vs. the control condition.

***In vitro* tubulogenesis assay**

An *in vitro* Matrigel™ (BD Biosciences, USA) model was used to evaluate the role of angiogenin on the angio-vasculogenic function of EPCs. Briefly, $4x10^4$ OECs were seeded in EBM (control media) or EBM plus angiogenin (100 ng/ml) into 24-well plates that were previously coated with 200 µl of cold Matrigel™ (growth factor reduced) for 30 min at 37ºC. Six representative images were obtained after 24 h using the Olympus IX71 microscope at 100X magnification. Finally, the number of rings (circular vessel-like structures), total tube length (perimeter of complete rings) and branching points (convergence of 3 or more tubes) were automatically measured using WimTube software from Wimasis Image Analysis®. The results are expressed as a percentage versus the control condition.

Statistical analysis

The SPSS 20.0 package was used for all statistical analyses. The normality of continuous variables was assessed using the Shapiro-Wilk test ($N<30$) or Kolmogorov-Smirnov ($N\geq30$). Normally distributed variables were analyzed using ANOVA or t-test, and the Mann-Whitney U-test or Kruskal Wallis tests were used for non-normally distributed variables. For the analysis of repeated measures, the paired t-tests were applied for normally distributed variables, and the Wilcoxon test was used for non-normal distributions. Bar 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 value less than 0.05 were considered statistically significant.

RESULTS

Treadmill increases neuroblast pools in angiogenin-rich SVZ areas of the ischemic hemisphere.

We evaluated the presence of DCX+ cells, as a marker of migrating neuroblasts from the dorsolateral SVZ area, after 12 days of rehabilitation. Our results demonstrate a significant increase in DCX in the ipsilateral hemisphere of the treadmill group compared to the non-rehabilitation ($p=0.047$) and pasta matrix ($p=0.014$) groups. Treadmill exercise also increased the DCX signal in the SVZ of the ipsilateral vs. contralateral hemispheres ($p=0.043$) (Figure 1A). In parallel, the presence of BrdU+ nuclei indicated the presence of proliferating new born cells in this neurogenic site (Figure 1A micrographs). Cell counts of proliferating DCX+ cells revealed increased neuroblast proliferation in the treadmill group, which was only significant versus the pasta matrix counts ($p=0.027$) (Figure 1A, right panel). Next, examined the presence of angiogenin in the SVZ neurogenic site, which was rich in neural stem cells. Protein homogenates of the SVZ and cultured NSCs exhibited angiogenin expression on western blot (Figure 1B, left panel). Immunohistochemistry images (Figure 1B, right panel) showed that the angiogenin expression occurred in the SVZ near nestin$^+$ cells and co-localized with DCX+ cells. These data support the hypothesis that angiogenin could be involved in the regulation of neural precursors in neurogenic sites.

Primary cell cultures of NSCs derived from the SVZ were exposed to exogenous angiogenin at different time points of neurosphere formation to further examine the impact of angiogenin on neural precursors (Figure 1C). The highest dose of angiogenin increased NSCs after the floating neurospheres were formed ($p<0.001$), and co-treatment with neomycin (the angiogenin inhibitor) abolished this proliferation ($p<0.05$)(Figure 1D). Indeed, no toxic evidence of neomycin was observed in NSCs ($p>0.05$; data not shown). We also treated NSCs with the highest angiogenin dose (200ng/ml) at the beginning of the culture prior to neurosphere formation (day 0), 3 days after seeding or both to quantify the number of proliferating cells and the neurosphere diameter. Figure 1E shows a significant increase in NSCs in all treated groups *vs.* control (day 0: $p=0.002$, day 3: $p=0.003$ and day 0-3: $p=0.004$). In this regard, we also observed an increase in neurosphere diameter. The maximum diameter achieved occurred when angiogenin was added to formed neurospheres on day 3 ($p=0.005$ vs. control) (Figure 1E).

Finally, we investigated whether angiogenin also triggered the proliferation and differentiation of a neuron-like cell line and axonal/neurite outgrowth in SH-SH5Y cells. Our results showed increased proliferation of undifferentiated cells at 200 ng/ml *vs.* control ($p<0.001$) (Figure 1F). However, differentiated cells exhibiting axonal sprouting did not respond to angiogenin treatment (Figure 1F). The capacity of axonal/neurite sprouting was confirmed in the presence of retinoic acid, as expected ($p<0.001$), but not with angiogenin treatment at any of the tested concentrations (Figure 1G).

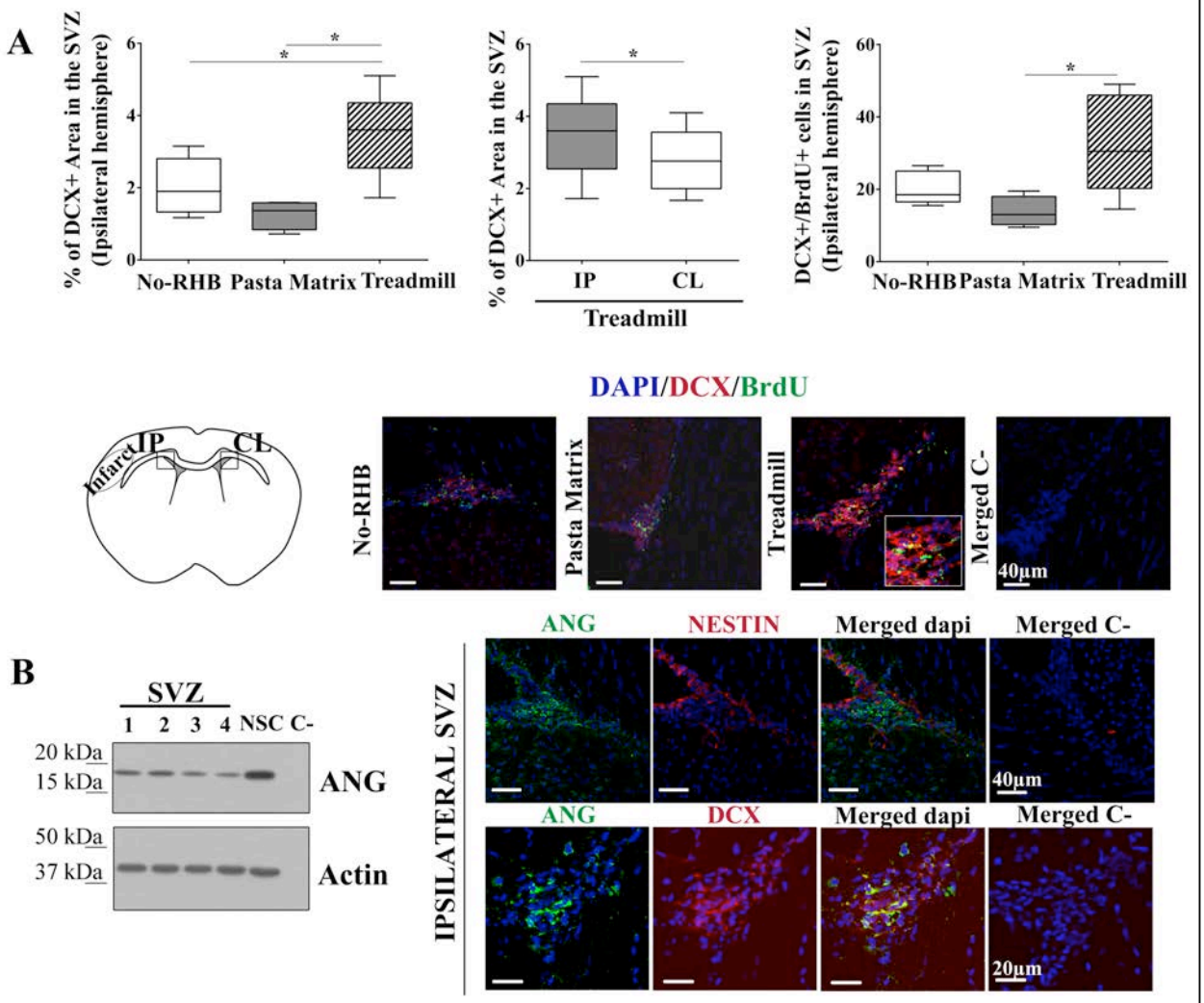

Figure 1 A and B: Modulation of SVZ neurogenesis after rehabilitation. (A) The DCX+ signal was measured in the SVZ together with BrdU. The left and middle panels represent DCX quantification and the right panel shows the number of positive proliferating cells (BrdU+)/neuroblasts (DCX+) in the SVZ (n=4-5). Box-plot graphs are shown together with representative images of immunostains. **(B)** Angiogenin expression evaluated by western blot in SVZ homogenates and derived NSCs, and representative immunostains of the SVZ showing NSCs (nestin+) in close proximity to neuroblasts (DCX+) expressing angiogenin. *$p<0.05$ as indicated by horizontal lines. Abbreviations: IP: ipsilateral, CL: contralateral, DCX: doublecortin, RHB: rehabilitation, SVZ: subventricular zone, ANG: angiogenin, C-: negative control of NSCs media.

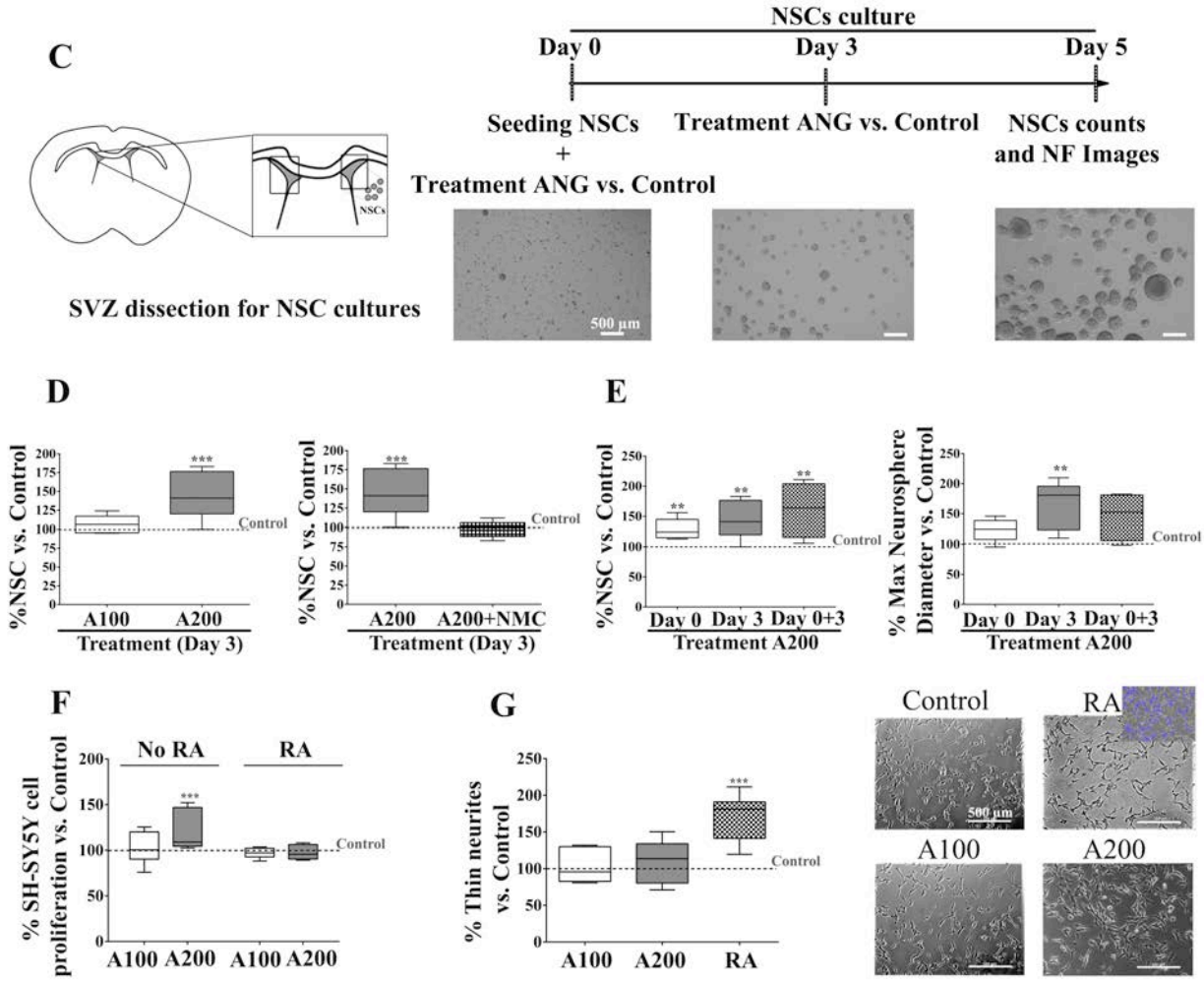

Figure 1 C-G: Angiogenin activity in SVZ neurogenesis. (C) Schematic figure of the SVZ dissection for NSC isolation and timeline of the experimental procedure of neurosphere cultures. **(D-E)** Box plots showing quantification of the NSC-forming neurospheres after angiogenin stimulation (100 or 200 ng/ml) at different time-points, the inhibition with neomycin, and the largest neurosphere diameters obtained with angiogenin treatment; n=4-9. **(F)** Box plots showing the angiogenin proliferation effect on SH-SY5Y cells, which was non-existent in retinoic acid (RA)-differentiated cells; n=5-6. **(G)** Box-plots and representative cell images showing axonal/neurite outgrowth in the presence of RA, but not under angiogenin stimulation; n=5-6. The insert in RA shows a micrograph representative of the WimNeuron analysis. **p<0.001 and ***p<0.001 vs. control. Box plots represent median (IQR) of the percentage vs. control values. Abbreviations: IP: ipsilateral, CL: contralateral, DCX: doublecortin, RHB: rehabilitation, SVZ: subventricular zone, ANG/A: angiogenin, NSCs: neural stem cells, NMC: neomycin, NF: neurospheres, RA: retinoic acid.

Rehabilitation does not modify remyelination.

Remyelination by myelin sheets from oligodendrocytes is one putative mechanism in post-stroke brain plasticity. Therefore, we aimed at testing whether rehabilitation therapies activated oligodendrogenesis after experimental ischemia and the impact of angiogenin on oligodendrocyte progenitors. Measures of white matter tracks thickness in the corpus callosum did not show any differences between rehabilitation groups (Figure 2A). MBP protein quantification confirmed this result (Figure 2B).

Angiogenin does not enhance oligodendrogenesis but may contribute to preservation of a stemness status in neurogenic sites.

SVZ NSCs give rise to OPCs and oligodendrocytes *in vivo.* Therefore*,* we explored the influence of the angiogenin detected in SVZ areas on OPC differentiation *in vitro* (Figure 2C). Six-day differentiated OPCs expressed specific OPC markers such as nestin, A2B5, PDGFR□ and MBP (Figure 2D).

Six days of differentiation with exogenous angiogenin did not further increase OPC proliferation (Figure 2E). We hypothesized that angiogenin altered OPC fate in terms of maturity to an oligodendrocyte phenotype. OPC markers for ICC did not reveal differences in OPC status (Figure 2F). However, protein quantifications by western blots revealed an increase in nestin protein when OPCs were differentiated in the presence of angiogenin ($p=0.014$ *vs.* control group; Figure 2G). Further quantification of PDGFRα and MBP proteins was not significantly different in these cells (data not shown).

Contralateral angiogenesis after rehabilitation therapies.

Cortical peri-infarct vascular density was not different between groups after 12 days of rehabilitation ($p>0.05$) (Figure 3A, left panel). Unexpectedly, contralateral cortical areas from the pasta matrix and treadmill groups exhibited increased vessel density compared to the ipsilateral hemisphere ($p=0.015$ and $p=0.041$; respectively) (Figure 3A, right panel).

Angiogenin triggers *in vitro* tubulogenesis and migration in endothelial progenitor cells.

We investigated the role of angiogenin on EPC function by assessing the proliferation, tubulogenesis and migration capacity. EPCs were phenotyped for vWF, Flk-1 and CD133 as previously described[28] (see Supplementary Figure 2). Angiogenin did not induce EPC proliferation after a 24 h treatment ($p>0.05$ vs. control; data not shown). However, angiogenin stimulated the formation of tubular structures via increasing the number of branching points, loops and network perimeters compared to the control condition (all $p=0.005$) (Figure 3B). Conversely, angiogenin enhanced the migration speed of EPCs ($p=0.005$, Figure 3C), but the total covered area in wound healing assays was similar between the angiogenin and control groups ($p>0.05$; data not shown).

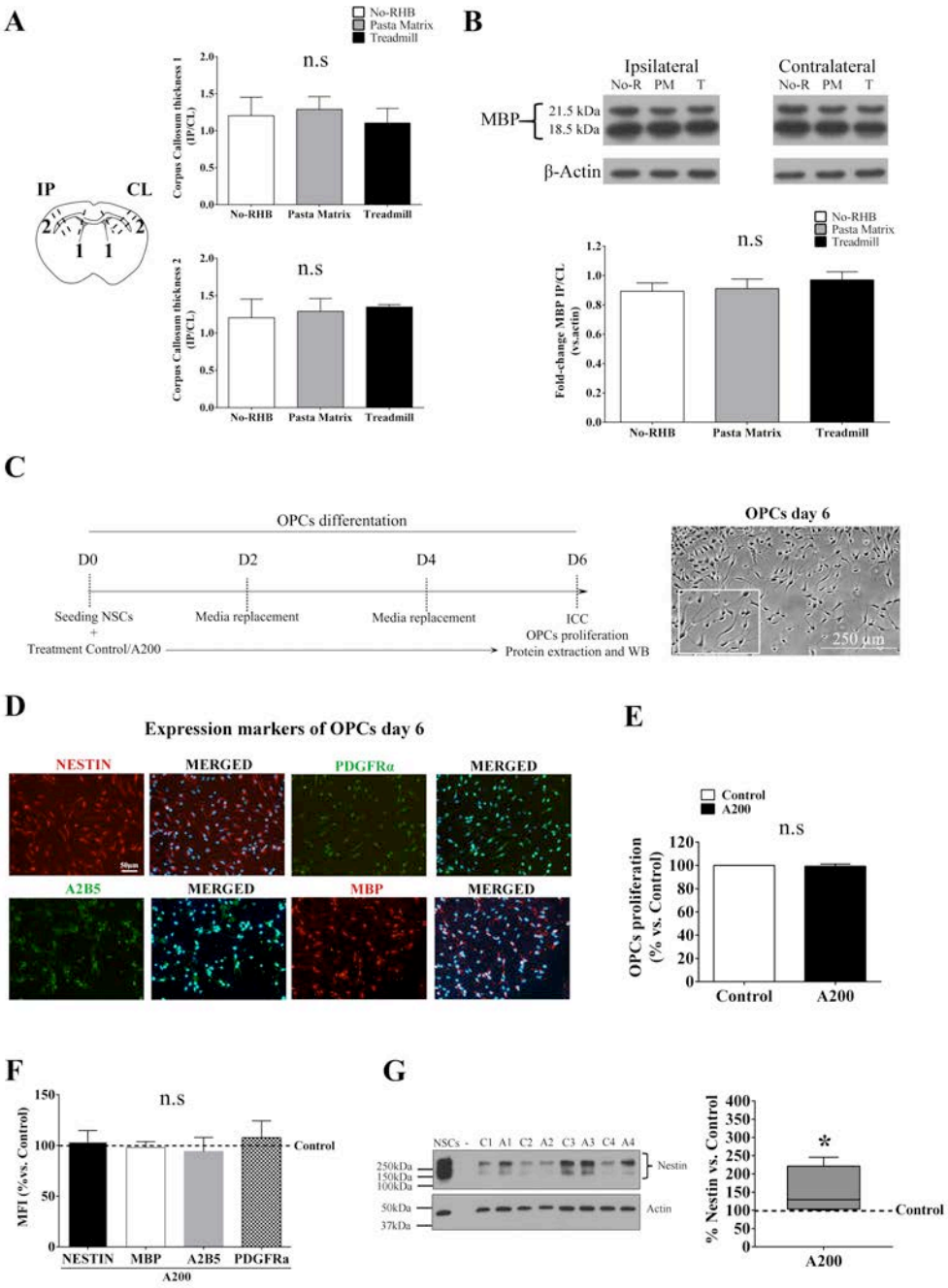

Figure 2: *In vivo* remyelination after rehabilitation and *in vitro* oligodendrogenesis after angiogenin stimulation. (A) Bar graphs showing the white matter thickness (MBP+ immunostain) in medial and lateral corpus callosum sections, n=5. **(B)** Additional MBP quantification using western blotting in cortical tissue after 12 days of rehabilitation. Bar graphs representing the MBP expression corrected by actin, n=5. **(C)** Study design of OPC differentiation from NSC cultures and representative image of OPC yields on day 6. **(D)** Immunofluorescence of OPC markers on day 6 showing positive signals for nestin, A2B5, PDGFR□ and MBP. **(E)** Bar graphs showing no changes in OPC yields after differentiation treatment with angiogenin; n=3. **(F)** Bar graphs representing the mean fluorescence intensity (MFI) of different OPC maturation markers; n=3-8 **(G)** Representative western blot (corrected by actin) and quantification (in box-plots) showing nestin expression after 6 days of differentiation of OPCs with (A) or without (C) angiogenin treatment. Expected double band within the 198-260 kDa range; *p<0.05; n=4. Bar graphs represent the mean±SEM, and box plots represent the median (IQR) of the percentage vs. control. Abbreviations: IP: ipsilateral, CL: contralateral, RHB/R: rehabilitation, n.s: non-significant, PM: pasta matrix, T: treadmill, OPC: oligodendrocyte precursor cells, NSC: neural stem cells, A: angiogenin.

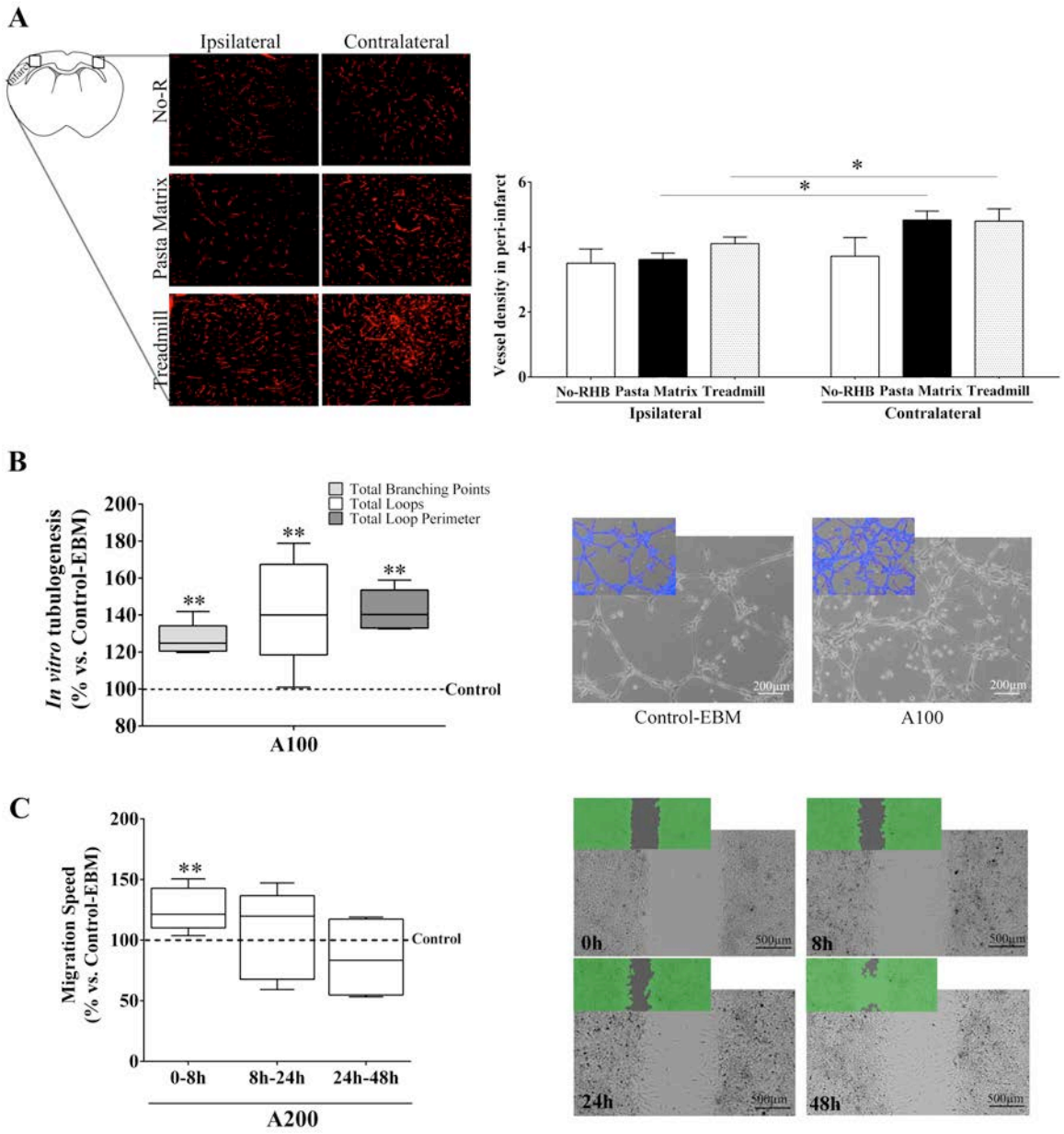

Figure 3: *In vivo* angiogenesis after rehabilitation and modulation of the angiogenic responses of EPCs *in vitro*. **(A)** Angiogenesis was evaluated after 12 days of rehabilitation in cortical peri-infarct areas. Bar graphs showing vessel density in peri-infarct ipsilateral and corresponding contralateral areas (n=4-5) together with images of representative immunostains, *p<0.05 as indicated by horizontal lines. **(B)** Box plots representing the *in vitro* tubulogenesis quantifications of the total branching points, total loops and total network perimeter, and representative matrigel images. **(C)** EPC migratory speed after angiogenin treatment is represented in box-plots and imaged in representative micrographs of migration assays. Inserts in B and C show micrographs representative of the WimTube and WimScratch analyses. **p<0.01 and n=5. Bar graphs represent the mean±SEM, and box plots represent the median (IQR). Abbreviations: R/RHB: rehabilitation; EBM: endothelial basal medium, A: angiogenin.

DISCUSSION

The present investigation showed that physical exercise after cerebral ischemia increased the neuroblast pool of cells in the neurogenic SVZ and that physical and task-specific exercises increased angiogenesis in the contralateral hemispheres of ischemic brains. Moreover, we demonstrated for the first time that angiogenin was expressed in neurogenic SVZ niches, which are rich in NSCs, specifically in migrating neuroblasts. Cell cultures showed that angiogenin stimulated the proliferation of NSCs and SH-SY5Y but did not stimulate the differentiation to oligodendrocytes or neural-like phenotypes, respectively. Angiogenin also promoted the angiogenic functions of EPCs.

Many people survive stroke but exhibit physical and motor deficits that limit functional independency and quality of life. Current rehabilitation programs are implemented in developed countries to reduce stroke-related disabilities to ultimately compensate for the impaired functions[7,8]. Several studies demonstrated that exercise improved functional recovery and activated cerebral repair-associated processes within a plastic brain[9,29]. These data are supported by preclinical rehabilitation models that are an emerging strategy to investigate underlying mechanisms during the recovery phase of stroke and elucidate the molecular and cellular pathways activated during the rehabilitation therapies received by stroke patients in the clinical setting[13,30,31].

Previous investigations in experimental stroke described neurogenesis enhancement such as increased neurogenesis in the SVZ and subgranular zone (SGZ) of the hippocampus and reported that neuroblasts from the SVZ migrated to infarct boundaries in response to ischemic injury[32,33]. Studies in rodents under exercise conditions showed enhanced neurogenesis in the hippocampus[18,34,35] that was related to memory recovery. According to these data, our results demonstrated that physical exercise after cerebral ischemia (treadmill running) enhanced DCX pools in the SVZ of ipsilateral hemispheres. Additionally we reported the presence of the trophic factor angiogenin in neuroblast cells in active SVZ closely associated with neural stem cells. Angiogenin is present during mouse embryogenesis and neuroectodermal differentiation[36], and it is also localized in axonal growth cones and neurites, where its inhibition impacts neural path-finding (but not in embryonic cell differentiation). Flores and colleagues recently suggested that angiogenin together with other proteins participated in the prevention of neural differentiation of neuroepithelial stem cells[37]. Other authors demonstrated increased neurosphere formation in an embryonic carcinoma cell line after the addition of angiogenin in culture[38]. Our study confirmed the neurogenic actions of angiogenin in primary cell cultures from SVZ niches. Angiogenin has been recently found to be present in the secretome of EPCs[19], 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[39,40]. 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. We used NSCs derived from the SVZ, and our results with neurosphere cultures supported the role of angiogenin in NSC proliferation but not differentiation in a more mature neuronal phenotype in the SH-SY5Y model. However,

additional studies under hypoxic conditions could modify our differentiation observations. Moreover, the ability of neomycin (inhibitor of angiogenin) to suppress primary NSC growth in neurospheres confirmed the specific effects on cells derived from neurogenic SVZ.

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[41], nestin, under undifferentiated conditions. These cells differentiate into neurons in the presence of retinoic acid[42], which allows for investigation of differentiation via the addition of drugs or molecules. SH-SY5Y cells in our experiments were treated with angiogenin, and we observed an increase in proliferation, whereas differentiated cells stopped proliferating, as expected. We also evaluated the capacity of angiogenin to induce neuronal differentiation. However, none of the measured parameters, such as circuit or neurite length, were modulated in the presence of this stimulus. These results are consistent with a previous[37] report that angiogenin was involved in the prevention of neural precursor maturation.

Myelin damage/degeneration is often associated with stroke and demyelinating diseases. Oligodendrogenesis is an essential repair mechanism to restore myelin loss in both scenarios[19,43,44]. Some studies reported an activation of oligodendrogenesis mechanisms after cerebral ischemia in animal models with white matter injury[45,46], and a recent report demonstrated an enhancement of OPC proliferation and differentiation in a rat model of chronic hypoperfusion[47]. Unfortunately, we did not find any indication of myelin synthesis in any of our treatment groups, perhaps because the minor impact on white matter injury in our cortical ischemia model, or the studied time-point. For example, others[48] reported activation of oligodendrogenesis in an animal model of cerebral ischemia that combined pMCAo and common carotid artery occlusion or chronic hypoperfusion models with selected white matter damage[19,47]. In parallel to our *in vivo* experiments, we also examined the role of angiogenin on OPC yields during NSC differentiation. In our previous collaborative study we reported that angiogenin did not increase OPC proliferation[19]. Also, in consonance with the aforementioned publications regarding the effects of angiogenin in neural differentiation prevention[37], our data showed an increase in nestin protein levels in OPCs under angiogenin treatment, which support its role in the maintenance of NSC stemness.

Several studies demonstrated that rodents with cerebral ischemia show increased vessels and angiogenic molecules in the ipsilateral cortex from minutes to several days or weeks after stroke and in combination with exercise[15,49–51]. Other authors described a reduction in vessel density after 2 weeks in the infarct boundary or an increase in angiogenic molecules in the contralateral hemisphere[52,53]. Our results showed an increase in vessel density in the contralateral hemispheres of the rehabilitated groups, which suggest that vascular remodeling also occurred in the contralateral side in response to the exercise after ischemic injury. This observation was an unexpected result. However, several factors must be considered to better define the angiogenic profile after rehabilitation, such as exercise intensity, time of analysis after ischemia and the fact that the contralateral hemisphere responded to ischemia with compensatory mechanisms after injury[54].

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[20]. We demonstrated that this molecule promoted tubulogenesis and migration speed in mouse EPCs, but we did not observe changes in EPC proliferation, as for OPCs in the current study.

The present study showed that physical exercise increased neurorepair-associated processes involving SVZ neurogenesis or angiogenesis in neurorehabilitation animal models. We also identified a new role for angiogenin in neural precursor maintenance lineage and proliferation, and it also participated as a pro-angiogenic molecule in endothelial progenitor cells.

Conflict of Interest Statement

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.

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SUPPLEMENTARY MATERIAL

Physical exercise enhances sub-ventricular zone neurogenesis and cortical angiogenesis while angiogenin stimulates primary neural stem cells and endothelial progenitor cells function.

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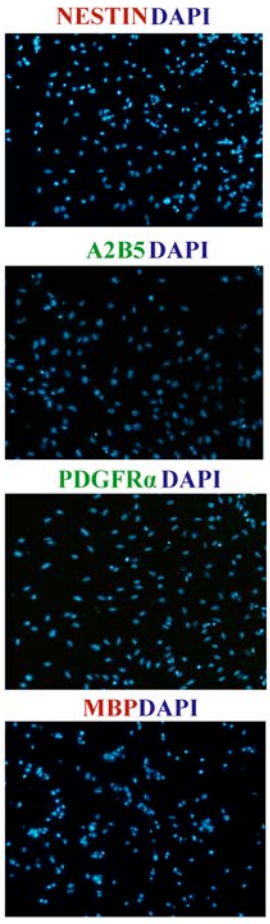
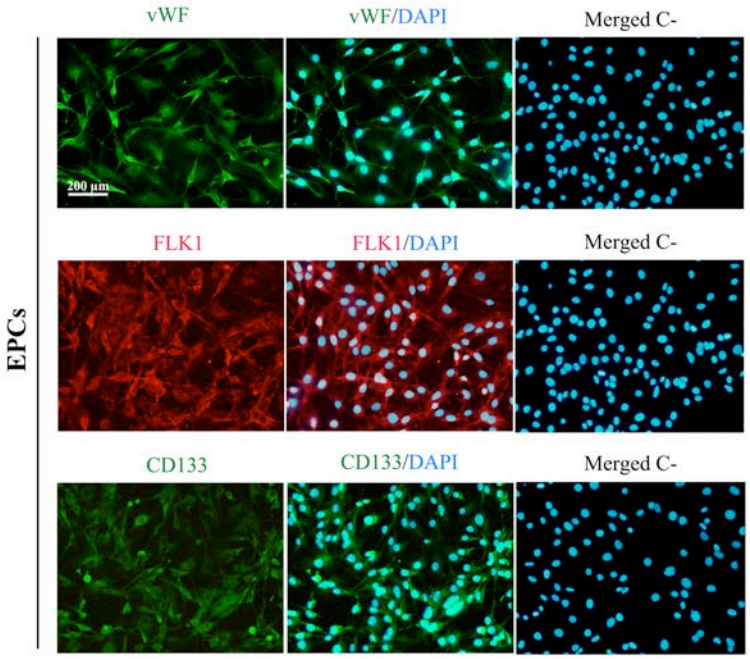
Supplementary Figure 1: Negative controls from Figure 2D.



Supplementary Figure 2: Immunocytochemistry of EPCs and negative controls.

Abbreviations: C-: negative control.



4.DISCUSSIÓ

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4.1 La importància dels models preclínics de rehabilitació.

Un gran número de supervivents a l'ictus presenten afectacions motores i funcionals, que fan que la seva vida diària es trobi compromesa a dependre d'altres persones per realitzar les seves activitats quotidianes com menjar, beure, vestir-se, etc. Per tal d'entendre quines vies es troben implicades durant les teràpies de rehabilitació, estudis clínics i preclínics en aquest camp són necessaris. Gràcies a aquests, podem arribar a entendre què passa al cervell a nivell cel·lular i molecular durant les teràpies de rehabilitació, ja que fins ara no hi ha cap fàrmac aprovat per tal de potenciar els efectes de la rehabilitació i poder recuperar les funcions motores danyades.

En aquest context, a falta d'un fàrmac aprovat durant la fase de recuperació, l'ús de models animals en els laboratoris és indispensable, ja que ajuden a entendre els mecanismes subjacents implicats durant la fase de recuperació i així avançar en la pràctica clínica. Diferents investigacions amb models preclínics de rehabilitació han demostrat que la funció motora dels animals pot arribar a millorar després d'una isquèmia mitjançant exercicis com el de rehabilitació per tasca específica[238]. Tanmateix, existeixen elements crítics a l'hora d'extrapolar els resultats dels mètodes de rehabilitació del laboratori a la clínica, ja que els pacients presenten condicions mèdiques diferents als animals, com la variabilitat genètica entre individus o els diferents tipus i localització de la lesió.

Hi ha estudis amb pacients que presenten controvèrsies sobre quan, quina intensitat i durant quant de temps és necessària la teràpia de rehabilitació, per això l'ús de models animals ajuda a conèixer el perfil temporal de les rutes que s'activen durant les teràpies. La majoria d'estudis estan d'acord en que es segur i beneficiós iniciar la rehabilitació el més aviat possible, acompanyada de la màxima intensitat que el pacient pugui tolerar[98,239,240]. En canvi, l'assaig clínic AVERT va reportar i posar en dubte que l'inici primerenc de la mobilització (aixecar-se, asseure's, caminar) pogués tenir efectes beneficiosos[241]. En aquest estudi la mobilització es va començar en les primeres 24 hores de la lesió, quan els pacients encara no havien mostrat un mínim de recuperació. És important no confondre els conceptes mobilització i rehabilitació, ja que la mobilització té lloc durant les primeres 24 h de l'ictus, on el pacient intenta realitzar petits moviments o incorporacions. En canvi, la rehabilitació és una intervenció molt més complexa, intensa i extensa, on el pacient realitza una sèrie d'activitats i exercicis motrius a diari.

Davant d'aquest escenari, els models de rehabilitació actuals poden ajudar a respondre les preguntes que planteja la clínica. Els experiments on es fa servir una tasca específica o un ambient enriquit, s'acosten més a les actuals teràpies que son realitzades a les unitats de rehabilitació[242,243]. S'ha vist

que rosegadors que exerciten l'extremitat afectada mitjançant una tasca específica arriben a millorar les funcions motores. Recentment, El Amki *et al.* van demostrar que una millora a nivell motor després de 7 dies de rehabilitació (tasca d'agafar pellets), no s'associava amb la reducció de l'infart[179]. En un altre estudi, es va comparar la tasca específica amb un exercici voluntari en rodes d'exercici. Els resultats demostraven que un tractament durant 5 setmanes, mitjançant una tasca específica, millorava les habilitats motores dels animals després de la isquèmia, en canvi l'exercici voluntari no presentava cap canvi a nivell motor ni en mecanismes de plasticitat cerebral[176]. A més de conèixer els efectes a nivell motor, és important saber quins canvis anatòmics, de plasticitat o moleculars, s'estan produint sota aquestes estratègies de recuperació. Diferents autors descriuen una millora motora, després de realitzar una tasca específica associada a un increment de l'activitat dels mapes motors corticals. En aquests experiments, la teràpia era capaç d'incrementar els processos de plasticitat cerebral sense observar una disminució del volum d'infart[244,245]. També, estudis que combinen un ambient enriquit amb tasques específiques van observar una millora en l'habilitat motora, on s'incrementava l'arborització de les dendrites[246]. Aquesta estratègia també afavoria l'expressió de diferents factors neurotròfics[247].

Per tal de respondre a la incògnita de quan començar la teràpia i com d'àmplia pot arribar a ser la seva finestra terapèutica, Biernaskie i col·laboradors van demostrar que el potencial terapèutic de la rehabilitació es troba reduït en el temps. És a dir, quan més aviat és comença a rehabilitar, els animals presenten marges de millora més grans, incrementant la complexitat de les seves xarxes neuronals[238]. De manera destacada, l'assaig clínic CPASS (de l'anglès Critical Periods After Stroke Study) ha estat dissenyat a partir dels resultats de Biernaski et al. En aquest assaig, hi ha diferents grups amb una intensitat de teràpia diferent i diferent temps d'inici d'aquesta. A més, s'avaluen els dèficits funcionals i motors conjuntament amb l'estudi de biomarcadors en sang dels pacients[248].

En els últims anys, les tècniques per aplicar protocols de rehabilitació en rosegadors han millorat gràcies al disseny i a la fabricació d'aparells robotitzats, capaços de simular la rehabilitació de les extremitats superiors que es fan a la clínica. Aquest nou mètode[180,249] ha demostrat una millora de l'habilitat motora i somatosensorial dels animals isquèmics. A més amb aquests nous instruments s'amplien les possibilitats d'estudi per esclarir els mecanismes estimulats durant la teràpia rehabilitadora, ja que els resultats obtinguts amb aquests nous mètodes podrien tenir un gran impacte translacional en els propers anys.

Des d'una altra perspectiva, l'exercici físic és utilitzat en la pràctica clínica com a estratègia de recuperació després d'un ictus en pacients que presenten problemes motors en la marxa. Mitjançant la cinta de córrer, alguns pacients han pogut millorar la seva habilitat al caminar[250]. També és un

mètode àmpliament utilitzat en els laboratoris que estudien les vies activades durant l'exercici, ja sigui mitjançant una cinta de córrer o rodes d'exercici. Els beneficis de l'exercici físic han estat descrits des de les primeres 24 hores fins als 7 dies després del dany cerebral, on s'ha vist una disminució de les vies apoptòtiques i un increment de l'angiogènesi i de la neurogènesi juntament amb l'estimulació de factors tròfics[174,175,251–253].

En definitiva, l'ús d'aquests models permet estudiar els mecanismes subjacents i poc coneguts associats a la rehabilitació i així poder garantir en un futur, una millor estratègia terapèutica per a cada individu.

4.2 La teràpia de rehabilitació és capaç de modular els mecanismes angiogènics endògens augmentant els nivells d'angiogenina i d'EPCs.

Diversos estudis han demostrat que la teràpia rehabilitadora es capaç de modular les respostes endògenes neuroreparadores després d'una isquèmia cerebral. En aquesta tesi doctoral s'ha observat que una teràpia de rehabilitació intensiva (TRI) incrementa els nivells d'angiogenina en sang i manté elevades les EPCs circulants en pacients isquèmics mesos després d'haver patit l'ictus. Paral·lelament a aquestes investigacions, en un model preclínic de rehabilitació, ratolins sotmesos a 12 dies d'exercici presentaven un augment de l'expressió d'angiogenina al cervell. A més, els nivells de cèl·lules endotelials derivades de cultius primaris de la melsa, també es trobaven incrementats a l'inici de la rehabilitació en el grup de rehabilitació amb exercici per tasca específica.

Es postula que la rehabilitació és capaç d'incrementar la plasticitat neuronal i promoure canvis estructurals a nivell cerebral en pacients i també en animals[94,254]. Tal i com s'ha comentat en l'apartat anterior de la discussió, no es coneix cap fàrmac aprovat per potenciar l'efecte d'aquestes teràpies en els pacients que no milloren amb els programes actuals, o per accelerar-ne els seus resultats. És per això que l'interès investigador en l'ús de biomarcadors, mitjançant tècniques no invasives, en aquestes teràpies es troba a l'alça. El fet d'implementar biomarcadors que defineixin l'estat del pacient o la seva resposta al tractament ens permetria respondre preguntes que ara no tenen resposta com: Quina és la millor estratègia de rehabilitació per aquest pacient? Quin és el potencial de recuperació d'aquest? Conèixer perquè un pacient millora i un altre no és encara un repte.

Són molts els estudis clínics de biomarcadors en la malaltia de l'ictus però escassos aquells que tenen en compte les intervencions terapèutiques rehabilitadores que reben aquests pacients i les associacions entre marcadors i l'estat o pronòstic del pacient durant la rehabilitació. Un estudi publicat pel nostre laboratori va descriure el perfil de les metal·loproteïnases de matriu (MMPs) en sang durant la TRI. En aquesta publicació es va reportar que nivells de MMP3 es trobaven associats amb una millora motora en els tests MRC i FMA, mentre que la MMP12 i MMP13 es trobaven estretament relacionades amb la severitat i els pacients que presentaven nivells més alts mostraven una pitjor recuperació[113]. En un altre estudi, es van analitzar els nivells de 8-OHdG (8-hydroxydeoxyguanosine; marcador d'estrès oxidatiu i de dany al DNA) en orina i es va situar aquest biomarcador com a possible indicador del pronòstic dels pacients[114]. Però l'ús d'aquests biomarcadors en combinació amb les actuals teràpies encara es troba en fases molt inicials i experimentals.

En el nostre estudi, l'objectiu és analitzar biomarcadors que es trobin modulats per les teràpies de rehabilitació i que ens permetin avaluar l'estat del pacient. Els nostres candidats són l'angiogenina i les EPCs ja que han estat associats directament amb els processos de remodelació vascular durant la fase de recuperació de l'ictus, i mai han estat estudiats en el context de la rehabilitació.

Tal i com s'ha comentat a l'apartat 1.5 de la introducció, l'angiogenina participa en processos de proliferació, migració i invasió necessaris per l'angiogènesi. A més, aquesta molècula es troba secretada per les cèl·lules endotelials, on el seu secretoma té importants funcions de reparació, incrementant processos d'angiogènesi i oligodendrogènesi[122,255]. També se li atribueixen funcions de resposta a estímuls adversos com ara situacions d'estrès cel·lular o d'hipòxia, on actua protegint les motoneurones[194,256]. En altres patologies com la síndrome coronària aguda, aturada cardíaca o càncer els nivells d'angiogenina es troben elevats en sang[194,257,258]. En estudis amb d'altres models de dany cerebral o dany en la medul·la espinal, s'ha observat que augmentant els nivells d'angiogenina ja sigui per teràpia cel·lular o amb vectors virals, promou que els animals presentin una millor recuperació[259,260]. Es per això que l'estudi d'aquesta molècula durant les fases de recuperació després de la isquèmia es fa encara més necessària i interessant.

En primer lloc, es va analitzar l'expressió d'angiogenina al cervell en la fase aguda de l'ictus, a partir de teixits post-mortem de pacients, identificant la presència d'aquesta proteïna en teixit infartat localitzada en cèl·lules neuronals i en microvasos. A continuació, es va analitzar el perfil de l'angiogenina en sang en pacients isquèmics abans i durant la TRI, on els nivells d'angiogenina pre-teràpia no eren diferents respecte els controls. En la mateixa línia, Huang *et al.* van descriure en pacients, un increment agut dels nivells d'angiogenina seguits d'una disminució als 14 dies[199]. Els mateixos investigadors van validar els seus resultats clínics en un model d'isquèmia en rata, on van descriure l'expressió de l'angiogenina en el nucli de les neurones[200]. De manera diferent a les investigacions de Huang et al, els pacients del nostre estudi no presentaven una associació entre els nivells d'angiogenina i el volum d'infart. Aquestes diferències poden ser degudes al fet que el temps pre-teràpia en la nostra cohort de pacients sigui referent a la fase sub-aguda/crònica de la malaltia, un cop ja ha passat la fase hiperaguda i per tant els nivells d'angiogenina anirien disminuint en el temps.

Un cop iniciada la teràpia es va observar un increment d'angiogenina al mes, moment en que de manera destacada aquests nivells es trobaven associats a un millor estatus neurològic i a un pronòstic més favorable dels pacients en puntuacions de les escales funcionals i motores Barthel, MRC i Rankin. Aquests resultats evidencien el potencial de l'angiogenina com a possible biomarcador que avaluaria la resposta dels pacients a la teràpia. Paral·lelament i per complementar el nostre estudi clínic es van analitzar els nivells d'aquesta molècula en un model d'isquèmia cerebral de ratolí sotmès a rehabilitació de tasca específica o exercici físic durant 2 (curt termini) o 12 dies (llarg termini).

Com s'ha discutit en el primer apartat, existeixen controvèrsies entre els estudis a l'hora d'establir quan començar a rehabilitar els animals i durant quants dies, per tal de que els resultats siguin el més

translacional possibles. Hi ha estudis que demostren que iniciar un exercici entre les primeres hores fins als 7 dies de la isquèmia és capaç de reduir el volum d'infart i promoure una millora dels dèficits neurològics[174,179,261]. Per això, els animals del nostre estudi van començar la teràpia 48 hores després de la lesió.

Per tal de realitzar l'exercici per tasca específica, els animals havien de ser entrenats *a priori*. Els resultats obtinguts durant l'entrenament van en la mateixa línia que els descrits per Kerr *et al.*, on els animals utilitzats van aprendre correctament la tasca específica (*pasta matrix*) en el temps esperat. El protocol per aquest tipus d'exercici requeria una petita restricció de la ingesta, per això es van controlar els nivells de glicèmia en sang. Els animals presentaven nivells baixos de glucosa tal i com esperàvem, però no es trobaven associats al volum d'infart. Aquest últim, tampoc era diferent entre els grups després de dos dies de rehabilitació, garantint unes condicions similars entre grups de tractament en termes de severitat de la isquèmia. Tanmateix, hi ha estudis que reporten que no són necessàries modificacions a nivell d'extensió de l'infart per tal de veure una recuperació o alteracions estructurals al cervell dels animals[176,179].

Posteriorment, es van analitzar els nivells d'angiogenina al cervell 24 hores post-pMCAo i els nivells de proteïna es trobaven elevats al nucli de l'infart respecte l'hemisferi no infartat. A l'inici de la rehabilitació (després de 2 dies, corresponents a 4 dies post-pMCAo), els nivells d'RNA de l'angiogenina es trobaven elevats en l'hemisferi ipsilateral vs. el contralateral de tots els grups, segurament com a resposta aguda de la malaltia. Però després de 12 dies de tractament, cal destacar que l'expressió d'angiogenina només es mantenia elevada en l'hemisferi ipsilateral del grup de tasca específica respecte el no-rehabilitat i els contralaterals d'ambdós grups rehabilitats també es trobaven incrementats respecte al inici de la rehabilitació. Recentment, Zhang et al.[172] han publicat un estudi on comparen la capacitat d'incrementar la plasticitat cerebral d'un exercici per tasca específica vs. un exercici físic (rodes d'exercici) després d'una isquèmia. En aquest estudi, la teràpia rehabilitadora mitjançant una tasca específica promovia una major estimulació de la plasticitat axonal i un increment en la millora de la funció motora en comparació amb l'exercici físic amb rodes[172]. Aquest resultats conjuntament amb el nostre estudi fan pensar que cada tipus d'exercici presenta diferents mecanismes i rutes d'activació. Els nivells elevats d'angiogenina en el grup tasca específica del nostre estudi poden estar incrementats pel fet que aquest grup podria presentar una estimulació de la plasticitat cerebral i d'aquesta manera es promouria l'activació de diferents factors i vies que donarien suport a diferents processos de neuroreparació, entre ells l'augment de molècules assocades amb l'angiogènesi. L'increment d'angiogenina en el grup *pasta matrix* va ser validat per immunohistoquímica, on s'observava un augment de l'expressió d'angiogenina en l'hemisferi infartat *vs.* no infartat. Aquest experiment, també va revelar la localització de l'angiogenina al

citoplasma de les neurones, en canvi, en la publicació de Huang *et al.*, la localitzaven al nucli. El paper d'aquesta molècula al citoplasma es desconegut, alguns autors li atribueixen aquest rol durant els processos de migració cel·lular[189]. En les nostres preparacions, l'angiogenina també es trobava expressada en altres cèl·lules que no presentaven un fenotip neuronal, per tant, futurs experiments són necessaris per tal d'identificar-les.

Tal i com s'ha fet en l'estudi clínic, també es van analitzar els nivells d'angiogenina en sang. Tot i no poder demostrar l'increment significatiu d'angiogenina en les primeres hores després de la isquèmia cerebral, després 12 dies de tractament, el grup no rehabilitat presentava nivells significativament més baixos respecte a l'inici de la rehabilitació, mentre que els altres dos grups mantenien els nivells elevats. Segurament es necessiten cohorts més grans per validar aquests resultats.

Per tal d'avaluar la funció motora dels animals, es va utilitzar un test motor (de l'anglès, grip strenght test) que mesura la força d'ambdues extremitats superiors. Els animals presentaven una disminució significativa de la força 24 hores post-pMCAo, però no es van poder observar diferències en la recuperació de la força entre els grups de tractament després de la rehabilitació. Aquest resultat es pot atribuir a que els animals mostraven una recuperació espontània molt ràpida tal i com ha estat descrit per alguns autors[262] i també a que aquest test només permet mesurar la força motora conjunta de les dues extremitats, podent així emmascarar els efectes dels tractaments en cadascuna de les extremitat superiors.

Finalment, es van estudiar els nivells d'EPCs com a possibles marcadors cel·lulars de l'estat del pacients durant la rehabilitació. Tal i com s'ha comentat en l'apartat 1.6 de la introducció, aquestes cèl·lules participen en la formació de vasos sanguinis nous durant l'etapa adulta i poden ser alliberades després d'un estímul com la isquèmia i dirigir-se a les zones on es necessiti reparar el dany. També han estat estudiades com a possibles biomarcadors relacionats amb la severitat, volum d'infart i pronòstic de l'ictus[219,220,237] però no han estat estudiades en el context de les teràpies de rehabilitació post-ictus.

En l'estudi clínic, els pacients mostraven un increment de les EPCs en comparació amb els controls sans, tal i com ja estava descrit per alguns autors, però a més s'ha observat que es aquesta població cel·lular es mantenia elevada durant la rehabilitació als 3 i 6 mesos. Paral·lelament, en els animals, la tècnica utilitzada per comptar EPCs va ser a partir de cultius primaris de melsa, ja que el poc volum sanguini dels animals dificultava la mesura per altres tècniques, com la citometria[263]. A l'inici de la rehabilitació, es va veure un augment de les EPCs en el grup d'exercici per tasca específica amb una posterior davallada als 12 dies. Malauradament, no es van observar diferències en el número d'EPCs després de l'exercici físic, on sí que esperàvem veure un augment. Altres estudis en models animals d'exercici voluntari en rosegadors i en humans, han descrit un increment en el número d'aquestes

cèl·lules després d'un exercici físic[55]. En el nostre estudi s'ha utilitzat un model animal d'exercici físic forçat, això podria arribar a explicar les diferències en els nivells d'EPCs amb els altres autors que fan servir un exercici voluntari o al fet d'utilitzar altres tècniques per mesurar les EPCs. A més, els animals de l'estudi es trobaven restringits parcialment de menjar, fet que incideix en el pes d'alguns òrgans com la melsa, i podria afectar al nostre comptatge d'EPCs[264].

En resum, els resultats presentats per aquest estudi demostren que la rehabilitació és capaç de modular els processos d'angiogènesi i reflectir-ho mitjançant marcadors moleculars i cel·lulars en sang. Per això, l'angiogenina i les EPCs podrien ser futurs biomarcadors per monitoritzar l'estat dels pacients durant les teràpies de rehabilitació i, potser formar part de teràpies futures en aquells pacients que no responguin adequadament al tractament i tinguin uns nivells baixos d'aquests marcadors.

4.3 L'exercici físic, en un model animal d'isquèmia cerebral, incrementa el pool de cèl·lules precursores neurals en nínxols neurogènics com la SVZ, on també s'expressa angiogenina. En la mateixa línia, *in vitro*, l'angiogenina estimula la proliferació de precursors neurals i incrementa l'expressió de nestina en NSCs diferenciades a OPCs.

Per tal d'estudiar els processos modulats durant la rehabilitació, es van utilitzar dos tipus de models animals: tasca específica i exercici físic. Els resultats d'aquesta tesi mostren que després de 12 dies d'exercici físic hi ha un augment de l'expressió de cèl·lules doublecortin positives en la SVZ de l'hemisferi infartat, identificades com a neuroblast. A més, s'ha descrit per primera vegada la localització de l'angiogenina al nínxol neurotròfic de la SVZ, on hi ha una gran abundància de cèl·lules mare neurals. En paral·lel, i mitjançant experiments *in vitro*, l'angiogenina augmenta la proliferació de les NSCs i de les SH-SY5Y, però no la seva diferenciació. Finalment, cultius d'OPCs prèviament diferenciats a partir de NSCs i tractats amb angiogenina expressaven més nestina però no augmentaven els pools d'OPCs diferenciats.

La neurogènesi és un procés que també té lloc durant l'etapa adulta i que pot ser impulsat per diferents estímuls, entre ells la isquèmia. És en aquest moment quan les zones neurogèniques es troben molt actives i comencen a incrementar la seva taxa de proliferació de cèl·lules neurals progenitores, d'on esdevindran diferents llinatges cel·lulars. Aquesta resposta es produeix per substituir les neurones i cèl·lules de la glia de la zona afectada[54,265–267] com també els pools de progenitors d'oligodendròcits[83]. Tanmateix, moltes d'aquestes noves neurones generades moriran, per això les estratègies de neuroreparació tenen com a objectiu mantenir el procés de la neurogènesi actiu bé sigui evitant la mort d'aquestes noves generacions de cèl·lules o bé mantenint la seva proliferació activa fins que es resolgui el dany i es recuperin les funcions afectades[56,119]. En aquest sentit sembla que l'angiogenina pugui ser una molècula clau en el manteniment dels pools de progenitors neurals.

Diferents laboratoris han descrit que l'exercici físic *per se* té la capacitat de modular les respostes de reparació endògenes, com la neurogènesi. Aquest tipus d'exercici, és una de les estratègies més comunes per estudiar els mecanismes de recuperació quan es produeix un dany en el sistema nerviós central o perifèric. A més, l'exercici actua promovent la recuperació funcional i augmenta el número de neurones a les zones de l'hipocamp[268,269]. La majoria dels estudis que es coneixen en aquest camp han estat realitzats en un model d'isquèmia en rata mitjançant un filament intraluminal. En canvi, en el nostre estudi es va utilitzar un model distal d'oclusió permanent de l'arteria cerebral mitja, ja que aquest model presenta una baixa mortalitat, i així és possible estudiar els mecanismes de reparació després de dies o setmanes de la rehabilitació.

Els efectes de l'exercici físic poden estar condicionats per factors implícits, com per exemple la intensitat de l'activitat. Alguns autors han descrit que un exercici d'alta intensitat impedeix l'augment de factors neurotròfics si es compara amb un de baixa intensitat[253]. En aquesta tesi es va tenir en compte aquest factor i es va portar a terme un exercici d'intensitat baixa-moderada. També s'ha de tenir en compte el tipus d'exercici; si és voluntari o forçat. En aquest àmbit hi ha molts estudis amb resultats diferents. Efectes neuroprotectors, com la disminució del volum d'infart o activació de vies associades als processos de neurogènesi, han estat descrits en animals que portaven a terme un exercici de manera forçada[174,270,271]. D'altres, demostren que l'exercici voluntari incrementa encara més els nivells de factors neurotròfics com el BDNF en comparació amb el grup d'exercici forçat, i també observen una millora motora sense canvis en el volum d'infart[272]. Alguns investigadors atribueixen aquestes diferències al fet que l'exercici de manera forçada pot incrementar l'estat d'estrès de l'animal i això impediria que algunes vies es poguessin activar. Hayes et al. van comparar ambdós tipus d'exercici i van veure que els animals sota un exercici forçat presentaven nivells més elevats de cortisona en sang[270]. Ara bé, l'avantatge de l'exercici forçat és que els paràmetres de velocitat i distancia recorreguda per cada animal es troben estrictament controlats. Per tal d'evitar que els animals poguessin estar sotmesos a els efectes de l'estrès, a més d'habituar-los i entrenar-los per la tasca específica, es va realitzar una habituació a la cinta de córrer (o *treadmill*). En aquesta habituació l'animal romania durant 3 sessions en dies consecutius (10min/sessió) en la cinta de córrer sense que estigués encesa, així s'evitaven futures respostes d'estrès i por.

Pel que fa al model de tasca específica, només hi ha un estudi en rata que descrigui un augment en la neurogènesi després de 14 dies de teràpia utilitzant el mètode de *constraint-induced movement* com a model de rehabilitació, ja que la majoria és centren en canvis a nivell de plasticitat neuronal i recuperació de la funció motora[169]. D'acord amb les publicacions discutides anteriorment, es va iniciar la rehabilitació a les 48 hores post-isquèmia. El grup *treadmill* presentava un augment significatiu de l'expressió de marcadors de neuroblasts, en la SVZ de l'hemisferi infartat, en comparació amb el seu propi contralateral, el grup no rehabilitat i el grup de tasca específica. A més, també es van quantificar les cèl·lules de nova generació mitjançant el doble marcatge per neuroblasts i BrdU. Malgrat que els resultats no van ser significatius, es va observar un increment de cèl·lules doble positives a l'hemisferi ipsilateral del grup *treadmill*.

Tal i com s'ha descrit anteriorment, l'exercici físic té la capacitat d'incrementar la proliferació de les cèl·lules en les zones neurogèniques, però els factors implicats en aquests procés encara són desconeguts. Es postula, que els senyals que promouen aquesta activació provenen de cèl·lules del seu propi nínxol, ja que les NSCs es troben envoltades d'elements vasculars, glials i ependimals[54]. Es

coneix que les cèl·lules endotelials són capaces d'influir en els nínxols neurogènics, ja que el fet de modular l'angiogènesi afecta a la neurogènesi[76]. Leventhal et al. van descriure que en co-cultius de NSCs de la SVZ amb cèl·lules endotelials, les NSCs diferenciaven més cap a fenotip neuronal[273]. En aquesta mateixa línia, s'ha vist que co-cultius de NSCs amb cèl·lules endotelials o el seu secretoma de rates controls, mantenien les NSCs augmentant la seva proliferació. Tanmateix, cèl·lules endotelials de rates isquèmiques o el secretoma de cèl·lules endotelials exposades a hipòxia augmenta la migració i diferenciació de les NSCs[74,274]. Tots aquests estudis fan pensar que les cèl·lules endotelials, o el seu secretoma, són capaces, d'alguna manera, de regular l'activitat de les NSCs. Un estudi publicat recentment pel nostre laboratori, ha reportat que el secretoma de les EPCs era ric en proteïnes associades als processos d'angiogènesi, i entre elles es trobava l'angiogenina[255].

L'angiogenina s'ha vist expressada durant el desenvolupament embrionari i durant la diferenciació del neuroectoderm en una línia embrionària de carcinoma. La inhibició d'aquesta molècula afecta a la ruta/direcció que han de seguir les cèl·lules embrionàries però no a la seva diferenciació[275]. També, en aquestes mateixes cèl·lules, l'angiogenina incrementa el diàmetre de les neuroesferes[276]. Un estudi de l'any 2017 també reportava el paper de l'angiogenina actuant amb altres molècules evitant la diferenciació d'una línia de cèl·lules mare neuroepitelials[277].

En relació a aquestes investigacions, una de les qüestions que es va voler adreçar en aquesta tesi va ser identificar si l'angiogenina es trobava expressada en les zones subventriculars implicades en la migració de neuroblast i diferenciació de les NSCs en el context de la isquèmia, i si aquesta era capaç de modular el creixement i diferenciació de les NSCs provinents d'aquestes zones.

En primer lloc, es va identificar l'expressió d'angiogenina a les zones subventriculars mitjançant dues tècniques diferents, western blot i immunohistoquímica. Per aquesta última, es va identificar la localització de l'angiogenina per primera vegada a les SVZ a prop de cèl·lules nestina positives (NSCs) i en el citoplasma de cèl·lules progenitores neurals com són els neuroblasts. A continuació, cèl·lules provinents de la SVZ es van cultivar amb presència d'angiogenina exògena i es va observar que aquesta augmentava la seva proliferació i el diàmetre de les neuroesferes.

Per tal de concloure que l'angiogenina era capaç de produir un efecte en les cèl·lules precursores de neuroblasts, es va fer servir una altra línia cel·lular amb característiques similars. La SH-SY5Y és una línia de neuroblastoma que expressa marcadors de cèl·lula mare, com la nestina, quan no es troba diferenciada[278]. Aquesta mateixa línia ha estat molt utilitzada en estudis de diferenciació neuronal, ja que en presència d'àcid retinoic comença a perdre els marcadors de cèl·lula mare i

genera prolongacions o neurites, pròpies d'un fenotip neuronal[279]. Finalment, l'angiogenina també augmentava la proliferació de les SH-SY5Y però no es van observar canvis a nivell de diferenciació. Aquests resultats confirmarien el paper d'aquesta molècula promovent la proliferació de precursors neuronals, però no la seva diferenciació que si que es produïa en presència d'àcid retinoic.

En paral·lel hem estudiat els processos d'oligodendrogènesi *in vitro* i de remielinització en els models animals de rehabilitació. Després d'un dany cerebral, els oligodendròcits poden veure's afectats, produint-se una pèrdua de mielina[83]. Mitjançant diferents estratègies de neuroreparació s'ha observat que es possible incrementar la generació de nous oligodendròcits. Maki *et al.* demostren que el secretoma de les EPCs incrementa l'expressió de marcadors d'oligodendròcits madurs en un model animal isquèmic d'hipoperfusió en ratolí[255]. Un altre estudi reporta que l'exercici físic augmenta el número d'oligodendròctis al sistema nerviós perifèric després d'un dany a la medul·la espinal[280].

Per a poder respondre si els models de rehabilitació exercien un efecte en el procés d'oligodendrogènesi, es va mesurar la longitud del cos callós a la zona lateral i medial juntament amb l'expressió mielina en els dos hemisferis (ipsilateral i contralateral). No obstant, no es van observar diferències estructurals en els feixos de mielina del cos callós ni en l'expressió de mielina (marcador d'oligodendròcits madurs) entre els grups de rehabilitació. Tot i això nostre model d'isquèmia cortical afecta de forma relativa al cos callós mentre que en els estudis on s'ha reportat un augment d'oligodendròcits es feien servir models d'isquèmia que afectaven a l'estriat i a la substància blanca[281,282].

En paral·lel a aquesta part *in vivo*, es van diferenciar NSCs a cèl·lules precursores d'oligodendròcits en presència de l'angiogenina. Els resultats van mostrar que les OPCs tractades durant la diferenciació, presentaven més expressió de nestina, però no s'observaven diferències en l'expressió del PDGFRα (marcadors d'OPCs) entre el grup angiogenina i el control. Aquest resultat, segueix en la mateixa línia que els anteriors, on sembla que l'angiogenina tindria un rol en el manteniment de les NSCs i no en la seva diferenciació.

4.4 L'exercici per tasca específica i l'exercici físic augmenten la formació de vasos al còrtex contralateral a l'infart i l'angiogenina activa l'angio-vasculogènesi de les EPCs.

L'increment de l'angiogènesi després d'una isquèmia ha estat descrit per diferents investigadors tant en models experimentals com en alguns estudis en pacients. L'estímul isquèmic activa la secreció de diferents factors, entre ells angiogènics i estimula la migració de cèl·lules endotelials que es desplaçaran fins a les zones d'alta demanda, en conjunt tota aquesta resposta orquestrarà la formació de vasos nous[283,284]

La remodelació vascular ha estat molt investigada en les fases posteriors de la isquèmia, però existeixen controvèrsies entre les investigacions. Diferents autors descriuen un increment de l'expressió de gens relacionats amb l'angiogènesi a les 24 h i un augment de nous microvasos a la zona del peri-infart[59,60]. Aquests vasos, es mantindran a la zona afectada durant 14-21 dies fins que es produeixi una disminució dels mateixos[59,62]. En referència a aquesta disminució dels vasos, Manoonkitiwongsa *et al.* proposen la hipòtesi del "clean-up", on postulen que aquest augment i posterior desaparició de l'angiogènesi es produeix per facilitar l'arribada de macròfags per tal de fagocitar el teixit necròtic[285]. Un cop els macròfags ja han exercit la seva funció, l'angiogènesi ja no és necessària. Altres estudis descriuen un augment de vasos a la zona del còrtex contralateral, Morancho et al.[286] observen una disminució dels microvasos a la zona de l'infart 24 h després de la reperfusió i altres investigadors observen un augment en l'expressió de gens i de l'angiogènesi en la zona contralateral conjuntament amb el peri-infart, suggerint que l'hemisferi contralateral té un paper actiu en la remodelació vascular[287,288].

De manera destacada, l'augment de l'angiogènesi ha estat associada amb una millora funcional tant en la clínica com en models animals[54,63]. Ara bé, com podem mantenir els vasos que s'han originat o fins quan hem de mantenir l'angiogènesi activa, per tal de reparar el teixit danyat per la isquèmia, és una pregunta que encara roman sense resposta.

Sabem que els mecanismes endògens de reparació poden ser modificats mitjançant factors tròfics, teràpia cel·lular, diferents fàrmacs o l'exercici físic[56,119,289] . Tal i com s'ha comentat en anteriors apartats, hi ha diferents estudis evidenciant els efectes beneficiosos de l'exercici físic. A partir d'aquests, sabem que l'exercici després d'una isquèmia experimental promou un augment del flux sanguini cerebral i del número de vasos als 14 dies, amb una disminució d'aquests en els dies posteriors[171,289]. En aquesta tesi s'ha avaluat l'angiogènesi després de 12 dies de rehabilitació (18 dies post-isquèmia), en un model distal d'isquèmia cerebral permanent. Els resultats mostren un augment de vasos a l'hemisferi contralateral en comparació amb l'ipsilateral en ambdós grups de

rehabilitació. Amb aquests resultats, la controvèrsia continua ja que les nostres investigacions no mostren un increment de vasos al peri-infart tal i com alguns autors descriuen. Aquest fet pot ser degut a que ens trobem en el moment de la davallada dels vasos (dia 18 post-isquèmia). L'augment en la densitat de vasos a l'hemisferi contralateral en els dos grups rehabilitats, fa pensar en la possibilitat de que aquest estigui participant activament en els processos de remodelació vascular després d'un dany cerebral.

En aquesta tesi el paper de l'angiogenina ha estat àmpliament estudiat, com a possible marcador associat a les teràpies de rehabilitació o bé exercint un efecte en l'augment de proliferació sobre les cèl·lules precursores de neuroblasts. A més de totes aquestes investigacions, vam voler provar la seva funció angiogènica *in vitro* en EPCs de ratolí. Els cultius tractats amb aquesta molècula no van mostrar cap efecte a nivell de proliferació cel·lular, en canvi si que es va observar un augment en la formació de vasos i un increment de la velocitat de migració. La participació de l'angiogenina en la proliferació cel·lular ha estat descrita en diferents línies cel·lulars, tal i com es comenta a l'apart 1.6 de la introducció. A més, en un article publicat pel nostre laboratori, es va reportar que l'angiogenina promovia la proliferació en una línia endotelial cerebral d'origen humà[255]. En cultiu, l'angiogenina també produeix canvis augmentat la migració en la línia cel·lular HeLa i promou la formació de tubs en diferents línies endotelials de retina o de cordó umbilical[189,195,290], tal i com s'observa en els nostres experiments.

Finalment, són necessàries noves investigacions per tal d'esclarir com interactua l'angiogenina en cada tipus cel·lular, ja que des del descobriment d'un receptor putatiu en cèl·lules endotelials humanes a l'any 1997 per Hu i col·laboradors[184], només se n'ha descrit un altre en astròcits, el sindecan 4[291].

4.5 Perspectives de futur.

Els resultats d'aquesta tesi proposen nous biomarcadors, l'angiogenina i les EPCs, capaços de mesurar l'estat funcional de pacients isquèmics en una rehabilitació de caràcter intensiu. Aquestes investigacions han de validar-se en una cohort més gran de pacients i observar els efectes específics de les teràpies de rehabilitació intensiva (versus no intensives). Per això, actualment des del grup de recerca i en col·laboració amb el la Unitat clínica de neurorehabilitació de l'HUVH, s'ha iniciat un estudi multicèntric on participen diferents centres hospitalaris de l'Estat Espanyol, els centres es troben distribuïts en rehabilitació intensiva o rehabilitació moderada. D'aquesta manera, amb l'ajuda de biomarcadors en sang, es podran observar les diferències, si n'hi ha, entre els dos tipus de teràpia i així poder arribar a adaptar les necessitats específiques per a cada pacient.

Els resultats de la nostra cohort de pacients han estat traslladats en un model animal de rehabilitació per tasca específica. A més, l'exercici físic també ha demostrat tenir un paper rellevant incrementant la neurogènesi en la SVZ. Per això, es proposa la combinació d'ambdós exercicis com a futura estratègia, i així estudiar l'efecte sinèrgic dels dos tipus de teràpia.

A més, models animals *knockout* deficients per l'angiogenina són necessaris per investigar en detall el paper d'aquesta molècula en la isquèmia i la seva implicació en els processos de neuroreparació, més concretament en els nínxols neurogènics.

Els dos models de rehabilitació utilitzats han demostrat exercir un increment de densitat de vasos a la zona contralateral del còrtex i les diferents controvèrsies sobre l'angiogènesi i la isquèmia han estat discutides. Es necessari planificar nous experiments tenint en compte el tipus d'exercici (forçat/voluntari), el temps de repòs fins que es comença la rehabilitació i durant quants dies es rehabiliten als animals per tal de descriure quan i on es produeix l'angiogènesi i com la podem potenciar.

Cada dia s'avança més en els coneixements i tractaments durant les fases de recuperació de l'ictus, demostrant que encara hi ha oportunitats terapèutiques per aquells pacients que han quedat greument discapacitats. Recentment un estudi publicat a la revista *Science*, a l'abril de 2018, reporta que un component anomenat *edonerpic maleate* augmenta la recuperació de la funció motora en ratolins i primats no-humans durant una rehabilitació per tasca específica i també actua promovent mecanismes de plasticitat neuronal[292]. A més, cal destacar que aquest component ja ha passat la fase I en el que es va provar la seguretat. Així que tot indica que en uns anys es podran veure els efectes d'aquest compost en un assaig clínic.

Per últim, per poder traslladar satisfactòriament els resultats del nostre model animal a la pràctica clínica, són necessàries més investigacions tenint en compte l'ús d'altres models d'isquèmia cerebral, animals d'edat més avançada, l'ús de femelles i animals que presentin comorbiditats.

5.CONCLUSIONS

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Les conclusions d'aquesta tesi són:

1. Els models de rehabilitació de tasca-específica i l'exercici físic reprodueixen alguns dels mecanismes biològics modulats durant les teràpies de rehabilitació.
2. L'Angiogenina i les EPCs podrien ser nous biomarcadors per avaluar l'estat i el pronòstic funcional del pacients durant les teràpies de rehabilitació.
3. Els resultats obtinguts en la nostra cohort de pacients han estat traslladats en bona part a un model preclínic d'exercici per tasca-específica.
4. L'exercici físic i l'exercici de tasca específica actuarien a nivell cerebral activant mecanismes de reparació endògens diferents.
5. *In vitro,* l'angiogenina té la capacitat d'exercir un efecte en diferents línies cel·lulars associades amb els processos de neuroreparació.

6. BIBLIOGRAFIA

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7.ANNEXOS

ANNEX 1

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Matrix metalloproteinase-13 participates in neuroprotection and neurorepair after cerebral ischemia in mice

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ABSTRACT

New neuroreparative and neuroprotective therapies are being sought to treat stroke patients. One approach is the remodeling of extracellular matrix, which participates in both brain injury and neurovascular repair when matrix metalloproteinases (MMPs) are thought to be key players. Our aim was to investigate the role of MMP-13 (collagenase-3) in the acute (24 h and 3 days) and delayed (2 weeks) phases of stroke. Permanent and transient cerebral ischemia models involving the cortex were induced in MMP-13 knock-out (KO) and wild-type (WT) mice. In the transient model, MMP-13 deficiency reduced the amount of TTC-stained infarct tissue, reduced hemorrhagic events and improved functional outcomes ($p < 0.01$). At two weeks, normal neuroblast (DCX+) migration from the subventricular zone toward the peri-infarct area was observed. However, MMP-13 deficiency significantly reduced the number of newborn neuroblasts (DCX+/BrdU+) in the cortical peri-infarct area ($p < 0.01$). This result occurred in parallel with aberrant cortical vascular remodeling: post-stroke peri-infarct vessel density increased in the WT mice ($p < 0.01$) but this increase was blocked in the MMP-13 KO mice. Prior to these vascular alterations, the levels of pro-angiogenic factors, including G-CSF, VEGF-A and angiopoietin-2, were lower in the ischemic cortex of MMP-13 KO mice than in WT mice ($p < 0.05$). *In vitro*, gene-silencing of MMP-13 in endothelial progenitor cells (EPCs) confirmed the reduced ability of these cells to build tubulogenic networks in Matrigel™ substrate. Together, our results indicate that MMP-13 is a central protease in infarct development and cortical remodeling during post-stroke neurorepair, which is critical for optimal angiogenic and neurogenic responses.

1. Introduction

Stroke is one of the most common causes of death and disability worldwide. Our investigation focused on ischemic stroke, which occurs when blood flow into the brain is interrupted by an artery occlusion. Recombinant tissue plasminogen activator (rtPA) and mechanical thrombectomy interventions are the only therapies that have shown efficacy, but they must be administered within a short therapeutic time window (Hacke et al., 2008; Lees et al., 2010, Jovin et al., 2015). New post-stroke therapies are therefore needed to diminish damaging molecular events and cellular death, which are responsible for further damage, at the same time that new opportunities to recover tissue are investigated. Of special interest are treatments that induce neurological repair with the aim of improving functional outcomes and rescuing the limited capability the damaged brain has to self-recover, with the potential to be administered in a wider therapeutic time window (Zhang and Chopp, 2009).

One approach to potentiating neurological repair is to target angioneurogenesis, which is the formation of new vessels and neural cells. Adult neurogenesis occurs primarily in the subventricular zone (SVZ) and the subgranular zone (SGZ), from which newly born neural cells migrate to the olfactory bulb or to the dentate gyrus under physiological conditions (Ming and Song, 2011). However, ischemic stroke alters this pattern of neurogenesis and stimulates neuronal cells to migrate toward the damaged area (Ohab and Carmichael, 2008). In this environment, the neurovascular niche has been described as linking angiogenesis and neurogenesis, demonstrating that, after stroke, neurogenesis occurs following angiogenesis in the peri-infarct areas and showing strong support for the idea that trophic factors are secreted by endothelial cells (Ohab et al., 2006) and glial cells (Hayakawa et al., 2013).

In this context, extracellular matrix remodeling is a key step in angiogenesis and neurogenesis during which matrix degradation is required to allow cell migration and capillary reorganization (Bovetti et al., 2007; Senger and Davis, 2011). Matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes, are involved in the

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0969-9961/

regulation of the composition of the cell-matrix. Interestingly, some MMPs have been described not only as the focus of many neuroprotective therapies aimed during the acute phase of stroke, but also as playing a role in neurovascular remodeling during neurological recovery in rodent models of stroke (Zhao et al., 2006; Rosell and Lo, 2008), being MMP-9 the most studied MMP. Among all other MMPs, our group has studied the expression of MMP-13 (also named collagenase-3) that mainly cleaves collagen II and shows the highest gelatinase activity among the collagenases (Knäuper et al., 1996, 1997). We have reported that high blood levels of MMP-13 were associated with lesion expansion in the acute phase of stroke (Rosell et al., 2005), whereas nuclear MMP-13 was found to be expressed in neurons and glial supporting cells in human and rodent ischemic brain tissue (Cuadrado et al., 2009a). Other authors have observed an increased expression of MMP-13 in rat neurons, with a peak on day 7 (Nagel et al., 2005) after ischemia. It is known that MMP-13 and has a central position in the activation of other MMPs, through which it plays a key role in angiogenesis and the re-epithelialization of the skin during wound healing (Hattori et al., 2009), but its specific role in neurorepair and vascular remodeling is still unknown.

With this background, we aimed to determine whether MMP-13 could acutely modulate neuroprotective and neurological repair functions during the recovery phase of ischemia, and we focused our interest on post-stroke vascular remodeling. For this purpose, both permanent and transient ischemic stroke models were induced in MMP-13 deficient mice, demonstrating that MMP-13 is involved in acute infarct expansion at the same time that it is required for neurorepair during recovery. The role of MMP-13 in the angio-vasculogenic function was additionally tested in tubulogenesis assays by the transient silencing of MMP-13 expression in outgrowing populations of endothelial progenitor cells (EPCs). Overall our data suggest that MMP-13 is a key protease that can be therapeutically modulated during both the acute and the recovery phases of stroke.

2. Materials and methods

2.1. Animals

Age-matched 8- to 15-week-old male MMP-13 knockout mice (MMP-13 KO in a C57BL/6 background) and wild-type (WT, C57BL/6) were bred in-house in a temperature and humidity controlled room and maintained on a 12 hour light/dark cycle. The offspring were used for these experiments. The founder mice for our WT and KO colonies were the kind gifts of Dr. Lopez-Otin and were generated as previously described (Inada et al., 2004) and crossed into congenic C57BL/6 mice for several generations. Genotyping analyses were performed to confirm that the mice were MMP-13 null, as described below. A total of 99 animals (49 WT and 50 MMP-13 KO) were used in the present study, and no mortality was observed during the study period. All procedures were approved by the Ethics Committee of Animal Experimentation (CCEA 67/11) of the Vall d'Hebron Research Institute and were conducted in compliance with Spanish legislation and in accordance with the Directives of the European Union.

2.2. Genotyping analysis

Freshly obtained mouse tail tissue was used for DNA purification to confirm the genotypes of randomly selected mice in our colonies. Genomic DNA was collected and purified using a Gentra Puregene Mouse Tail Kit (Qiagen, Germany) according to a protocol provided by the manufacturer. Polymerase chain reaction was performed using Platinum® Taq DNA polymerase (Life Technologies, USA). The following pair of exon primers were used to genotype the wild-type mice: (Exon 5F) 5′TTTATTGTTGCTGCCCATGAG3′ and (Exon 6R) 5′AGTTTCTCCTCGGAGACTGGT3′. For the MMP-13 KO mice, a primer for the neomycin resistance gene, (neo) 5′GACCCA CCCCTTCCCAGCCTCT3′, was used as the oligonucleotide primer. The approximate fragment sizes of each band were 1300 Kb for the wild-type and 1485 Kb for the MMP-13 KO on agarose gels.

2.3. Focal cerebral ischemia models (MCAo)

The mice were given free access to food and water before surgery. Permanent electrocoagulation and transient compression of the distal part of the middle cerebral artery (MCA) were performed as previously described by our group (Morancho et al., 2012). Briefly, the animals were anesthetized using 5% isoflurane followed by 1.5–2% isoflurane for maintenance anesthesia *via* a facemask in medical air (79% N_2/21% O_2; Abbott Laboratories, Madrid, Spain). A small craniotomy was performed in the left temporal bone (between the retro-orbital and ear area) to expose the MCA. This artery was compressed for 60 min with a 30-G needle using a micromanipulator to perform transient MCA occlusion (tMCAo) or electrocauterized for permanent MCA occlusion (pMCAo). A heating pad connected to a rectal probe was used to maintain body temperature between 36.5–37.5 °C during all surgical procedures. Cerebral blood flow (CBF) was monitored using laser-Doppler flowmetry (Moor Instruments, UK) to ensure appropriate occlusion in both models and reperfusion in the tMCAo mice (Morancho et al., 2012). Only the mice that showed a reduction in CBF to below 80% and subsequent reperfusion to above 75% of the baseline CBF were included in this study. Buprenorphine (0.05–0.1 mg/kg), an analgesic, was administered subcutaneously immediately after the procedure. The animals in the sham group ($n = 6$ for each genotype) were subjected to the same procedures (including 60 min of anesthesia for the tMCAo mice), except for electrocauterization or compression of the MCA.

A total of 17 mice were subjected to pMCAo (9 WT and 9 MMP-13 KO), 70 to tMCAo (35 WT and 35 MMP-13 KO) and 12 to sham surgery (6 WT and 6 MMP-13 KO), according to the design of the study, as presented in Fig. 1.

2.4. Grip strength neurological test

The grip strength test is designed to assess the peak forelimb force executed by a mouse when the mouse releases its front paws from a grid. A computerized grip strength meter (Harvard Apparatus, USA) was used. The specific protocol is described elsewhere (Rosell et al., 2013b). A total of 6 trials were conducted for each test, and the mean value was calculated as the average force used at one time point. This test was conducted blindly by a trained researcher before the surgery (pre-surgery) and then repeated at 24 h, 3 days, 7 days and 14 days post-ischemia (according to the study groups, A and B, as detailed in Fig. 1).

2.5. Infarct volume and cortical atrophy

The mice that were subjected to 60 min of occlusion (tMCAo) and the mice in the pMCAo group that were included in the infarct volume protocol (study A in Fig. 1) were transcardially perfused with ice-cold saline and euthanized at 24 h or 3 days after ischemia. The dissected brains were cut into 1 mm-thick coronal sections and then stained with 2.5% 2,3,5-triphenyl-2H-tetrazolium chloride (TTC; Sigma-Aldrich, USA) for 15 min at room temperature and digitalized to obtain the measurements. The extension of the pale-colored ischemic lesion was calculated blindly, and the results are presented as percentage of the infarct volume (as the % of the ipsilateral hemisphere volume), as described previously (Morancho et al., 2012).

Cortical atrophy was evaluated using cresyl violet staining at 14 days after tMCAo (as shown in study B in Fig. 1). Briefly, the mice were deeply anesthetized and then euthanized after the brain was perfused

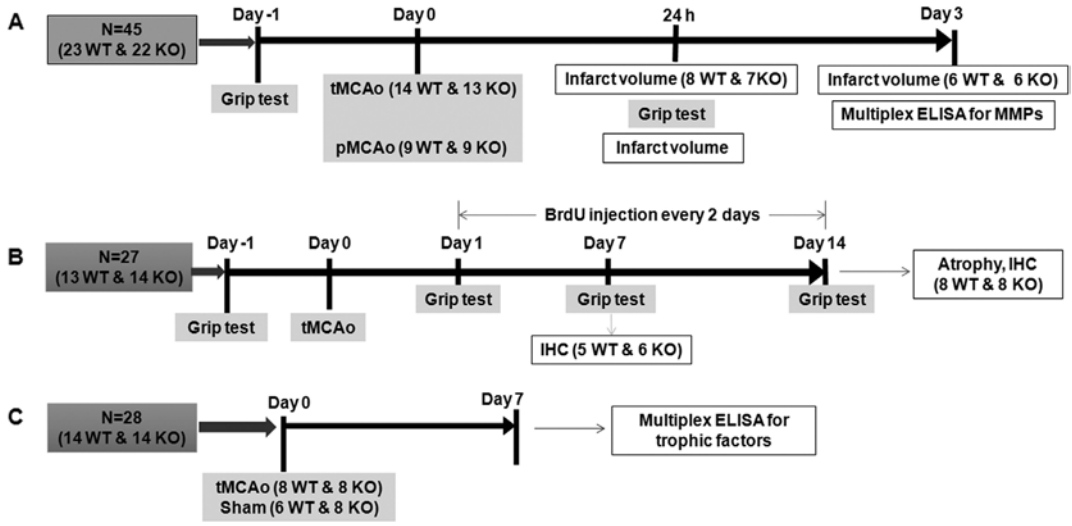

Fig. 1. The study design. Three experimental protocols (A, B and C) were followed to study the role of MMP-13 in neuroprotection and neurological repairs after cerebral ischemia.

with ice-cold 4% PFA. The brains were harvested and post-fixed in PFA overnight at 4 °C, followed by cryoprotection in 30% sucrose in PBS until the brains sank. Afterward, the brains were embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Fisher Scientific, USA) and stored at −80 °C until used. Twelve-μm-thick coronal sections including the lateral ventricles and the hippocampus were collected. Five sections from bregma positions matching with the TTC stained slices 2 to 6 were selected per animal from the WT and MMP-13 KO ischemic mice. The sections were stained with 0.1% freshly-filtered cresyl violet containing 0.2% acetate and 0.15% acetic acid for 15 min at 37 °C. The areas of ipsilateral cortex and of the whole ipsilateral hemisphere were measured blindly and the results are presented as cortex percentage vs. ipsilateral hemisphere.

2.6. Immunohistochemistry (IHC)

The ischemic animals in protocol B received 5-Bromo-2′-deoxyuridine (BrdU, 50 mg/kg in saline, B9285, Sigma-Aldrich, USA) intraperitoneally every 2 days after ischemia (Rabenstein et al., 2015). On day 7 or day 14, the animals were euthanized, and the brain sections were prepared for immunohistochemistry as described for the atrophy study. Later, the sections (bregma between 0.7–0.2) were fixed in ice-cold acetone and then blocked in 0.1% PBS-tween containing 1% BSA and 10% goat serum after acid denaturation with 2 M HCl PBS for 1 h. Slides were incubated with the following primary antibodies overnight at 4 °C (DCX) or for 3 h at room temperature (BrdU or NeuN): rabbit anti-doublecortin antibodies (DCX, 1:1000, ab18723, Abcam, UK), rat anti-BrdU antibodies (BrdU, 1:100, ab6326, Abcam, UK) and anti-NeuN antibodies conjugated to Alexa Fluor® 488 (NeuN, 1:200, MAB377X, Merck Millipore, Germany). Alexa Fluor 488 goat anti-rabbit IgG (H + L) and Alexa Fluor 647 goat anti-rat IgG (H + L) (1:500, A-1108 & A-21247, Invitrogen, USA) were the secondary antibodies, which were applied, where required, at room temperature for 1 h. The samples were then mounted in Vectashield™ with DAPI (H-1200, Vector Laboratories, USA) to stain the cell nuclei.

To determine the number of neuroblasts migrating from the SVZ to the peri-infarct tissue, the DCX+/DAPI+-labeled area in the SVZ and corpus callosum (CC) regions of one slide was quantified. In addition, triple-labeled cells (DCX+/BrdU+/DAPI+), which indicated the new proliferating neuroblasts, were also quantified in the SVZ and peri-infarct areas (three selected regions of interest (ROIs) in the infarct boundary area). The images were obtained at 400× magnification using a confocal laser scanning biological microscope (FV1000, Olympus, Japan) and analyzed using FV10-ASW software. Finally, the mature (NeuN+/DAPI+) neurons in the peri-infarct cortical areas adjacent to the damaged tissue were imaged at 100× magnification and counted using ImageJ software (NIH, USA). The results are presented as the neurons per area. All analyses were conducted by a researcher who was blinded to the genotype and group.

To study angiogenesis, the mice from study B (see Fig. 1) were injected intravenously (retro-orbitally) with Dylight 594-labeled tomato lectin (80 μg/mouse, DL-1177, Vector Laboratories, USA) 10 min before euthanized. Two similar isotopically oriented slides per animal were selected from the brain sections. One image from each section that contained the infarct boundary area was randomly imaged at 100× magnification using the 10× objective. Angiogenesis was evaluated as the lectin-positive blood vessel density in the selected ROIs and the corresponding contralateral areas. The total area of the lectin-positive perfused vessels was calculated using ImageJ software (NIH, USA) by an investigator blinded to the genotype group. Vessel co-localization with CD-31 endothelial marker was evaluated at 2 weeks in some sections, a rabbit anti-CD31 antibody (1:20, ab28364, Abcam, UK) was used overnight at 4 °C and the Alexa Fluor 488 goat anti-rabbit IgG (1:500) was used as secondary antibody at room temperature for 1 h.

2.7. Multiplex ELISA

Both the WT and the MMP-13 KO mice (see study protocol C in Fig. 1) were transcardially perfused with ice-cold saline and euthanized at 7 days after ischemia. The mouse brain tissue obtained from both the ipsilateral and contralateral cortex was homogenized 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, Switzerland) and 0.5% aprotinin (Sigma-Aldrich, USA). Cortical lysates were collected from the supernatant after centrifugation at 12,000 rpm for 12 min at 4 °C.

A MILLIPLEX® map mouse angiogenesis/growth factor magnetic bead panel (MAGPMAG, EMD Millipore, Germany) was used to detect angiogenesis-related proteins at day 7 after ischemia. Briefly, 25 μl of the diluted lysate (1/2 in assay buffer) was loaded into the sample wells to quantify the levels of angiopoietin-2, granulocyte-colony stimulating factor (G-CSF), amphiregulin, fibroblast growth factor (FGF)-2, vascular endothelial growth factor-A (VEGF-A) and stromal cell-derived factor-1α (SDF-1), according to the manufacturer's instructions. The assay sensitivity for the different proteins was as follows:

angiopoietin-2, 5.2 pg/ml; G-CSF, 0.8 pg/ml; amphiregulin, 2.2 pg/ml; FGF-2, 10.6 pg/ml; VEGF-A, 0.7 pg/ml and SDF-1, 24.6 pg/ml. Each sample was assayed in duplicate, and the coefficients of variation were <20%. The results are presented as the ratio of the amount of protein in the ipsilateral cortex to the amount of protein in the contralateral cortex.

To evaluate the potential MMP protein imbalance in a MMP-13 knockdown, a MILLIPLEX® map mouse MMP magnetic bead panel 3 (MMMP3 MAG, EMD Millipore, Germany) was run to detect the expression of MMP-3, MMP-8 and MMP-9 in brain homogenates obtained at 3 days after reperfusion (tissues were homogenized right after TTC staining), as described above. In brief, 25 μl of the brain homogenate (1/10 in lysis buffer) was loaded into the sample wells. The assay sensitivity of the different MMPs was MMP-3, 2.5 pg/ml; MMP-8, 3.4 pg/ml and MMP-9, 3.1 pg/ml. Each sample was assayed in duplicate, and the coefficients of variation were <20%.

2.8. EPC cultures and MMP-13 silencing

Outgrowth endothelial cells (OECs) with an endothelial-like phenotype and a highly proliferative nature were obtained from FVB wild-type male mice spleens for a previous study (Morancho et al., 2013) and stored in liquid nitrogen until use. These cells behave as competent endothelial cells both *in vitro* and *in vivo* (Fadini et al., 2012).

MMP-13 gene silencing was achieved using delivery of passive siRNA *via* Accell SMART pool siRNA (Thermo Scientific, USA) according to the manufacturer's protocol. Briefly, 2×10^4 EPCs were seeded in 48-well plates and treated separately after 24 h with the respective siRNA solutions corresponding to each siRNA pool (2 μmol/l). Non-targeting/non-coding siRNA was used as a negative control to measure the minimal toxicity and side effects of the assay. Cyclophilin B (PPIB) siRNA was used as a positive control to test the efficiency and specificity of MMP-13 silencing. After 48 h of treatment, the cells were thoroughly washed with PBS, trypsinized and collected in basal EBM media (Lonza, Spain) to perform Matrigel assays, which are described below. To confirm the mRNA expression in each condition, real-time PCR was conducted using the mRNA that was obtained from Matrigel-disaggregated cells, which were isolated using the Cell Recovery Solution (BD Biosciences, USA) for 60 min at 4 °C. Briefly, an RNeasy® Minikit (Qiagen, Germany) was used, according to the manufacturer's instructions, and the quantity and quality of the RNA was measured using a Nanodrop Spectrophotometer and retrotranscribed as cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The incubation conditions for the retrotranscription were: 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C. Finally, the samples were incubated at 4 °C until use or frozen at −80 °C (Thermal Cycler 2720, Applied Biosystems, USA). We included a negative control (RNase-free water) for the reverse transcription to cDNA to detect possible contamination. RT-PCR was performed by mixing 5 μl of Taq Man Universal 2× PCR Master Mix (Applied Biosystems, USA), 0.5 μl of the Taq Man® Gene Expression Assay solution (PPIB: Mm 04208118_gl, GAPDH: Mm99999915_g1, MMP-13: Mm00439491_m1; Applied Biosystems, USA), 3.5 μl of RNase-free water and 1 μl of the sample (cDNA or RNase-free water). These experiments were performed in triplicate. A sample calibrator was used to compare the samples from different reading plates. Hybridizations and expression analyses were performed using a 7900 thermocycler HT Fast Real-Time PCR System (Applied Biosystems, USA), and the results were analyzed using SDS 2.3 software.

2.9. In vitro tubulogenesis

To assess the angiogenic and/or vasculogenic ability of MMP-13 silenced cells, Matrigel™ matrix (BD Biosciences, USA) was used for *in vitro* tube formation assays (also named tubulogenesis). The experimental groups consisted of MMP-13-silenced OECs, non-targeting/non-coding siRNA OECs and Cyclophilin B (PPIB) siRNA OECs. Briefly, 9000 cells were seeded in 96-well plates that were previously coated with 50 μl of Matrigel™ matrix and then maintained in a CO_2 incubator at 37 °C. After 24 h, two images per well were acquired using an Olympus IX71 microscope (100× magnification). Each experiment was run in duplicate in four independent experiments.

In addition, to monitor the formation of vessel-like structures by mouse EPCs, a time-lapse imaging assay was performed over 24 h, as described in a previous study (Morancho et al., 2013). A standard Matrigel™ assay was conducted as described above; however, continuous image acquisition was performed starting 2 h after seeding and then every 30 min (for up to 24 h). An Olympus multi-dimensional-TIRFM cell-R microscope (Olympus, Japan) with temperature, CO_2 and humidity control was used. Two images per well were acquired at 100× magnification.

For both assays, the number of complete rings, the number of branching points and the total tube lengths (the perimeter of complete rings) were automatically counted blindly using Wimasis® Image Analysis software. The results are expressed as a percentage of the control condition.

2.10. Statistical analysis

SPSS version 15.0 was used for statistical analysis. Normality was assessed for continuous variables using the Shapiro-Wilk test. The values from the normally distributed variables are expressed as the mean ± SD and represented as bar graphs. The values from the non-normally distributed variables are expressed as the median (interquartile range) and represented as box plots. The differences between the different groups were analyzed using an independent *t*-test and ANOVA and, if significant, followed by the Dunnett *post-hoc* test (all *vs.* the control condition) if normally distributed or the Mann-Whitney *U* test and Kruskal-Wallis if not normally distributed. To assess differences between time points, we used repeated ANOVA followed by Bonferroni *post-hoc* tests. Finally differences between categorical variables were analyzed using Fisher's exact tests or Chi Square tests. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. MMP-13 deficiency protects the brain from ischemia-reperfusion injury and improves functional outcomes

The WT and KO alleles for MMP-13 were confirmed using PCR (Fig. 2A).

The brain infarct was localized to the somatosensory and motor cortex in both the permanent and the transient model (Morancho et al., 2012). Unexpectedly, 4 WT mice presented some infarct expansion that affected the striatum at 3 days after MCAO. MMP-13 deficiency reduced infarct percentage in the ipsilateral hemisphere in the ischemia-reperfusion model at both 24 h and 3 days: 16.40 (12.38–22.53) % for WT vs. 7.12 (5.12–12.23) % for MMP-13 KO, $p = 0.009$; and 27.03 (22.01–30.07) % for WT vs. 8.90 (6.97–12.41) % for MMP-13 KO, $p = 0.002$, respectively, as shown in Fig. 2B–C. In this reperfusion model infarct expanded from 24 h to 3 days only in WT mice ($p = 0.014$). However, the difference between genotypes was not significant in the permanent occlusion model: 10.48 (7.08–13.51) % for WT *vs.* 8.61 (5.88–11.09) % for MMP-13 KO, $p = 0.387$. Finally, when comparing the permanent vs. the transient model only WT mice showed an increase in infarct lesion in the ischemia-reperfusion model at both 24 h ($p = 0.027$) and 3 days ($p = 0.003$).

An analysis of functional outcomes also confirmed a significant decrease in forelimb force at 24 h in the WT mice (decreased 83.8 ± 15.8% from baseline, $p < 0.01$); however this was not clearly observed in the MMP-13 KO mice despite a statistical trend (90.2 ± 23.9% from

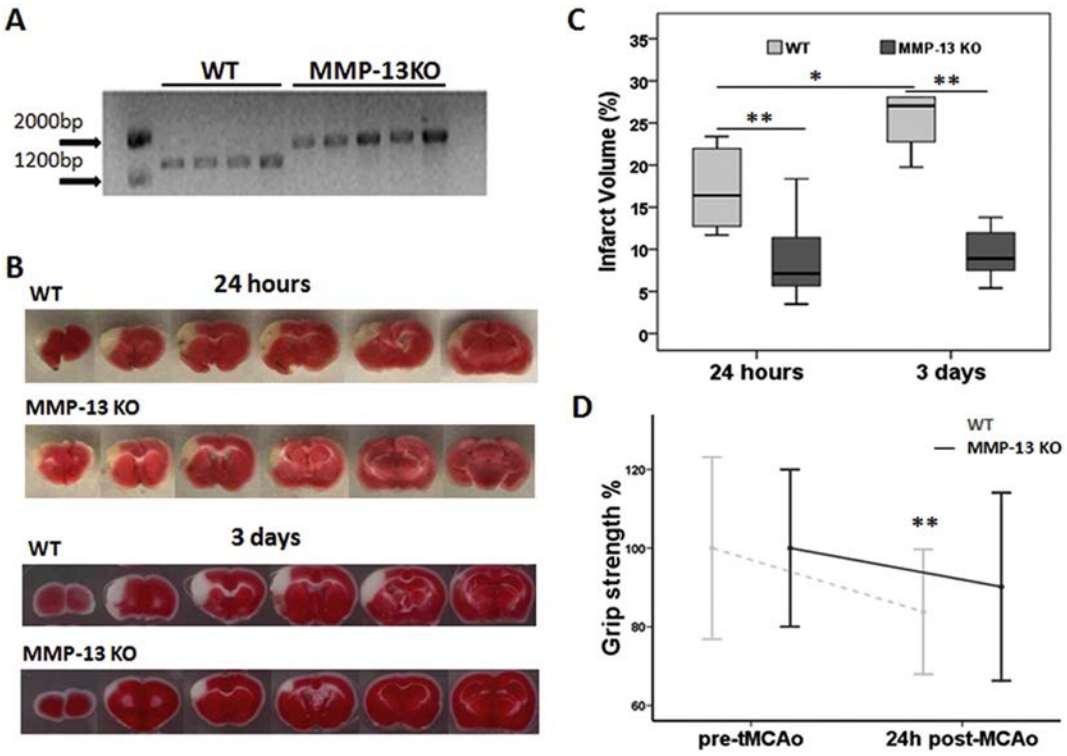

Fig. 2. MMP-13 deficiency protects brain tissue during cerebral ischemia reperfusion. Genotype characterization of wild-type (WT) and MMP-13 knockout (KO) mice: a 1300 Kb band indicates a WT allele, and a 1485 Kb band indicates a MMP-13 KO allele (A). TTC staining showing infarct areas at 24 h and 3 days after 60 min of transient ischemia (B). Bar graph showing infarct lesions (% of infarct volume *vs.* volume of the ipsilateral hemisphere) at 24 h and 3 days according to mice genotype; $n = 6$–8/group, $^{*}p < 0.05$ and $^{**}p < 0.01$ (C). Grip strength measurements confirming that the significant neurological impairment observed in WT mice was reduced in MMP-13-deficient animals; $n = 13$–14/group, $^{**}p < 0.01$ *vs.* pre-tMCAo (D).

baseline, $p = 0.083$); see Fig. 2D. No differences were observed between mice with different genotypes at either 24 h or 3 days (data not shown).

Interestingly, we observed more spontaneous intracranial hemorrhagic transformations at 3 days than at 24 h, and this was more common in the WT (6/6 and 4/8, respectively) than in the MMP-13 KO mice (3/6 and 1/7, respectively), as shown in representative images of the WT in Fig. 2B. The differences between genotypes or reperfusion day were not statistically significant, however if the only presence of hemorrhagic transformations was considered, regardless the duration of MCAo, MMP-13 deficiency protected brains from spontaneous hemorrhagic conversions (10/14 in WT vs. 4/13 in MMP13-KO, $p = 0.035$).

The animals studied over a longer time (two weeks, study B) showed significant differences in forelimb force between WT and MMP-13 KO at 24 h and 1 week after tMCAo ($p = 0.035$ and $p = 0.015$, respectively). When the neurological outcomes within the groups were analyzed, only the WT mice exhibited a significant decrease in forelimb force values *vs.* baseline at 1 week ($66 \pm 4.6\%$, $p < 0.001$) and 2 weeks ($70.2 \pm 5.7\%$, $p = 0.017$), as shown in Fig. 3A. At two weeks, differences in forelimb force were no longer observed.

3.2. MMP-13 and cortical damage

Cerebral ischemia leads to brain damage at the same time that it triggers neurorepair mechanisms. Cortical atrophy was evaluated at 14 days after tMCAo as a percentage of the remaining cortex in the ipsilateral hemisphere ($48.5 \pm 2.6\%$ for WT *vs.* $47.7 \pm 3.0\%$ for MMP-13 KO, $p = 0.578$) as shown in Fig. 3B, regardless of large differences in baseline infarct volumes.

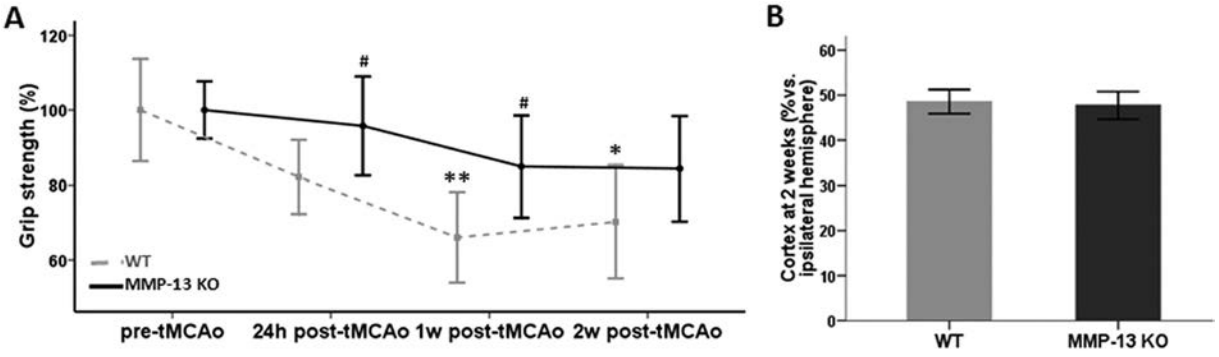

Fig. 3. Recovery from brain ischemia reperfusion. Grip strength performance: MMP-13 KO mice were acutely protected from functional impairment following cerebral ischemia. After two weeks, differences between genotypes were no longer observed. $n = 8$, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. pre-tMCAo in the same cohort; $^{\#}p < 0.05$ vs. WT at the same timepoint (A). Cortical atrophy of the ipsilateral hemisphere at 2 weeks, $n = 8$ (B).

3.3. A reduced number of newly born neuroblasts was observed in the peri-infarct areas of the MMP-13 deficient mice during recovery

DCX+ neuroblasts/immature neurons and proliferating cells were studied in the post-stroke brain during recovery (at days 7 and 14), with a focus on the SVZ dorsolateral area (DL), the corpus callosum (CC) and peri-infarct areas. At the studied timepoints, no enhanced neuroblast migration was detected at day 7 compared with the contralateral hemisphere in either the WT or the MMP-13 KO mice ($p > 0.05$, not shown). At day 14 we observed a higher number of neuroblasts in

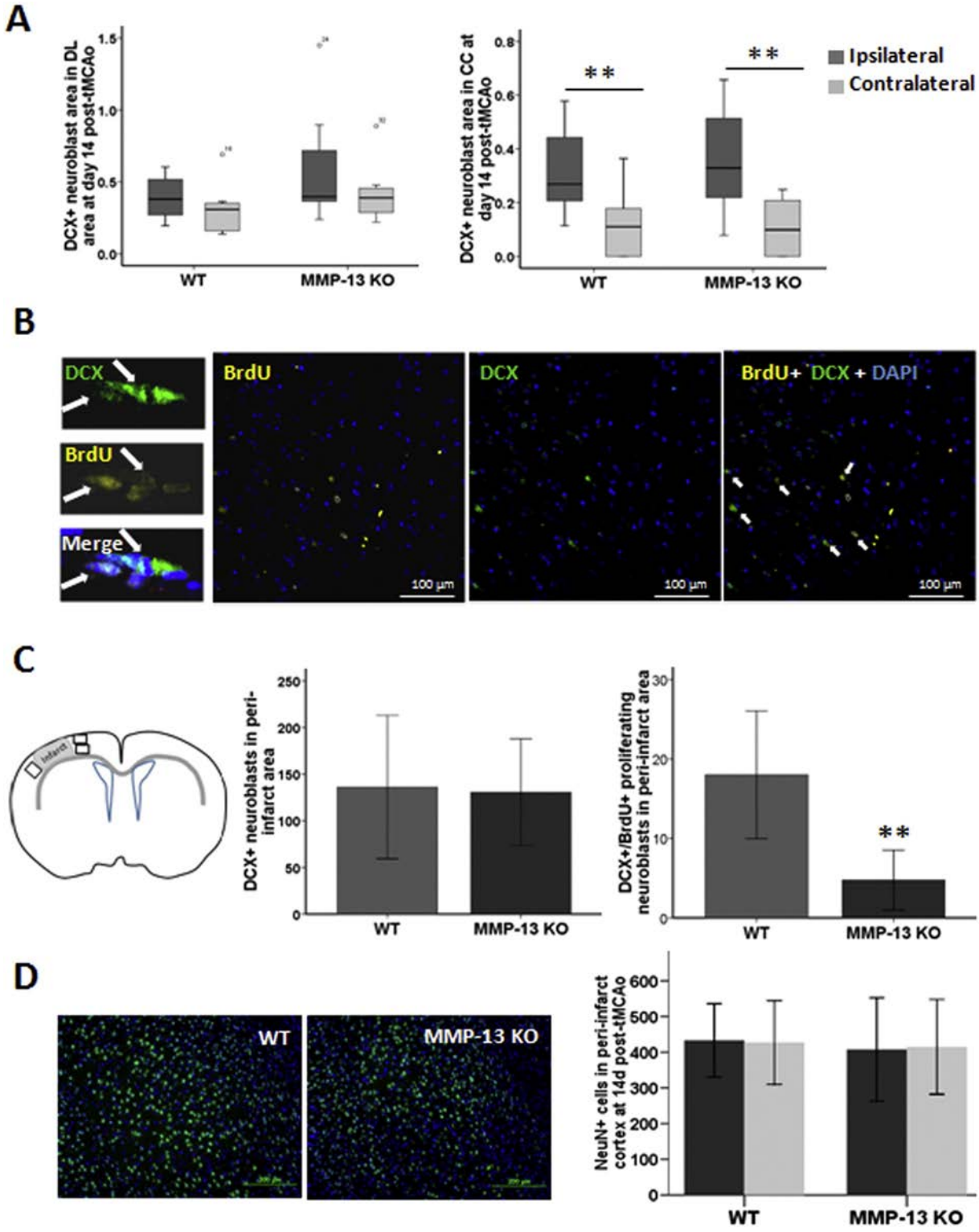

Fig. 4. Neuroblast migration from the SVZ through the CC to the peri-infarct cortex. DCX+ (neuroblast) area quantification in the dorsolateral ventricle (DL) area and the corpus callosum (CC) area at day 14 after tMCAo (A, $n = 8$); $^{**}p < 0.01$. The micrographs show peri-infarct neuroblasts under proliferation as merged signal for DCX and BrdU in DAPI nuclei on day 14 after tMCAo in a WT mouse (B), the white thick arrows point at newborn neuroblasts. Representation of the peri-infarct studied regions of interest and bar graphs showing that MMP-13 deficiency decreased the number of DCX+/BrdU + −proliferating neuroblasts in the peri-infarct cortex; $n = 8$, $^{**}p < 0.01$ (C). Tissue was stained for NeuN and the number of positive cells was quantified in the same peri-infarct areas, showing no differences in the number of mature neurons between genotypes at 14 days (D).

the ipsilateral CC area than in the contralateral in both genotypes ($p = 0.011$, respectively), but not in the DL area; see Fig. 4A. Newborn neuroblasts were identified as DCX+/BrdU+ cells as shown in Fig. 4B. Although the number of DCX+ cells did not change among genotypes in peri-infarct areas at 14 days ($p = 0.873$; see Fig. 4C), we observed a strong reduction in newly born neuroblasts in those areas of the MMP-13 KO mice compared to WT (4.7 ± 1.3 cells vs. 18.0 ± 2.8 cells, $p = 0.001$), as seen in Fig. 4C. Similarly, the percentage of newly born neuroblasts (DCX+/BrdU+) out of the total number of neuroblasts was reduced in MMP-13 deficient mice (16.9% in WT *vs.* 4.8% in MMP-13 KO, $p = 0.005$). However, the number of mature neurons (NeuN+) in the peri-infarct tissue were not different among genotypes (WT: 433 ± 103 cells in the ipsialteral vs. 427 ± 117 in the contralateral and MMP-13 KO: 407 ± 145 in the ipsialteral vs. 415 ± 132 in the contralateral; $p > 0.05$); see Fig. 4D.

3.4. MMP-13 is a key protease during post-stroke vascular remodeling

We sought to study the functional brain microvasculature using *in vivo* endothelial labeling with lectinin peri-infarct, as described. Lectin vessels studied in the peri-infarct areas show co-stain for CD-31 (Fig. 5A). Our data show that 7 days after cerebral ischemia, no difference in peri-infarct vessel density (the percentage of the lectin-stained area of the ROIs, as shown in Fig. 5A) occurred as a consequence of ischemic insult in either the WT (3.11 ± 0.35 in the ipsilateral *vs.* 3.15 ± 0.47 in the contralateral side, $p = 0.861$) or the MMP-13 KO mice (3.16 ± 0.64 in the ipsilateral *vs.* 3.37 ± 0.60 in the contralateral side, $p = 0.578$). Nevertheless, on day 14, the WT mice presented higher vessel density in the peri-infarct cortex than in the corresponding contralateral area (3.03 ± 0.41 *vs.* 2.45 ± 0.19, $p = 0.003$; Fig. 5B–C), whereas the MMP-13 KO mice did not display ischemia-enhanced angiogenesis in the peri-infarct area (2.53 ± 0.51 *vs.* 2.24 ± 0.20, $p = 0.160$; Fig. 5B–C). Accordingly, the WT mice presented a more extensive vessel area than the MMP-13 KO mice in the peri-infarct cortex (3.03 ± 0.41 *vs.* 2.53 ± 0.51, $p = 0.049$), and this was also observed, although less extensively, in the contralateral cortex, where it did not reach statistical significance (2.45 ± 0.19% in WT *vs.* 2.24 ± 0.20% in MMP-13 KO; $p = 0.05$; Fig. 5B–C). No differences were observed in the peri-infarct area in the striatum, as expected, in the distal cortical experimental model of stroke (data not shown).

Interestingly, we studied whether the number of proliferating neuroblasts (DCX+/BrdU+ cells) was related to vessel density. The correlation study showed a strong statistical trend ($p = 0.056$) to present increased vessel density and more proliferating neuroblasts at 2 weeks.

In agreement with this temporal profile, the levels of several angiogenesis-related growth factors were examined in the ischemic brain at day 7, which is just before the increase in vessel density observed in the peri-infarct tissue. Data obtained from immunodetection assays showed that G-CSF (shown as a ratio of ipsilateral/contralateral) was higher after ischemia in the WT mice than in the sham group (5.1 (1.9–10.5) *vs.* 1.1 (0.8–1.8), respectively; $p = 0.003$, not shown). At the same timepoint, the WT ischemic mice presented a higher cortical G-CSF than the corresponding MMP-13-deficient mice (5.2 ± 2.7 vs. 2.5 ± 1.5, $p = 0.031$; see Fig. 6A). In agreement, the expression of other growth factors associated with vascular remodeling was also significantly higher in the infarct cortex of WT mice than in the MMP-13 KO mice: VEGF-A (1.4 ± 0.3 vs. 0.9 ± 0.3, $p = 0.007$) and Ang-2 (1.1 ± 0.2 vs. 0.9 ± 0.2, $p = 0.049$; see Fig. 6A). Other growth factors, did not show significant differences when compared to the WT

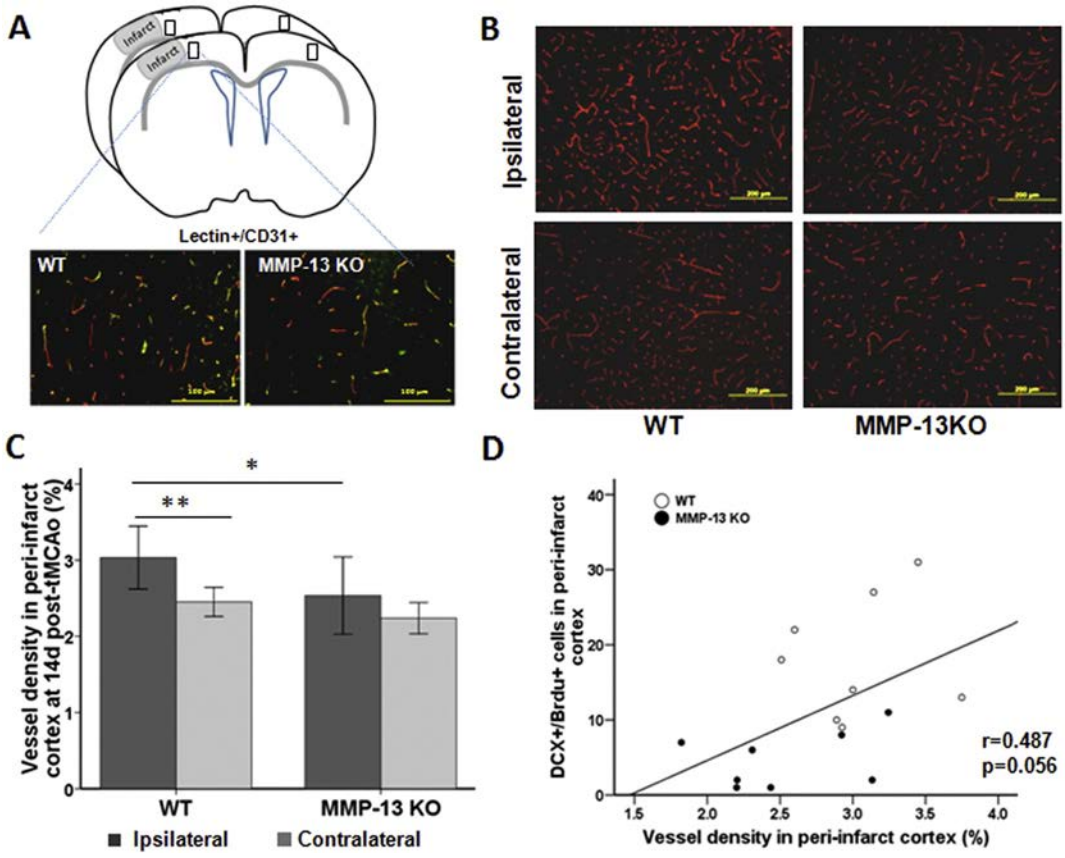

Fig. 5. Brain vessel density after ischemia reperfusion. Representation of the studied areas (indicated by ROIs) where peri-infarct vessel density was studied. Microvessels are shown as Lectin + (in red) and CD-31+ (in green) structures. (A). Micrographs of Lectin + (red) vessels in representative brains; scale bar represents 200 μm (B). Bar graphs representing vessel density (Lectin+) in the peri-infarct cortex and the corresponding contralateral area on day 14 after tMCAo; $n = 6$–8, $^{**}p < 0.01$ and $^{*}p < 0.05$ (C). Correlation plot between vessel density and the number of proliferating neuroblasts in peri-infarct areas on day 14 (D).

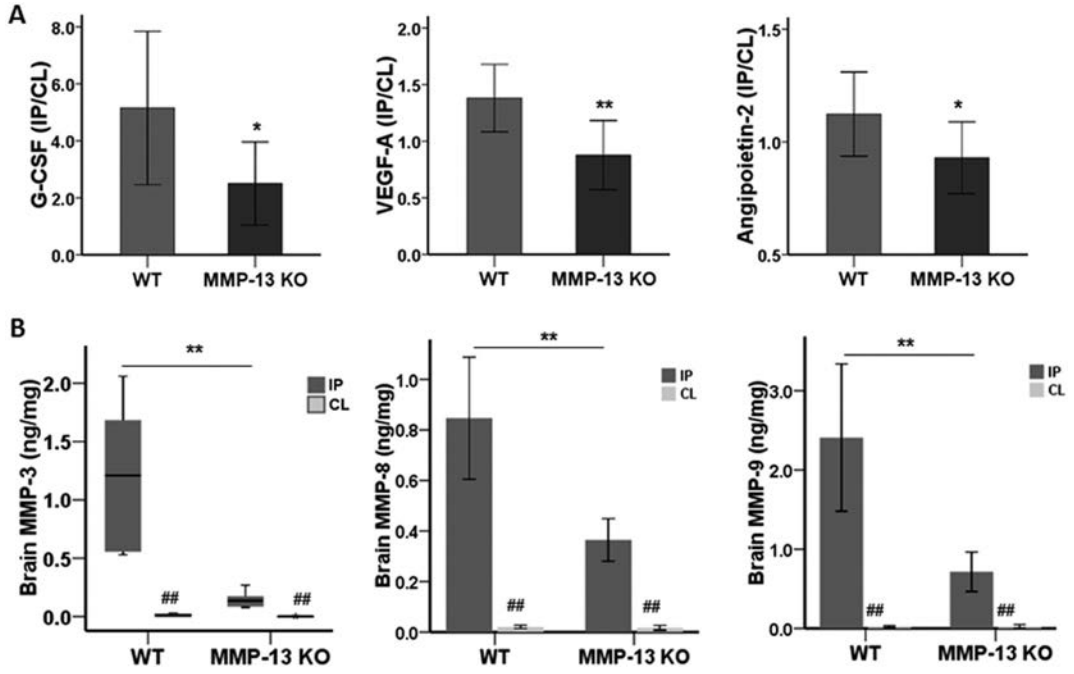

Fig. 6. Pro-angiogenic factors and MMPs in the cortex after ischemia. Bar graphs representing the ratio of MMP proteins between the ipsilateral (IP) and contralateral (CL) hemispheres in WT and MMP-13 KO ischemic mice at 7 days after ischemia; $n = 7–8$, $^{*}p < 0.05$, $^{**}p < 0.01$ (A). Box plots and bar graphs demonstrating the overexpression of other MMPs in the ipsilateral hemisphere of WT brains and their dysregulation in MMP-13-deficient mice on day 3. $^{**}p < 0.01$ and $^{\#\#}p < 0.01$ vs. ipsilateral (B). Data are expressed as ng of MMP per milligram of total protein.

(not shown), and exploring an earlier time point (3 days) only VEGF was increased in WT brains compared to MMP-13 KO ($p = 0.02$), but not G-CSF or Ang-2 ($p = 0.240$); not shown.

To investigate effects on other members of the MMP family of proteins that might potential compensate for MMP-13 knockdown, the levels of three MMPs (including another collagenase, MMP8) were quantified in the ischemic cortex and the corresponding contralateral tissue. Interestingly, our results show that the levels of MMP-3, MMP-8 and MMP-9 were significantly elevated in the damaged cortex in mice with both genotypes compared to the contralateral cortex, as shown in Fig. 6B. Moreover, the amount of these MMPs was considerable higher in the ipsilateral cortex of WT mice than in the MMP-13 KO mice: 1.21 (0.55–1.78) ng/mg vs. 0.14 (0.09–0.20) ng/mg for MMP-3, $p = 0.002$; 0.8 ± 0.2 ng/mg vs. 0.4 ± 0.1 ng/mg for MMP-8, $p = 0.001$; and 2.4 ± 0.9 ng/mg vs. 0.7 ± 0.3 ng/mg for MMP-9, $p = 0.002$. These results support the idea that MMP-13 plays a role as a central protease that regulates other MMPs after ischemia.

3.5. Transient silencing of the MMP-13 gene impairs tubulogenesis in vitro

To determine whether the angiogenic/vasculogenic functions of OECs are controlled by MMP-13, an *in vitro* Matrigel™ assay was conducted after MMP-13 expression was silenced using siRNA. As shown in Fig. 7A, introducing MMP-13 siRNA into OEC cell cultures reduced the expression of MMP-13 mRNA by 68.62% compared with the control cells ($p = 0.001$). This effect occurred without modifying the expression of house-keeping genes, such as PPIB. Additional experiments were conducted to demonstrate that the precise targeting of other genes, such as PPIB, successfully reduced their expression (up to 93.63, $p < 0.001$) without modifying MMP-13 expression, as shown in Fig. 7A. Nonspecific (non-targeting) siRNA did not modify any of the expression patterns of MMP-13 or PPIB.

Matrigel™ functional assays showed that OECs that did not endogenously express MMP-13 formed aberrant tubulogenic networks compared with all the other tested conditions (see Fig. 7B and Videos 1–4). The total number of branching points and loops and the total tube lengths of the vessel-like structures were quantified. Compared with the non-targeting siRNA (control group), the MMP-13-silenced OECs showed decreased numbers of vessel-like structures, as assessed by the analysis of all parameters. The difference in the percentage of loops (circular structures) reached statistical significance ($p = 0.019$), whereas the percentage of branches and the percentage of tube lengths are presented as statistical trends after the statistical analyses among the groups ($p = 0.132$ and $p = 0.052$, respectively), as shown in Fig. 7C.

4. Discussion

The present study demonstrates for the first time that collagenase-3 (MMP-13) participates in tissue damage during cerebral ischemia and is simultaneously required for neurovascular remodeling during the recovery phases. MMP-13 deficiency reduced infarct size and acutely diminished the deterioration of functional outcomes observed after experimental transient cerebral ischemia. However, the absence of MMP-13 also reduced the amount of newly born neuroblasts in the peri-infarct areas during neurological recovery and altered stroke-induced angiogenesis by reducing peri-infarct angiogenesis and suppressing the secretion of G-CSF, angiopoietin-2 and VEGF-A. Finally, endothelial progenitor cell function (demonstrated in their high potential for vascular remodeling) was abolished when MMP-13 gene expression was silenced *in vitro*.

Stroke leads to cell and tissue death within minutes/hours because the brain spontaneously activates both injury and repair mechanisms that compete for the recruitment of tissue after an ischemic insult (Lo, 2008). This state opens a new battlefield wherein neuroprotection is needed to minimize the expansion of the injury and neurorepair strategies are needed to enhance endogenous repair. Among the known neurorepair mechanisms is the process by which cerebral ischemia triggers spontaneous neurogenesis (Ohab et al., 2006; Kreuzberg et al., 2010), angiogenesis (Ergul et al., 2012) and gliogenesis (Tanaka et al., 2003; Miyamoto et al., 2010) in peri-infarct areas. From a mechanistic point of view, our efforts have been focused on MMPs, which are zinc-dependent endopeptidases that degrade components of the extracellular matrix, including the basal lamina surrounding brain

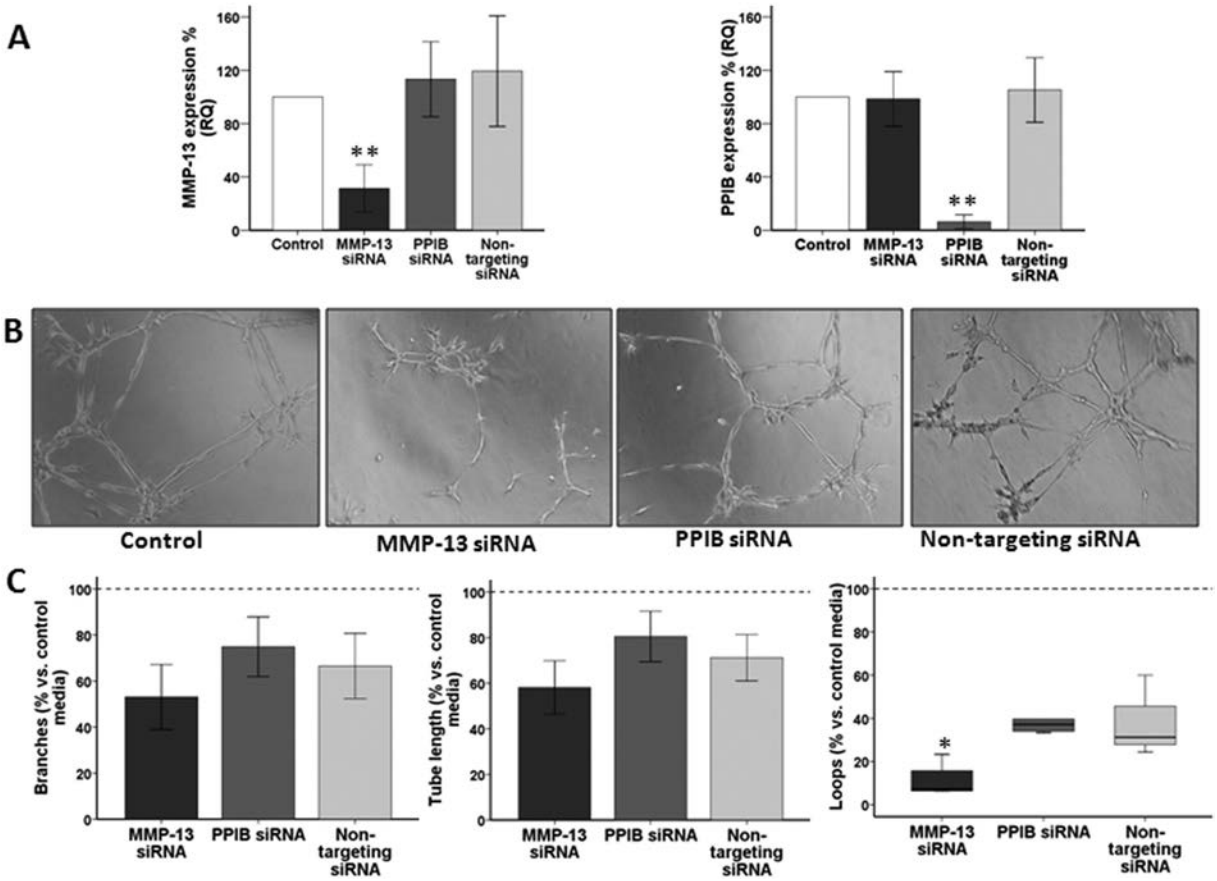

Fig. 7. Tubulogenesis network formation in Matrigel™ assays of mouse OECs after silencing MMP-13 expression. Bar graphs represent the level of MMP-13 and PPIB RNA expressed in each culture condition; $n = 4$, $^{**}p < 0.01$ vs. control (A). Representative micrographs showing vessel-like structures shaped by OECs in different treatment groups (B). The bar graphs show the quantification of the number of branches, the total tube lengths and the loops of vessel-like structures that were induced in non-silenced, MMP-13-silenced and PPIB-silenced OECs; $n = 4$, $^{*}p < 0.05$ vs. non-targeting siRNA EPCs (C).

microvessels, which are modified during angio-vasculogenesis. Some MMPs have been found to contribute to brain injury (Asahi et al., 2001; Gidday et al., 2005) and also to orchestrate vascular remodeling (Morancho et al., 2013; Zhao et al., 2006). Knowledge regarding the role of MMP-13 is limited in the context of stroke.

In our study, both MMP-13 KO and WT mice were subjected to permanent or transient ischemia; however, the infarct size was only significantly reduced in the MMP-13-deficient mice in the transient model at both 24 h and 3 days after MCA, indicating that the role of MMP-13 in tissue damage is most likely more related to the reperfusion phase. This observation was further supported by the increase in the infarct lesion in reperfused brains compared to permanent ischemia. In addition, we unexpectedly observed spontaneous hemorrhagic transformations in our model (especially at day 3), but the knockdown of MMP-13 partially protected mice from suffering these complications. Previous results from our laboratory revealed a relationship between a high plasma level of MMP-13 and early brain lesion growth in stroke patients (Rosell et al., 2005). We identified an increase in MMP-13 protein levels in the infarct/peri-infarct areas in human and rat tissues, and the activation of neuronal nuclei has been related to cell death in oxygen- and glucose-deprivation studies (Cuadrado et al., 2009a and Cuadrado et al., 2009b). Recently, increased MMP-13 mRNA and protein levels were observed in brain tissue during the acute phase of ischemia reperfusion by Lenglet and colleagues (Lenglet et al., 2014). Other authors have reported that injured endothelial cells release MMPs, including MMP-13, which attack the basal lamina and degrade the matrix components and tight junctions of endothelial cells (Hattori et al., 2003). Ueno and colleagues found that the expression of MMP-13 in hippocampal vessels was increased in rats with blood brain barrier (BBB)-damaged vessels compared with rats without BBB impairment, indicating the importance of MMP-13 recruitment to brain tissue during the healing process (Ueno et al., 2009). Indeed, it has been demonstrated that a high level of MMP-13 activity lasts for up to 14 days after ischemia, and this appears to be closely associated with an increase in aggrecan, suggesting their role in neuronal reorganization (Nagel et al., 2005). All these data indicates that MMP-13 might participate in the development of acute ischemic damage, while at the same time contributes to favorable neurovascular remodeling. As we will discuss in detail, the results in this report support these hypotheses.

The distal MCAo model was chosen for this study due to its association with low mortality rates and for the precise infarct location it induces in cortical areas. However, cortical damage tends to produce milder neurological deficits over the long-term. In our experience, the grip strength test has been proven to be a feasible method for short- and long-term evaluations after distal MCAo (Rosell et al., 2013b). Our data show that only the WT mice presented significant neurological deterioration in the first week, supporting a protective role for MMP-13. Moreover, the WT mice presented reduced forelimb force at one and 7 days of ischemia compared to MMP13 KO, but this difference disappeared at 2 weeks, when WT mice showed more vessel density and more new born neuroblasts in peri-infarct areas. These data would support the idea that MMP-13 is implicated in the post-stroke spontaneous-recovery phase at a functional level. In this regard, our results also showed that similar cortical atrophy occurred in MMP-13 KO and WT mice at 2 weeks, despite the larger infarct volumes observed

in WT mice, which could be a consequence of different capabilities related to neurorepair, the inhibition of cell apoptosis or the promotion of neuronal survival, among other mechanisms. It will be interesting to perform studies using longer timepoints to follow-up the state of atrophy in the damaged tissue.

Neurogenesis and angiogenesis were studied to further explore the mechanisms affected by the lack of MMP-13 that could influence cerebral plasticity. Our results confirm that at 2 weeks, neuroblasts had migrated to the peri-infarct areas, as has been described to occur in post-stroke brains (Arvidsson et al., 2002; Carmicheal, 2008). This effect occurred from the SVZ through the corpus callosum, as expected, and no difference was observed between the genotypes. Interestingly, the peri-infarct areas of the mice lacking MMP-13 contained a reduced number of proliferating neuroblasts (DCX+/BrdU+), which are responsible for the formation of new neural cells in the areas showing active neurorepair. Most neuroblasts die during migration and the generation of new neurons (Zhang et al., 2004). We hypothesized that either the microenvironment in peri-infarct areas and along the migration pathways in brains lacking MMP-13 did not support neuroblast survival or that lack of MMP-13 may result in decelerated standard migration patterns. In this regard, neurogenesis has been shown to be coupled to angiogenesis in a post-stroke neurovascular niche (Ohab et al., 2006), and it has been demonstrated that blood vessels in the peri-infarct cortex secrete soluble factors that maintain neurogenic potential and guide neurogenesis toward the damaged cerebral cortex (Shen et al., 2004; Thored et al., 2007).

Importantly, our study shows that at day 7 after ischemia-reperfusion injury, lower amounts of some nourishing factors, such as G-CSF, angiopoietin-2 and VEGF-A, were observed in the infarct cortex of the MMP-13 KO mice than in the WT brains. This is an interesting finding because the administration of some factors in the early stages of stroke has been shown to stimulate angio-neurogenesis and to lead to improved functional recovery (Schäbitz et al., 2003; Rivera and Bergers, 2014; Beck et al., 2000). These data suggest that MMP-13 could be indirectly contributing to an appropriate angiogenic micro-environment. In agreement with this assumption, our observations support a role for MMP-13 in vascular remodeling after stroke: two weeks after cerebral ischemia, increased vessel density in the peri-infarct area was found only in the WT animals and not in the MMP-13-deficient mice. The present study does not identify the source of the trophic factors that are affected by MMP-13 deficiency. These could be secreted by brain resident cells, such as astrocytes or mature endothelial cells, as has been described by others (Ohab and Carmichael, 2008; Kahle and Bix, 2013). However, they could also be released by stem/progenitor cells, such as EPCs or NPCs, which secrete multiple growth factors (Rosell et al., 2013a; Drago et al., 2013; Urbich et al., 2011).

EPCs have been demonstrated to have therapeutic potential in that they directly enhance angiogenesis and neurogenesis (Asahara et al., 1997; Carmeliet, 2003; Ma et al., 2015). These cells are able to differentiate into cells with an endothelial phenotype and have been proven to reduce the brain damage caused by ischemia and to enhance angiovasculogenesis in stroke models (Ohta et al., 2006; Moubarik et al., 2011; Rosell et al., 2013a). In the present study, we further demonstrated a role for MMP-13 in tubulogenic remodeling in experiments in which we silenced the expression of MMP-13 in EPCs. Our data demonstrate that EPCs develop abnormal tubulogenic remodeling patterns when MMP-13 expression is blocked, in which they failed to shape vessel-like networks in Matrigel™ substrate. This interesting observation should be considered for future therapeutic strategies based on EPC therapies.

In summary, our study highlights the role of MMP-13 in neuroprotection and neurorepair after cerebral ischemia. This central role is further supported by our findings regarding the regulation of other MMPs in the ischemic brain. We demonstrate that in WT mice, MMP-3, MMP-8 and MMP-9, are upregulated in the ischemic brain, as was reported by our group in human infarct tissue (Cuadrado et al., 2009b), but also that MMP-13 deficiency considerably diminished the overexpression of these other MMP family-members.

Therefore, lacking MMP-13 protects the brain and improves functional outcomes over the short term, whereas over longer periods of time, this protease might be required to support vascular remodeling and the production of growth factors that are linked to peri-infarct neurogenesis. However since the final neurorepair mechanisms and outcome can be also influenced by the observation of reduced infarct volumes in MMP-13 deficient mice, new studies are needed to elucidate the contribution of MMP-13 in angiogenesis and neurogenesis in other MCAO models related to infarct extension.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2016.03.016.

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ANNEX 2

Original Contribution

Endothelial Progenitor Cell Secretome and Oligovascular Repair in a Mouse Model of Prolonged Cerebral Hypoperfusion

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Background and Purpose—Endothelial progenitor cells (EPCs) have been extensively investigated as a therapeutic approach for repairing the vascular system in cerebrovascular diseases. Beyond vascular regeneration per se, EPCs may also release factors that affect the entire neurovascular unit. Here, we aim to study the effects of the EPC secretome on oligovascular remodeling in a mouse model of white matter injury after prolonged cerebral hypoperfusion.

Methods—The secretome of mouse EPCs was analyzed with a proteome array. In vitro, the effects of the EPC secretome and its factor angiogenin were assessed on primary oligodendrocyte precursor cells and mature human cerebral microvascular endothelial cells (hCMED/D3). In vivo, mice were subjected to permanent bilateral common carotid artery stenosis, then treated with EPC secretome at 24 hours and at 1 week, and cognitive outcome was evaluated with the Y maze test together with oligodendrocyte precursor cell proliferation/differentiation and vascular density in white matter at 4 weeks.

Results—Multiple growth factors, cytokines, and proteases were identified in the EPC secretome, including angiogenin. In vitro, the EPC secretome significantly enhanced endothelial and oligodendrocyte precursor cell proliferation and potentiated oligodendrocyte precursor cell maturation. Angiogenin was proved to be a key factor since pharmacological blockade of angiogenin signaling negated the positive effects of the EPC secretome. In vivo, treatment with the EPC secretome increased vascular density, myelin, and mature oligodendrocytes in white matter and rescued cognitive function in the mouse hypoperfusion model.

Conclusions—Factors secreted by EPCs may ameliorate white matter damage in the brain by boosting oligovascular remodeling.

Visual Overview—An online visual overview is available for this article. (***Stroke*. 2018;49:00-00. DOI: 10.1161/STROKEAHA.117.019346.**)

Key Words: angiogenin ■ carotid stenosis ■ endothelial progenitor cell ■ repair ■ white matter

White matter damage caused by chronic cerebral hypoperfusion is a histological feature of stroke and cerebrovascular disease. Recently, it has been proposed that tissue physiopathology is influenced by the dynamic balance between deleterious versus beneficial responses to the initial insult,[1] including neuroinflammation, demyelination, and cell death but also regenerative capacities such as neurogenesis, angiogenesis, and neuroplasticity.

In the context of white matter remodeling, an endogenous pool of oligodendrocyte precursor cells (OPCs) is widely distributed in the adult brain for physiological myelin renewal and for repair under pathological conditions.[2] In demyelinating disorders, residual OPCs tend to proliferate and differentiate into oligodendrocytes to alleviate white matter damage.[3,4] However, the regeneration of oligodendrocytes and myelin sheaths might fail because of an insufficient maturation of OPCs. Cell-based therapies are promising therapeutic options for enhancing oligodendrogenesis as bone marrow–derived mesenchymal stem cells and mononuclear cells have been reported to promote

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myelin repair after intravenous injection into rodent models of demyelination.[5,6] Also, the paracrine effects of transplanted mesenchymal stem cells have been reported to promote the OPC maturation and remyelination both in vitro and in vivo.[7,8] Other studies have demonstrated that the administration of endothelial progenitor cells (EPCs) enhances neurogenesis and angiogenesis in mouse models of stroke,[9,10] and EPC-secreted factors promoted cortical vascular remodeling after cerebral ischemia,[11] but its regenerative role in white matter damage is unknown. Among these secretome-remodeling factors, we have focused on angiogenin, a member of the Ribonuclease A superfamily which undergoes nuclear translocation in endothelial cells stimulating ribosomal RNA transcription, cell proliferation, and growth.[12,13] With known effects on tumor neovascularization and cancer progression,[14,15] it has been also described to promote hematopoietic regeneration of stem/progenitor cells,[16] being neurotropic and neuroprotective.[13] In this regard, mutations in angiogenin have been described in patients with familial and sporadic amyotrophic lateral sclerosis, affecting neuronal survival.[13]

In the present study, we used a combination of in vitro culture systems and in vivo mouse model[17] to ask whether EPC-secreted factors (the EPC secretome) can enhance oligovascular proliferation, maturation, and repair in damaged white matter. Our results demonstrate for the first time that the treatment with the EPC secretome preserves cognitive function and enhances oligo-angiogenesis in the injured white matter.

Materials and Methods

Ethics Statement

Experiments were performed following institutionally approved protocols in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Directives of the European Union. The article adheres the Transparency and Openness Promotion Guidelines, and all data supporting the findings of this study are available from the corresponding authors on reasonable request.

Cerebral Prolonged Hypoperfusion Model

Cerebral prolonged hypoperfusion was induced by bilateral common carotid artery stenosis using a microcoil in 24 male C57Bl/6 mice (2–3 months old, Charles River Institute) as described.[17] Follow-up for surgeries was performed by an investigator blinded for treatment. The mice were euthanized 28 days after the surgery; see the online-only Data Supplement.

Protocol for Conditioned Media Production

Mouse EPC cultures were used for the production of conditioned media (CM) in culture for 24 hours in endothelial basal media (CC-3156 from Lonza) as described[11] and filtered and concentrated using 10 KDa-membrane centrifuge tubes (Sartorius, V0601). Filtered and concentrated fresh endothelial basal media was used as vehicle treatment. See the online-only Data Supplement.

Secretome Therapy

Under anesthesia (1.5% isoflurane in 70% N_2O and 30% O_2), each animal received 160 µL of CM or vehicle intravenously (retroorbital) at 24 hours and at 7 days after bilateral common carotid artery stenosis procedure.

5-Bromodeoxyuridine Labeling

A cell proliferation marker 5-bromodeoxyuridine (Sigma-Aldrich) was dissolved in 0.9% saline (5 mg/mL) and injected intraperitoneally (50 mg/kg body weight) every 12 hours between day 5 and 14 after the bilateral common carotid artery stenosis surgery.

Y Maze Test

The Y maze test was performed 28 days after the surgery as described.[18,19] Memory and spontaneous activity indicators were evaluated blindly to treatment; see the online-only Data Supplement.

Isolation of Corpus Callosum and Western Blot

Nine anesthetized mice were transcardially perfused with ice-cold PBS. Brains were removed, and one half was placed in a brain tissue matrix and sectioned to obtain three 1-mm-thick coronal blocks. The corpus callosum was separated from the other half under a microsope as shown in Figure I in the online-only Data Supplement, frozen in dry ice, and stored at −80°C. For Western blot, lysates from the corpus callosum region were ultrasonically homogenized in 100 to 200 µL PRO-PREP Protein Extraction Kit (iNtRON Biotechnology). Samples were tested for anti-platelet-derived growth factor receptor-alpha (PDGFR-α), anti-CD31, MBP (myelin basic protein), or anti-β-actin as described in the online-only Data Supplement.

Immunohistochemistry

Mice were perfused with 4% paraformaldehyde in PBS, and the brain was postfixed for 24 hours in 4% paraformaldehyde in PBS at 4°C before cryoprotection with 30% sucrose. Frozen brains were cut into 16-µm-thick consecutive coronal sections at 0 to 0.5 mm anterior from the bregma and stained with anti-5-bromodeoxyuridine, anti-CD31 (for endothelial cells), anti-glutathione-S-transferase-pi (for mature oligodendrocytes), or anti-PDGFR-α (for oligodendrocyte progenitors) as detailed in the online-only Data Supplement.

Protein Profile of EPCs Secretome

CM were collected as described, and total protein content measured by the Comassie Protein Assay kit (Thermo Scientific). The protein profile (n=4) was analyzed using the Proteome Profiler Mouse Angiogenesis Array kit (R&D Systems); see the online-only Data Supplement.

Endothelial Cell Proliferation Assay

To test the angiogenic properties of CM on human cerebral microvascular endothelial cells, we assessed cell proliferation after treatment with CM or angiogenin (a potent proangiogenic factor identified in EPCs secretome) combined with Neomycin (an angiogenin inhibitor) with the Muse™ Cell Analyzer and the Cell Count and Viability Kit, as described.[20]

Endothelial Cell Viability Assay

Measurement of the reduction of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide by human cerebral microvascular endothelial cells (hCMEC/D3) treated with Neomycin at different doses was performed as described in the online-only Data Supplement.

OPC Culture

Primary cultured rat OPCs were prepared according to our previous report[21] from cerebral cortices of 1- to 2-day-old SD rat pups; see the online-only Data Supplement.

OPC Viability Assay

OPC viability was assessed by WST reduction assay (Cell Counting Kit-8, Dojindo), which is similar to 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide assay. The cells were incubated with 10% WST solution for 1 hour at 37°C when the absorbance of the culture medium was measured at 450 nm wavelength with a reference wavelength of 630 nm.

Evaluation of OPC Differentiation

After OPCs were treated with EPC-CM or vehicle diluted from 1/5 to 1/50 in OPC differentiation media (final concentration of CNTF [ciliary neurotrophic factor], triiodothyronine, and B27 was 10 ng/mL, 15 nmol/L, and 2%, respectively) for 24 hours, they were further cultured with the same OPC differentiation media for another 5 days.

Immunocytochemistry

Cultured EPCs or OPCs were immunostained for MBP or angiogenin, respectively, following standard procedures detailed in the online-only Data Supplement.

EPC-CM Western Blot for Angiogenin

Thirty-two microliter of reduced EPC-CM and endothelial basal media-vehicle were loaded in SDS-PAGE (12%) together with mouse recombinant angiogenin as positive control and transferred into polyvinylidene fluoride membranes as detailed in the online-only Data Supplement.

Statistical Analyses

Normality was assessed by the Shapiro–Wilk test. To assess differences between groups, *t* tests and one-way analysis of variance followed by Dunnett (bilateral) post hoc tests were used for normally distributed variables, while the Kruskal–Wallis and Mann–Whitney *U* tests were used to explore differences in non-normally distributed variables. Values are expressed and represented as mean±SD, and statistical analysis was conducted by the SPSS 15.0 software. A P value <0.05 was considered statistically significant.

Results

EPC Secretome Induced Endothelial and OPC Proliferation and Oligodendrocyte Maturation In Vitro

Mature endothelial cells and OPCs were cultured and exposed to EPC-CM or corresponding vehicle. Our results show that EPC-CM enhanced cell proliferation after treatment in both endothelial and OPC cells (P<0.05; Figure 1A and 1B). Additionally, EPC-CM-treated OPCs showed an increase in MBP expression (P<0.05; Figure 1C), indicating the switch to a more mature oligodendrocyte phenotype.

EPC Secretome Proteomic Profile

A proteome array identified 38 proteins, including angiogenin and other promoters of angiogenesis (SDF-1 [stromal derived factor-1], PDGFAA/AB/BB [platelet-derived growth factor], VEGF-B [vascular endothelial growth factor], or several MMP [matrix metalloproteinase]), but also some inhibitors (endostatin or thrombospondin-2); see Table I and Figure II in the online-only Data Supplement. At the same time, molecules critically involved in the proliferation, maturation, and survival of OPCs, such as PDGF, IGF-1 (insulin-like growth factor-1), EGF (epidermal growth factor), or IL-1β (interleukin-1β) were also detected in the EPC secretome.

Secreted Angiogenin as a Key Factor in the EPC Secretome

Western blots demonstrated that angiogenin was present in the EPC-CM (Figure 2A). Immunocytochemistry confirmed that EPCs were indeed positive for angiogenin (Figure 2B).

In parallel, cell cultures treated with recombinant angiogenin at different doses confirmed the induction on endothelial proliferation (P<0.05) but not on OPC cells at the same doses (Figure 3A and 3B), respectively. Functional assays showed that the effects of the EPC secretome on endothelial proliferation were blocked by the addition of neomycin (10 µmol/L), an aminoglycoside antibiotic which blocks angiogenin signaling by inhibiting its nuclear translocation for angiogenesis (P<0.05); however, these effects were not observed in OPC cultures as expected (Figure 3A and 3B). No toxic effects of

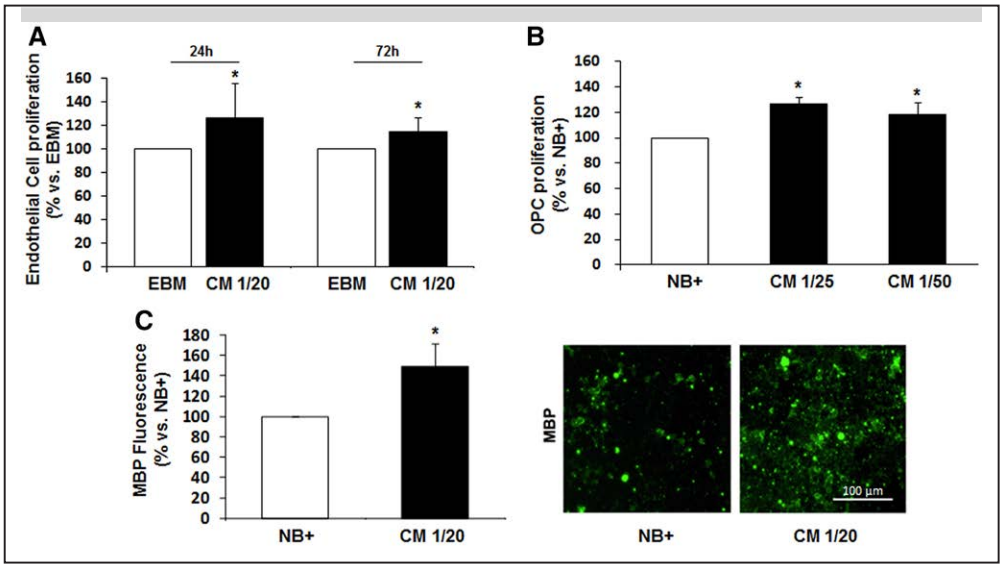

Figure 1. In vitro effects of endothelial progenitor cells (EPCs) conditioned media (CM) on mature endothelial cells and oligodendrocyte progenitors. **A**, The number of viable human cerebral microvascular endothelial cells (hCMEC/D3) increased at 24 and 72 hours after treatment with CM compared with cells cultured with endothelial basal media (EBM). **B**, Oligodendrocyte precursor cells (OPC) proliferation was also observed 24 hours after treatment with CM compared with cells cultured with neurobasal plus 2% B27 (basal media, NB+). **C**, Cultured OPCs showing maturation markers such as myelin basic protein increasing after 24 hours treatment with CM. Tested CM dilutions in basal media are indicated in each graph; n=3 to 6/group, *P<0.05.

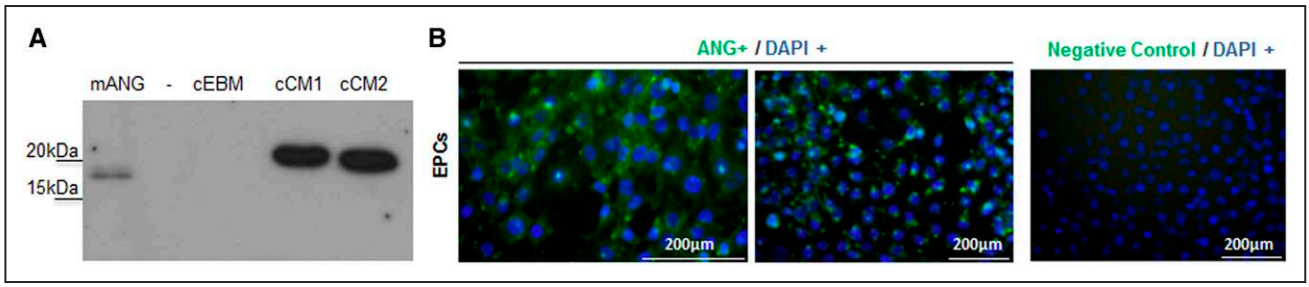

Figure 2. Endothelial progenitor cells (EPC) as a source of angiogenin. **A**, ANG immunoblotting. **B**, Immunocytochemistry images identifying ANG in endothelial progenitor cells. ANG indicates angiogenin; cCM1/2, concentrated conditioned media from mouse EPCs; cEBM, concentrated endothelial basal media; and mANG, recombinant mouse ANG.

neomycin were observed in endothelial cells (see Figure III in the online-only Data Supplement).

EPC Secretome Therapy Improved Cognitive Function In Vivo

Finally, we asked whether these oligovascular effects of the EPC secretome could be observed in vivo (Figure 4A). Mice were subjected to prolonged cerebral hypoperfusion via permanent bilateral occlusion of the carotid arteries. Cognitive impairment was then measured with the Y maze test. Alterations of entries were significantly increased in EPC-CM-treated mice (75.1±7.5%) compared with vehicle-treated mice (66.3±7.2%; $P<0.05$; Figure 4B). On the other hand, spontaneous activity, shown by the number of total arm entries, was not significantly different (Figure 4B).

Secretome Therapy Facilitated Oligovascular Remodeling in the White Matter

We next asked whether these beneficial effects of EPC-CM were associated with white matter remodeling in vivo. Vascular density in the corpus callosum area increased in those mice treated with EPCs-CM ($P<0.05$; Figure 5A), whereas Western blot analysis showed some nonsignificant increase in CD31 expression ($P<0.1$; Figure 5B). Similarly, the presence of OPCs (PDGFR-α marker) was not different between treatments (Figure 5C) when analyzed by Western blot, but the amount of MBP in those areas was significantly increased (by 97%) after EPC-CM treatment; $P<0.05$; Figure 5D.

We then identified OPC proliferation/maturation since the number of total proliferating oligodendrocyte lineage cells increased by 32% in the damaged corpus callosum in mice receiving EPCs-CM ($P<0.05$; Figure 6A). Of these, the number of proliferating OPCs (5-bromodeoxyuridine/PDGFR-a double-positive cells) was not different (Figure 6B), but there was a significant increase in the number of newly generated oligodendrocytes (5-bromodeoxyuridine/glutathione-S-transferase-pi double-positive cells) in EPC-CM-treated mice ($P<0.05$; Figure 6C), representing ≈78% increase. These results suggest that EPC secretome therapy facilitated

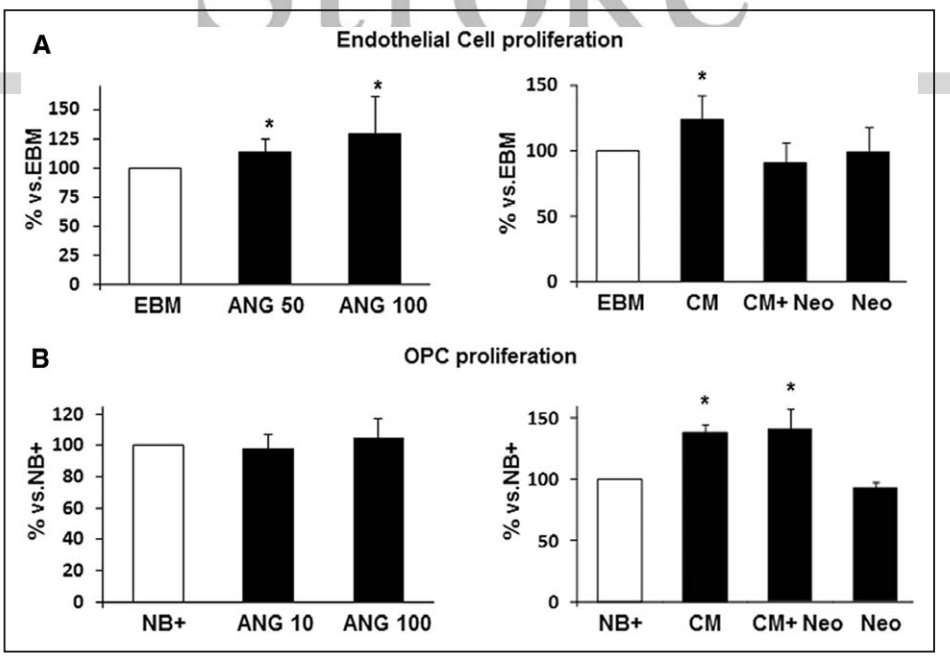

Figure 3. Study of angiogenin (ANG) as key factor in conditioned media (CM) for endothelial cell proliferation but not for oligodendrocyte precursor cells (OPCs). **A** and **B**, **left**, Treatment with ANG (50 and 100 ng/mL) induces endothelial cell proliferation dose-dependently but not in OPCs. **A** and **B**, **right**, Bar graphs showing that blocking ANG with Neomycin (Neo, 10 μmol/L) inhibits CM-induced proliferation in endothelial cells but not in OPCs; CM was diluted 1/20 and 1/5, respectively. Neomycin treatment alone did not reduce cell viability. n=3 to 6 per group, *$P<0.05$. EBM indicates endothelial basal media.

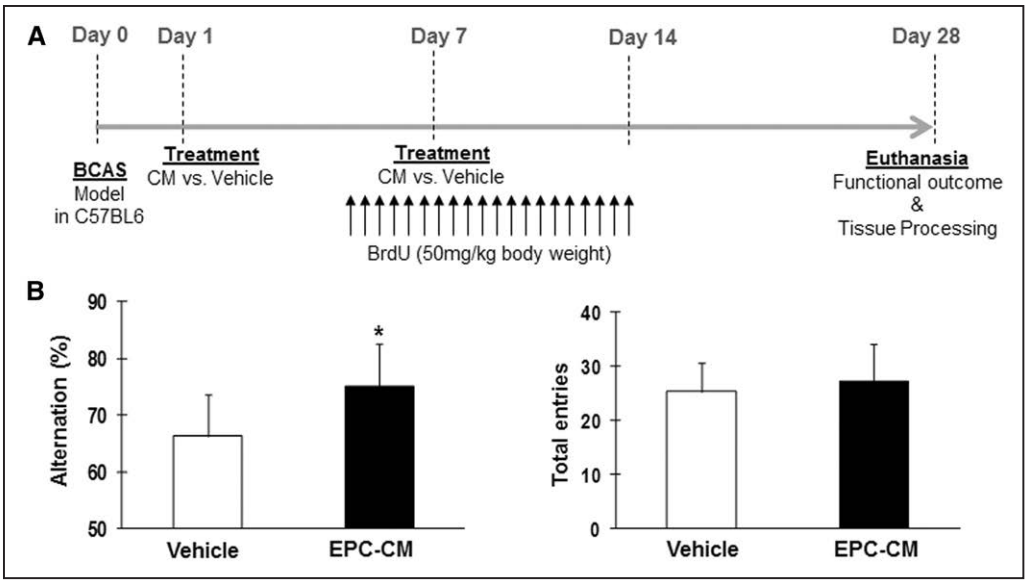

Figure 4. Preclinical model of cerebral hypoperfusion: study design and functional outcome. **A**, Study design: conditioned media (CM) from endothelial progenitor cells (EPCs) was administered 24 hours and 1 week after inducing cerebral hypoperfusion with the bilateral common carotid artery stenosis (BCAS) model. 5-Bromodeoxyuridine (BrdU) was intraperitoneally injected from day 5 to 14 every 12 hours, and functional outcome was assessed at day 28 before euthanasia when brains were collected. **B**, Cell-free conditioned media from EPCs improves cognitive function. Bar graphs showing number of total arm entries and alternation behavior in the Y maze test at day 28 of vehicle-treated (n=10) and EPC-CM-treated (n=8) mice, **P*<0.05.

oligovascular remodeling and maturation of newly generated OPCs after prolonged cerebral hypoperfusion.

Discussion

The failure of the myelination/remyelination process attributed to insufficient regenerative responses, as well as the suppression of oligodendrocyte maturation, accompanies white mater injury in stroke and cerebrovascular disease. Hence, treatments boosting endogenous myelin repairing responses would be effective to improve patients' outcome. Our findings in the present study support the therapeutic actions of factors secreted by EPCs, highlighting the importance of angiogenin, a known enhancer for angiogenesis. We finally demonstrate the regenerative actions of the EPC secretome in vascular and myelin remodeling in an experimental mouse model of white matter injury.

Cell transplantation is considered a valid therapeutic strategy to repair the injured tissue.[22] In this regard, several types

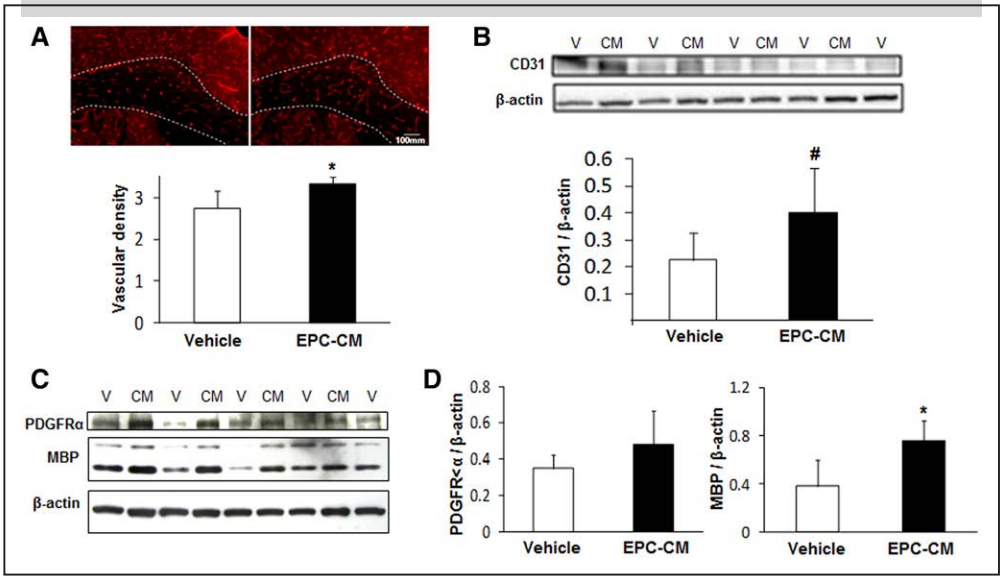

Figure 5. Cell-free conditioned media (CM) from endothelial progenitor cells (EPCs) increases endothelial and mature oligodendrocyte markers in the white matter. **A** and **B**, The corpus callosum was studied for CD31 protein expression (marker for blood vessel) by immunofluorescence or immunoblotting. **C** and **D**, Additional immunoblotting for PDGFR-α (marker for oligodendrocyte precursor cells [OPCs]), MBP (myelin basic protein; marker for mature oligodendrocytes), and β-actin were conducted and quantified; vehicle-treated (n=5) and EPC-CM-treated (n=4) mice, #*P*<0.1 and **P*<0.05.

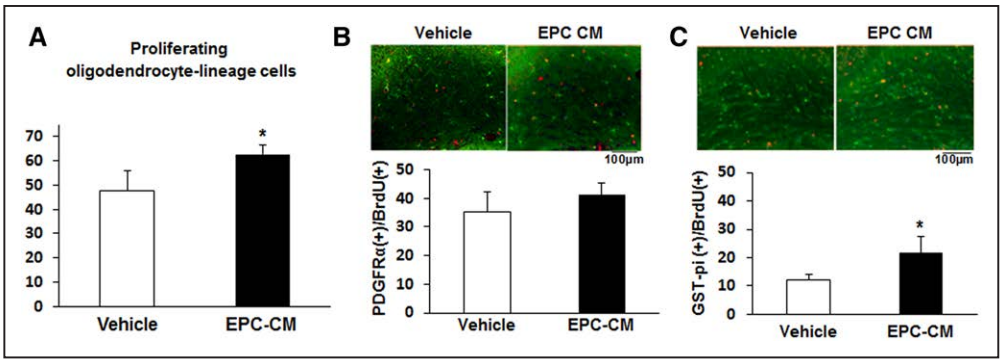

Figure 6. Immunohistochemistry study showing the maturation oligodendrocyte lineage cells. **A**, Bar graph showing the total number of proliferating oligodendrocyte-lineage cells in the corpus callosum area, including both PDGFR-α and glutathione-S-transferase-pi (precursor and mature oligodendrocytes, respectively) double-stained with 5-bromodeoxyuridine (BrdU). **B** and **C**, Representative images of the stained corpus callosum area and bar graphs representing the number of PDGFR-α(+)/BrdU(+) cells (**left**) and glutathione-S-transferase-pi(+)/BrdU(+) cells (**right**), in vehicle-treated (n=5) and EPC-CM-treated (n=4) mice. **P*<0.05.

of stem and progenitor cells have been successfully tested in preclinical models of stroke, multiple sclerosis, or neurodegenerative diseases.[23] However, the mechanisms of repair are still under investigation, including direct cell replacement, indirect secretion of nourishing factors, or the creation of a biobridge that connects the neurogenic sites of the brain with the injured areas with active remodeling.[24,25]

EPCs are bone marrow–derived cells capable of differentiating ex vivo into endothelial-phenotyped cells representing a model for endothelial generation and vascular repair.[26,27] Other studies have demonstrated the benefit of factors secreted by progenitor/stem cells, such as VEGF[28] or hepatocyte growth factor[8] or IL (interleukin)-8.[29] In this context, the secretome of EPCs is known to stimulate endothelial cell migration, growth, and function[30,31] and to protect from axonal degeneration in cultured cortical neurons exposed to oxygen–glucose deprivation.[32] In this regard, intravenously administered EPC-CM enhances vascular remodeling in a cortical model of stroke,[11] whereas intramuscular injections of similar products promoted tissue revascularization and recovery in a model of hindlimb ischemia.[33]

After white matter injury, Bai et al[8] have recently shown that CM obtained from mesenchymal stem cells is a valid therapeutic strategy to reverse the effects of inflammatory demyelinating diseases such as experimental autoimmune encephalomyelitis, a model of multiple sclerosis. The authors show that the effects of mesenchymal stem cells-CM are dependent on the presence of hepatocyte growth factor, accelerating remyelination, and functional recovery. To our knowledge, the present study is the first describing recovery from white matter injury after the administration of an EPC secretome in a cell-free approach.

The prolonged hypoperfusion model used in this study causes subcortical white matter injury affecting oligodendrocyte/myelin integrity, without apparent neuronal loss at 1 month.[17,19,34,35] Our study demonstrates for the first time the interaction of the EPC secretome with vascular and myelin remodeling by increasing vessel density, the number of proliferating oligodendrocyte lineage cells, and enhancing myelination in the corpus callosum. EPCs can be incorporated into neovessels,[26,36] and their restorative actions on vascular remodeling have been widely reported in vivo in stroke models[37–39] or traumatic brain injury.[40] At the same time, several nourishing factors have been reported to modulate EPCs function. For example, VEGF-C regulates the proliferation of neural progenitors expressing VEGFR-3[41] and stimulates the proliferation of EPCs in a hypoxia-ischemia in vivo model,[42] whereas VEGF-A strongly promotes OPC migration in a concentration-dependent manner, in vitro.[43]

We have identified the presence of several trophic, proteolytic, and signaling factors in the EPC-CM that support the therapeutic effects on white matter remodeling reported in this study. It is known that SDF1, VEGF, or MMPs could be responsible for maintaining the oligovascular niche in the normal and injured brain[44] and that hepatocyte growth factor is essential to accelerate remyelination and recovery in the experimental autoimmune encephalomyelitis model.[8] We have shown that several of these factors, among others, are present in our therapeutic EPC-CM. The relative contribution of each factor on oligovascular remodeling is unknown, but we pursue on exploring the role of angiogenin as a potent proangiogenic molecule that enhances endothelial angiogenesis,[12] which can be pharmacologically inhibited by antibiotics such as neomycin or neamine.[45,46] In this regard, we observe that brain endothelial cells proliferated with EPCs secretome treatment being reverted in the presence of neomycin, highlighting the angiogenin-dependent effects of secretome therapy in vascular remodeling. However, we acknowledge that our model systems cannot directly link angiogenin actions in the remodeling brain, especially on oligodendrocyte proliferation, and that other nourishing factors present in the EPCs secretome could be also contributing to the observed white matter repair. Nevertheless, others have suggested that angiogenin may have potent nonvascular effects such as neuroprotection against oxidative stress[47] or hypoxic injury.[48]

Importantly, we explore the cognitive function at the end of the study period when spatial working memory dysfunction has been observed,[19,49] allowing the evaluation of any therapeutic intervention. Our results show that the treatment with EPC-CM improves the functional outcome of mice exposed to cerebral hypoperfusion using the Y maze test. Moreover, the scores obtained for alternation rates by EPC-CM-treated mice

were similar to those scored by sham-operated mice in other studies of the group.[18] This finding seems to be selective for the cognitive function and not for motor function. The long-term cognitive status and the effect of prolonged treatments with EPCs secretome deserves to be further evaluated.

Summary

Treatment with the EPC secretome in a mouse model of prolonged cerebral hypoperfusion may improve cognitive function by enhancing oligovascular repair in white matter. These results highlight the importance of EPC-released proteins such as angiogenin in therapeutic strategies for white matter injury after cerebrovascular disease.

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Disclosures

None.

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Endothelial Progenitor Cell Secretome and Oligovascular Repair in a Mouse Model of Prolonged Cerebral Hypoperfusion

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SUPPLEMENTARY MATERIAL

METHODS

Cerebral prolonged hypoperfusion model

Briefly, male C57Bl/6 mice (25-30g, 2-3 months old, Charles River Institute) were anesthetized with 4.0% isoflurane and then maintained on 1.5% isoflurane in 70% N_2O and 30% O_2 using a small-animal anesthesia system. Through a midline cervical incision, both common carotid arteries were exposed. A microcoil with an internal diameter of 0.18 mm (Sawane Spring Co.) was applied to bilateral common carotid arteries. Rectal temperature was maintained between 36.5°C and 37.5°C. A total of 24 mice were initially subjected to the BCAS surgery. Since most of the BCAS-operated mice do not exhibit severe neurological dysfunction nor massive infarcts at least within 1 month after the surgery (1-3) we excluded animals that presented signs of paresis and convulsion, remarkable body weight loss (>20% from baseline during the follow-up period) and evident infarcts at the histological evaluation as follows: from 24 mice which were initially subjected to the BCAS surgery, 3 mice were euthanized during or immediately after the surgery after presenting bleeding from the CCA, left hemiparesis, and convulsion, respectively. The other 21 mice were divided into two groups; vehicle-treated (n=11) vs. EPC-CM-treated (n=10) mice. Three mice were excluded for further functional and histological evaluation because they showed remarkable body weight loss during the follow-up period (n=2) or an evident infarction at 28 days after the surgery (n=1).

Therefore, a total of 18 mice (vehicle-treated (n=10) and EPC-CM-treated (n=8) mice) were evaluated for functional outcome and white matter integrity.

Protocol for Conditioned Media Production

Mouse outgrowth Endothelial Cells (OECs) derived from EPC cultures of FVB mice obtained in a previous study (4) and stored in liquid nitrogen were used for the production of EPC-secretome as conditioned media (CM). Briefly, cells were thaw and seeded in fibronectin-coated 75cm^2 plastic flasks and grown in EGM-2MV OR EGM-2 media (consisting in EBM plus supplemental factors such as hEGF, Hydrocortisone, GA, VEGF, hFGF-B, IGF-1 and Ascorbic Acid, from Lonza) plus 10%FBS media. At confluence flasks contained about 4x10^6 cells, growing media was removed and cells were gently washed 3 times with PBS. Finally 12 mL of basal media (EBM) were added for 24 hours when CM was collected, transferred into a sterile tube and centrifuged at 1,500 rpm for 5 minutes to remove any cell debris. Finally the CM was concentrated using 10KDa-membrane centrifuge tubes (Sartorius, V0601) and spun for a total of 22 minutes at 4,000g at 21ºC (about 12x final volume). CM was obtained from 10 flasks at different days. EBM media was also concentrated as vehicle media but only for 3 minutes to obtain similar concentration volumes. Single aliquots of concentrated CM and concentrated basal media (BM, vehicle) were frozen at -80ºC until use.

Y Maze Test

The maze consists of 3 arms (40 cm long, 9.5 cm high, and 4 cm wide, labeled arm-A, -B, or -C) with equal angles between all arms. Experiments were performed in a dimly illumination room between 9:00pm to 11:00pm. Mice were initially placed within one arm and allowed to move in the maze freely. The sequence and number of arm entries were recorded for each mouse over an 8-minute period without reinforces such as food, water, or electrical foot shock. The task was videotaped with a video camera (Everio GZ-MG-77-S). The percentage of triads in which all three arms were represented (ABC, CAB, or BCA but not BAB) was calculated as an

alternation to estimate short-term memory of the last arms entered whereas the number of arm entries serves as an indicator of spontaneous activity.

Western Blot

Corpus callosum protein concentrations were quantified and adjusted to the same concentrations (0.5 μg/μl) by adding PBS, samples were mixed with equal volumes of sample buffer containing 91% SDS (Novex) and 9% 2-mercaptoethanol (Sigma). Subsequently, samples were heated at 95°C for 5 min and each sample (10 μg per lane) was loaded onto 4–20% Tris–glycine gels. After electrophoresis and transferring to nitrocellulose membranes (Novex), the membranes were blocked in Tris buffered saline with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk for 60 min at room temperature. Membranes were then incubated overnight at 4°C with anti-PDGFR-α (1:1000, Santacruz), myelin basic protein (MBP) antibody (1:500, Thermo scientific), anti-CD31 antibody (1:500, BD Pharmingen), or anti-β-actin antibody (1:10000, Sigma Aldrich) followed by incubation with peroxidase-conjugated secondary antibodies and visualization by enhanced chemiluminescence (Amersham).

Separately, EPC-CM were loaded in SDS-PAGE (12%) and transferred into PVDF membranes. Non-specific bindings were blocked with non-fat milk (10% in PBS-Tween), and membranes were incubated overnight with rabbit anti-ANG (NOVUS; 1:500) with non-fat milk (10% in PBS-Tween) at 4ºC. Secondary antibody was diluted 1:10.000 and membranes incubated at room temperature for 1 hour. The substrate reaction was developed with a chemiluminescent reagent and visualized with a luminescent image analyzer (Las-3000, FujiFilm; USA).

Immunohistochemistry

After fixation and cryoprotection, the brain was frozen and 16-μm-thick consecutive coronal sections at 0 to 0.5 mm anterior from the bregma were prepared using a cryostat. Brain sections were incubated at 37°C for 30 min in 1N HCl to detect BrdU labeling. Sections were incubated

overnight with anti-BrdU (1:50; Oxford Biotechnology). Double immunofluorescence staining was performed by simultaneously incubating the sections overnight at 4°C with anti-GST-pi (1:100, MBL) and anti-PDGFR-α (1:100, R&D systems) antibodies. For CD31 staining, the mouse was perfused transcardially with ice-cold saline, and the brain was removed, frozen on dry ice immediately and stored at -80℃ in light-shielded conditions. The brain was cut on a cryostat (20μm) and fixed with 4%PFA (15min), incubated with PBS-0.1%Tween (15min), and blocked with 10% blockase (AbD serotec, 60min) before sections were incubated at 4℃ overnight with anti-CD31 antibody (1:100, BD Pharmingen). Then sections were incubated with secondary antibodies with fluorescence conjugated secondary antibody (1:200; Jackson Immunoresearch Laboratories) at room temperature for 60 min. Subsequently, the slides were covered with Vectashield mounting medium with DAPI (H-1200, Vector Laboratories). Immunostaining was analyzed with a fluorescence microscope (Nikon) interfaced with a digital charge-coupled device camera. The number of the BrdU- and GST-pi- immunoreactive (BrdU+/GST-pi+), and BrdU- and PDGFR-α- immunoreactive (BrdU+/ PDGFR-α+) cells in the corpus callosum was counted by an investigator blinded to the experimental groups**.** Total number of proliferating oligodendrocyte lineage cells (which included both GST-pi+ and PDGFR-α+ / BrdU+) were also analyzed according to treatment.

Protein Profile of EPCs Secretome

Briefly, $2x10^6$ OECs were cultured as described above in 75 cm^2 flasks. After 24 hours they were washed twice and 12 ml of fresh basal media (EBM) were added to obtain the CM 24 hours later. The media were concentrated with 10K-membrane centrifugal filters (Amicon Ultra-0.5ml 10K Ultracel, Millipore, Germany), obtaining 0.4 to 0.5 ml of concentrated CM. The total protein amount in the concentrated CM was determined by Comassie Protein Assay kit (Thermo Scientific, USA). Next, the profile of proteins present in the OECs conditioned

media (n=4) was analyzed using the Proteome Profiler Mouse Angiogenesis Array kit (R&D Systems, USA) which can detect the expression of 53 mouse angiogenesis-related proteins. Briefly, 150ug of total protein of concentrated CM mixed with the biotinylated detection antibodies provided in the kit were incubated with the nitrocellulose membrane containing the capture antibodies (n=4). After an overnight incubation the membranes were washed and the Streptavidin-HRP and chemiluminescent detection reagents applied. The spot-signal was detected with the Luminescent Image Analyzer LAS-3000 (Fujifilm Medical systems, USA) and densities were analyzed with the Searchlight Array Analyst (Axiocor, Canada). Concentrated EBM was used as negative control. Results are expressed as neat chemilluminiscent units (by subtracting the background of the negative control).

Endothelial Cell Proliferation Assay

To test the pro-angiogenic properties of EPCs conditioned media on human cerebral microvascular endothelial cells (hCMEC/D3) we assessed cell proliferation after treatment with EPCs conditioned media, Angiogenin (a potent pro-angiogenic- mitogenic factor identified as aprt of EPCs secretome) and Neomycin (an inhibitor of Angiogenin). Briefly, $1x10^4$ human endothelial cells were seeded in 24/well plates in EGM-2 media (cointaining EBM, ½ of the supplemental factors hEGF, Hydrocortisone, GA, VEGF, hFGF-B, -IGF-1, Ascorbic Acid, Heparin) and 2% FBS) during 3 days. At day 4th wells were gently washed with PBS twice and treatments added in corresponding wells: conditioned media diluted 1/20 in EBM, Angiogenin or Neomycin at the mentioned dilutions. As controls concentrated EBM 1/20 or EBM were used, respectively. Each experiment was run in duplicates and data is expressed as percentage of control condition. The number of total and viable cells was counted with the Muse™ Cell Count and Viability Kit.

Endothelial Cell Viability Assay

Measurement of the reduction of 3-(4,5-dimethylthiazol- 2-yl)2,5-diphenyl-tetrazolium bromide (MTT) to produce a dark blue formazan product was performed to assess the integrity of mitochondrial function as a measure of cell viability in hCMEC/D3 treated with Neomycin at different doses for 24 hours when MTT was diluted basal media and added to cells for 60 minutes. Two wells per condition were run in each experiment and the obtained blue formazan was measured per duplicate to obtain a mean value. Results for treatments are expressed as a percentage of the control (basal media) group absorbance.

Oligodendrocyte Precursor Cell Culture

OPCs were prepared from cerebral cortices of 1-2 day old SD rat pups. Dissociated cortex cells were plated in poly-d-lysine-coated flasks, and cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 20% fetal bovine serum and 1% penicillin/streptomycin. After the cells were confluent, the flasks were shaken for 1 hour on an orbital shaker (220 rpm) at 37°C to remove microglia. The medium was changed and shacked overnight when the medium was collected and plated on non-coated tissue culture dishes for 1 hour at 37°C to eliminate possible contamination by astrocytes and remaining microglia. The non-adherent cells were collected and maintained in Neurobasal (NB) media containing glutamine, 1% penicillin/streptomycin, 10 ng/mL PDGF-AA, 10 ng/mL FGF-2 and 2% B27 supplement onto poly-dl-ornithine-coated plates.

Immunocytochemistry

Cultured EPCs or OPCs were immunostained for Myelin Basic Protein (MBP) or Angiogenin respectively. Briefly, cells were washed with ice-cold PBS (pH 7.4), treated with 4% PFA for 15 min, incubated with 0.1% triton for 10 min and further incubated with 3% BSA for 1 hr. Subsequently, cells were incubated with primary antibody against MBP (1:200, Thermo Scientific) or ANG (1:50) at 4°C overnight. After being washed with PBS, they were incubated with secondary antibodies with fluorescence conjugations for 1 hour at room temperature.

Finally, nuclei were counterstained with DAPI. Image was analyzed with a fluorescence microscope (Nikon) interfaced with a digital charge-coupled device camera and an image analysis system.

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TABLE & FIGURE LEGENDS

Supplementary Table I. List of proteins detected in the EPCs secretome.

Supplementary Figure I. Representation of the analyzed regions of the corpus callosum studied for by western blot and immunohistochemistry studies.

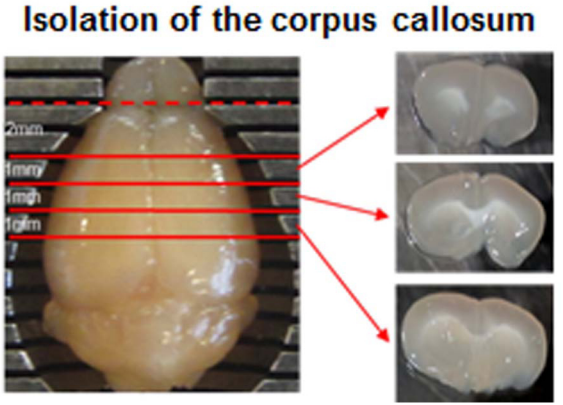
Supplementary Figure II. Chemilluminiscent signal of representative Proteome Profiler™ membranes at different acquisition times including both conditioned media (CM) and vehicle treatments. White circles show membrane positive controls, all other bright signal corresponds to expressed proteins.

Supplementary Figure III. Cell viability of mature human endothelial cells (hCMEC/D3) treated with neomycin. The potential toxic effects of Neomycin (used as an inhibitor for the ANG present in the Conditioned media) were tested by the MTT assay. No significant differences were observed at the tested concentrations (n=4).

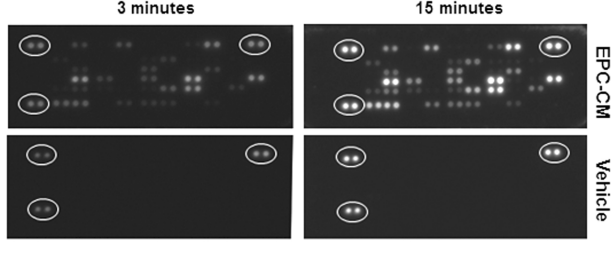
Supplementary Table 1. List of proteins detected in the EPCs secretome.

PROTEIN	Signal Intensity	PROTEIN	Signal Intensity
ADAMTS1	**79.2±40.6**	**IP-10/CXCL10**	**212.5±35.8**
Amphiregulin	n.d	**KC/CXCL1**	**101±46**
Angiogenin	**103.6±44.0**	Leptin	n.d
Angiopoietin-1	n.d	**MCP-1/CCL2**	**110.7±25**
Angiopoietin-3	n.d	MIP-1a/CCL3	6.5±5.8
Cogulation factor III	**37.8±25.5**	**MMP-3**	**203.6±59.1**
CXCL16	**170.5±55.8**	**MMP-8**	**13.2±8**
Cyr61	**44.3±28.2**	**MMP-9**	**7.7±2**
DLL4	n.d	**NOV**	**203.1±43.5**
DPPIV	n.d	**Osteopontin**	**31.7±18.7**
EGF	n.d	**PD-ECGF**	**57.7±38.8**
Endoglin	**21±12.9**	**PDGF-AA**	**26±15.6**
Endostatin/Collagen XVII	**84.8±34.2**	**PDGF-AB/PDGF-BB**	**6.7±5.5**
Endothelin-1	**10.7±7.8**	**Pentraxin-3**	**36.7±29.2**
FGF acidic	**10.5±6.5**	**Platelet factor 4/CXCL4**	**123.2±36.8**
FGF basic	n.d	**PlGF-2**	**197.1±57.6**
FGF-7/KGF	**5±4.5**	Prolactin	n.d
Fractalkine	**58.2±29.7**	**Proliferin**	**74.1±31**
GM-CSF	n.d	**SDF-1/CXCL12**	**159±56.7**
HB-EGF	**2.7±2.84**	**Serpin E1**	**165.3±49.7**
HGF	80.8±26.8	Serpin-E1/PAI-1	n.d
IGFBP-1	n.d	**Thrombospondin-2**	**63.25±24.5**
IGFBP-2	**20.7±12.4**	**TIMP-1**	**66.25±25.6**
IGFBP-3	n.d	**TIMP-4**	**41.25±26**
IL-1 alpha	n.d	VEGF	n.d
IL-1b	**10.2±5.8**	**VEGF-B**	**28.5±18.6**
IL-10	**7.8±5.9**		

Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.

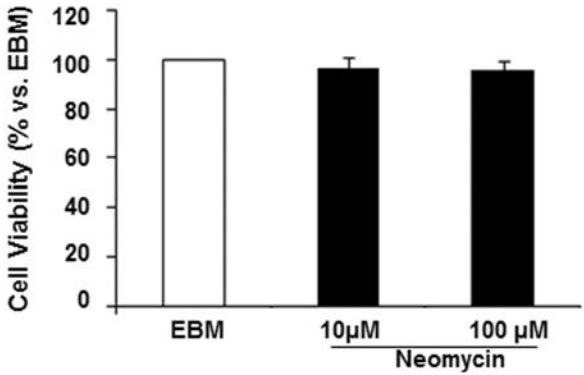


Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☑ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☑ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☑ An overall study timeline is provided.
Inclusion and exclusion criteria	☑ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.
Randomization	☑ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☐ Type and methods of randomization have been described. ☐ Methods used for allocation concealment have been reported.
Blinding	☑ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☑ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☐ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☑ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☐ Baseline data on assessed outcome(s) for all experimental groups have been reported. ☑ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. ☑ Statistical methods used have been reported. ☑ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☑ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☐ Different sex animals have been used. If not, the reason/justification is provided. ☑ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☑ Statements on funding and conflicts of interests have been provided.