eman ta zabal zazu

Universidad del País Vasco / Euskal Herriko Unibertsitatea
Facultad de Medicina y Enfermería
Departamento de Neurociencias

Microglial Phagocytosis of Apoptotic Cells Triggers a Neuromodulatory Program that Supports Neurogenesis

Tesis doctoral para optar al grado de Doctor, presentada por:

Irune Diaz Aparicio
2018

Directores de Tesis:

Dra. Amanda Sierra

Dr. Jorge Valero

TABLE OF CONTENTS

TABLE OF CONTENTS

1. ABBREVIATIONS

1. ABBREVIATIONS

µm	Micron
18S	18S ribosomal subunit
28S	28S ribosomal subunit
AD	Alzheimer's Disease
AFE	Agilent Feature Extraction
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ANP	Amplifying neuroprogenitor
AP	Anteroposterior
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ARTN	Artemin
Ascl	ASC1 like protein
Atf1	Activating transcription factor 1)
ATP	Adenosine triphosphate
ATP11C	ATPase Phospholipid Transporting 11C
ATPase	Adenosine triphosphatase
Aβ	Amyloid β
BAD	BCL2 associated agonist of cell death
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BLAST	Basic Local Aligment Search Tool
BLBP	Brain lipid binding protein
Bp	Base pair
BrdU	5-bromo-2 -deoxy-uridine
BSA	Bovine serum albumin
C1	Complement protein 1
C1q	Complement protein 1q
C3	Complement protein 3

3

ABBREVIATIONS

C3AR1	C3A receptor-1
C3b	Complement protein 3b
CA	Cornus Ammonis
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CD11b	Cluster of differentiation molecule 11B
CD68	Cluster of differentiation 68
cDNA	Complementary DNA
CM BV2 LPS	LPS treated BV2 CM
CM BV2	CM from control BV2
CM microC	CM from control microglia
CM microLPS 6 + 18h	CM from microglia treated with LPS for 6h and 18h of fresh media
CM microLPS	High dose LPS-treated microglia CM
CM microPH	CM from phagocytic microglia
CM	Conditioned media
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CR3	Complement receptor 3
CRT	Calreticulin
CSF	Colony stimulating factor
Csf3	Colony stimulating factor 3
CX3CL1	Microglial-specific CX3C motif chemokine ligand 1
CX3CR1	Microglial-specific CX3C motif chemokine receptor 1
Cy3	Cyanine 3
D	Day
DAP12	DNAX-activation protein of 12 kD
DAPI	4',6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DCX	Doublecortin
DG	Dentate gyrus
Dll	Delta-like
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium with F12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DNAse	Deoxyribonuclease
DV	Dorsoventral
E	Embryogenic day
E2F1	E2F transcription factor 1
EAE	Experimental autoimmune encephalomyelitis
ECL	Enhance chemiluminiscence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	EGF receptor
EIF2	eukaryotic initiation factor 2
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ErbB	EGF receptor
FACS	Fluorescence-activated cell sorting
FAS	Fas cell surface death receptor
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable region
FDR	False discovery rate
FE	Feature Extraction
FGF	Fibroblast growth factor
FGF-2	Fibroblast growth factor-2
FGF-8	Fibroblast growth factor 8
FGFR	Fibroblast growth factor receptor
FOXO3	Forkhead box O3
FSC	Fordward scatter
FU	5'-Fluorouridine
Fura-2 AM	Fura-2-acetoxymethil ester
Fwd	Forward
G2A	G protein-coupled receptor 2A
Gal-3	Galectin-3
GAS1	growth arrest specific 1
GAS6	Growth arrest-specific 6
gC1qR	Globular C1q receptor
G-CSF	Granule-colony stimulating factor

5

ABBREVIATIONS

G-CSFR	Granule-colony stimulating factor receptor
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFL	GDNF family ligands
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor receptor
GO	Gene ontology
gp130	Glycoprotein 130
GPR34	G protein-coupled receptor 34
GPR81	G protein coupled receptor 81
Gria	Glutamate receptor ionotropic AMPA
Grik	Glutamate receptor ionotropic kainate
Grin	Glutamate receptor ionotropic NMDA
GRM	Glutamate receptor metabotropic
H	Hours
H3K27Ac	Acetylation at the 27th lysine residue of the histone H3· protein
H3K4me	Methylation at the 4th lysine residue of the histone H3· protein
H3K4me1	Mono-Methylation at the 4th lysine residue of the histone H3· protein
H3K4me2	Bi-Methylation at the 4th lysine residue of the histone H3· protein
HBSS	Hank's balanced salt solution
HCL	Hierarchical Clustering
HCl	Hydrochloride acid
HD	Huntington's disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hif-1α	Hypoxia-inducible factor-1-α
HPRT	Hypoxanthine guanine phosphoribosyl transferase
HRP	Horseradish Peroxidase
HSPG	Heparin sulfate proteoglycans
IFC	Integrated fluidic circuit
IFN	Interferon
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor1
IL	Interleukins

IL-10	Interleukin-10
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RI	Type I IL-1 receptor
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin 1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
iNOS	Inducible nitric oxide synthase
IPA	Ingenuity Pathway Analysis
iPLA2	Phospholipase A2
Jag1	Jagged1
k score	Kappa score
KA	Kainate
KCl	Potassium chloride
kDa	Kilo dalton
KEGG	Kyoto encyclopedia of genes and genomes
KO	Knock out
L27A	60S ribosomal protein L27A
LL	Laterolateral
LPC	Lysophosphatidylcholine
LPS	Bacterial lipopolysaccharide
LRP	Low-density lipoprotein receptor related protein
LysoPS	Lysophosphatidylserine
M	Molar
MANGO	Mammalian Adult Neurogenesis Gene Ontology
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein
M-CSF	Macrophage colony stimulating factor
MEGF10	Multiple epidermal growth factor-like domains protein 10
MEM	Minimum Essential Medium
MerTK	Mer tyrosine kinase
MFG-E8	Milk fat globule EGF factor 8
MgSO4	Magnesium sulfate
MIAME	Minimum Information About a Microarray Experiment

ABBREVIATIONS

MicroC	Control microglia
Min	Minutes
MIP-1a	Macrophage-inflammaroty protein
MMLV	Moloney Murine Leukemia Virus
MMP3	Matrix metallopeptidase 3
MMPs	Matrix metalloproteinases
Mo	Month old
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NaH2PO4	Sodium phosphate monobasic
NaHCO3	Sodium bicarbonate
NBOR	Neighborhood-based ordering of single cells
NeuN	Neuronal nuclei
NGF	Neuronal growth factor
NLGN3	Neuroligin 3
NMDA	N-methyl-D-aspartate)
NO	Nitric oxide
NPC	Neuroprogenitor cell
NPC	Neuroprogenitor cells
NRG1	Neuregulin 1
NRTN	Neurturin
NSC	Neural stem cell
NT-4	Neurotrophin-4
OAZ-1	Ornithine decarboxylase antizyme
P	Postnatal day
p53	Tumour protein 53
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular patterns
PANTHER	Protein Analysis Through Evolutionary Relationships
PBS	Phosphate buffered saline
PCA	Principal Component Analysis
PD	Parkinson's disease
PDGF	Platelet-derived growth factor

PDGFR	Platelet-derived growth factor receptor
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
Ph M dead	Apoptotic cell media
Ph	Phagocytosis
Ph+LPS	Ph24h followed by LPS
Ph24h	24h phagocytic microglia
Ph-index	Phagocytic index
PI	Propidium iodide
PI3K	Phosphatidylinositol3 kinase
PLA	Phospholipases
Plcγ	Phospholipase C γ
PNS	Peripheral nervous system
Pol	Polymerase
Poly(I:C)	Polyinosinic:polycytidylic acid
Prox1	Prospero homeobox 1
PS	Phosphatidylserine
PSA-NCAM	Polysialated neural cell adhesion protein
p-Smad	Phospho-Smad
PSPN	Persephin
PTEN	Phosphatase and tensin homolog
REST	RE1 silencing transcription factor 1
Rev	Reverse
RIN	RNA integrity number
RNA pol	RNA polimerase
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
rNSCs	Radial neural stem cells
ROS	Reactive oxygen species
Rpm	Revolutions per minute
Rrm1	Ribonucleotide reductase catalytic subunit M1
Rsq	R-squared
RT	Room temperature
RT-qPCR	Real Time-Quantitative Polymerase Chain Reaction
S100a8	S100 calcium binding protein A8

9

ABBREVIATIONS

S100a8/a9	S100a8 and S100a9 heterodimer
S100a9	S100 calcium binding protein A9
S100β	S100 calcium binding protein b
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
SCG2	Secretogranin 2
Sec	Seconds
SEM	Standard error of the mean
SGZ	Subgranular zone
sIL-6R	Soluble fraction of interleukin-6 receptor
SIP	Solution of Isotonic Percoll
SNARE	Soluble NSF Attachment Protein Receptor
Sox2	SRY box 2
SSC	Side Scatter
STB	Standard tris buffer
STP	Staurosporine
SVZ	Subventricular zone
TAM	Tyro3, Axl, and Mer
TBS-T	Tris buffer saline tween20
TCA	Trichloroacetic acid
TGF	Transforming growth factors
TGFBR	Transforming growth factor receptor
TGFα	Transforming growth factor β
TGFβ	Transforming growth factor β
TLR	Toll-like-receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TNF-R	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells-2
tSNE	t-Distributed Stochastic Neighbor Embedding
U	Units
Ucp2	Uncoupling protein 2
UTP	Uridine triphosphate
vATPases	Vacuolar ATPases

VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VGF	Non acronym
VNR	Vitronectin receptor
WGCNA	Weighted gene co-expression network analysis
Wnt	Wingless
Xkr8	Xk-related protein 8

2. RESUMEN/SUMMARY

2. RESUMEN / SUMMARY

2.1. RESUMEN

La neurogénesis, o formación de nuevas neuronas, continúa durante la vida adulta en roedores y humanos en dos regiones bien definidas: la región subventricular, que genera interneuronas destinadas al bulbo olfativo; y la región subgranular (SGZ) del hipocampo, que genera neuronas granulares que se integran en el circuito hipocampal y participan en los procesos de memoria y aprendizaje. Múltiples son los factores endógenos que regulan la proliferación, supervivencia, diferenciación e integración de las nuevas neuronas en el hipocampo adulto. Entre ellos, nuestro grupo ha descrito recientemente el papel de la microglía, que se encarga de eliminar mediante fagocitosis el exceso de células recién nacidas (neuroprogenitores, neuroblastos, y neuronas) que entran en apoptosis o muerte celular programada. La microglía es el macrófago residente del sistema nervioso, y además de coordinar la respuesta inflamatoria es el fagocito cerebral profesional. Mientras que la respuesta inflamatoria está muy bien descrita, la fagocitosis microglial es una gran desconocida. Las prolongaciones microgliales son altamente móviles, lo que permite el "escaneado" constante del tejido cerebral en busca de alteraciones de la homeostasis. Además, la microglía está equipada con receptores para las señales "find-me" y "eat-me" producidas por las células apoptóticas, hacia las que se induce una respuesta quimiotáctica positiva que termina en el englobamiento del residuo celular en un fagosoma, que posteriormente se fusionará con lisosomas para su completa degradación. En el hipocampo, la microglía "en reposo" del nicho neurogénico es capaz de reconocer y eliminar de manera muy eficaz las células apoptóticas recién nacidas, en un proceso que dura menos de 1.5h. La fagocitosis impide la liberación de contenidos intracelulares y además es activamente anti-inflamatoria, por lo que es esencial para evitar la alteración del tejido circundante. En este estudio, proponemos que la fagocitosis microglial no termina con la eliminación física de las células apoptóticas si no que, además, **la fagocitosis inicia la producción de factores neuromoduladores que contribuyen directamente al correcto mantenimiento de la cascada neurogénica hipocampal adulta.**

Para demostrar nuestra hipótesis, nuestro **Objetivo 1** ha sido poner a punto un modelo in vitro de fagocitosis que recopilase el mayor número de características posibles de la fagocitosis in vivo. Nos enfocamos especialmente en el tipo de cargo, en un englobamiento

temprano y en la presencia de moléculas opsonizadoras clave. La microglía es capaz de fagocitar diversos cargos, pero para la presente tesis, nos centramos en concreto en la fagocitosis de células apoptóticas. Como diana fisiológica para este estudio se utilizaron líneas celulares de ratón (NE-4C) y humanas (SH-SY5Y), a las que inducimos apoptosis. Otra característica de la fagocitosis in vivo es que las células apoptóticas son rápidamente eliminadas, por lo que para este estudio nos aseguramos que la inducción de apoptosis de las líneas celulares fuese corta, de manera que la microglía únicamente fagocitase células apoptóticas tempranas y no células que ya estuviesen perdiendo la integridad de su membrana (necróticas secundarias). Por último, la presencia o ausencia de moléculas opsonizadoras como la molécula del complemento C1q se han descrito como claves para determinar la respuesta inmune de la fagocitosis microglial, por lo que analizamos su papel en relación a las células apoptóticas y a la microglía antes de llevar a cabo el ensayo de fagocitosis. Una vez estudiadas en detalle todas estas características, finalmente pusimos a punto un ensayo de fagocitosis in vitro que consistía en alimentar microglía primaria murina con líneas celulares (NE-4C o SH-SY5Y) previamente tratadas con estaurosporina para la inducción de apoptosis, en presencia de C1q en el suero. Para los siguientes objetivos, utilizamos el modelo xenogénico (microglía murina, SH-SY5Y humanas), lo que nos permitió detectar de forma específica los transcritos de microglía de ratón en los posteriores análisis transcripcionales.

Nuestro **Objetivo 2** ha sido el estudio de los cambios transcripcionales de la microglía producidos tras la fagocitosis de células apoptóticas. Para ello, utilizamos el modelo de fagocitosis in vitro detallado en el párrafo anterior y realizamos un estudio comparativo de la transcripción en microglía naïve vs. microglía fagocítica usando matrices de expresión génica ("gene arrays"). Tras una serie de filtrados estadísticos, aquellos genes que presentaban cambios significativos fueron clasificados acorde a su perfil de expresión: genes con expresión incrementada o reducida a lo largo del tiempo, y genes con expresión aumentada o disminuida de forma transitoria. A continuación, realizamos un análisis funcional de los patrones de expresión utilizando tres plataformas bioinformáticas distintas: DAVID, ClueGO e IPA. Los tres análisis revelaron la presencia de diversas funciones relacionadas con la neurogénesis que estaban significativamente alteradas en la microglía fagocítica. Asimismo, el análisis funcional también mostró cambios en numerosas vías de señalización como las metabólicas por ejemplo, que abren las puertas para futuros estudios. Con todo esto, nuestros resultados sugerían la existencia de un programa neuromodulador en la microglía iniciado por la fagocitosis.

Nuestro **Objetivo 3** ha sido el estudio de la identidad de aquellas moléculas potencialmente neuromoduladoras producidas por la microglía fagocítica. Para esta tarea realizamos un primer filtrado de los genes de la base de datos de MANGO (The Mammalian Adult Neurogenesis Gene Ontology), separándolos en dos categorías: genes autólogos, cuya función es actuar en la misma célula que lo produce; y genes heterólogos, que actúan fuera de la célula que los produce. Debido a que la modulación de la neurogénesis por la microglía fagocítica sólo se podría llevar a cabo mediante genes heterólogos, nuestro siguiente paso fue buscar todos los genes heterólogos en nuestro array relacionados con la neurogénesis con ayuda de los identificadores de función (GO terms) obtenidos de MANGO. Finalmente, se realizó un filtrado manual para seleccionar aquellos genes que estuviesen posicionados de manera extracelular en la microglía. Varios de los genes seleccionados que presentaban grandes cambios o que habían sido clásicamente descritos como neuromoduladores fueron analizados mediante RT-qPCR para validar su expresión.

Nuestro **Objetivo 4** ha consistido en analizar los efectos en la neurogénesis in vitro de las moléculas producidas por la microglía fagocítica. Para llevar a cabo este objetivo, pusimos a punto un modelo de neurogénesis in vitro utilizando neurosferas. Las neurosferas obtenidas de cerebros postnatales de ratón fueron expandidas durante una semana y posteriormente disociadas y sembradas sobre cubreobjetos de cristal en los que se les permitió proliferar durante 48h. Tras este periodo, los neuroprogenitores se trataron con los medios condicionados de microglía control y fagocítica durante 3 y 5 días. Tras el tratamiento observamos la presencia de diferentes fenotipos celulares: células estrelladas, que expresaban altos niveles de la proteína ácida fibrilar glial (glial fibrillary acidic protein, GFAP); células ramificadas, que expresaban altos niveles de doblecortina (DCX) y finalmente células bipolares, que expresaban altos niveles de nestina y GFAP. En los cultivos tratados con medio condicionado de microglía fagocítica, casi el 90% de las células eran bipolares y no había ninguna célula que expresase DCX; mientras que en los cultivos tratados con medio condicionado de microglía control, la mayoría de las células eran estrelladas y un pequeño porcentaje eran células DCX. Debido a su morfología y expresión de marcadores, clasificamos las células estrelladas como astrocitos y las ramificadas como neuroblastos. Sin embargo, la clasificación de células bipolares requería mayor caracterización.

Dado que las células bipolares no expresaban DCX y poseían altos niveles de nestina y GFAP, concluimos que se podían tratar o bien de astrocitos o bien de células

17

neuroprogenitoras que se mantenían indiferenciadas. Para estudiar la primera posibilidad, realizamos una inmunofluorescencia con S100β, marcador de astrocitos maduros, en los neuroprogenitores tratados con medio condicionado de microglía control o fagocítica. En los cultivos tratados con medio condicionado de microglía control, sólo un pequeño porcentaje de células estrelladas presentaba marcaje de S100β, lo que sugería que la mayoría de los astrocitos del cultivo aún eran inmaduros. También descubrimos un pequeño porcentaje de células que únicamente se marcaban con S100β, y que por su morfología identificamos como oligodendrocitos. En los cultivos tratados con medio condicionado de microglía fagocítica, cerca de la mitad de las células bipolares presentaban marcaje débil de S100β, lo que podría sugerir que las células bipolares pertenecen al linaje astrocitario. Para estudiar la posibilidad de que las células bipolares fuesen neuroprogenitores aún sin diferenciar, realizamos un ensayo de pluripotencia en el que tras tratar a las células con sus correspondientes medios condicionados, el medio fue reemplazado por el medio habitual de diferenciación de las neurosferas con objeto de sacar del arresto celular al fenotipo bipolar. Tras 5 y 9d de diferenciación, el cultivo tratado con medio de microglía fagocítica presentaba un porcentaje invertido entre células bipolares y estrelladas, encontrando un número mayor de estas últimas, y nunca presentaba células DCX, lo que, una vez más, sugería que este fenotipo pertenece a un linaje astrocitario. Además, para una caracterización más profunda de los distintos fenotipos, realizamos estudios de imagen de calcio de los cultivos tratados con microglía control y fagocítica. Estos experimentos demostraron que las células bipolares compartían características tanto con las células estrelladas como con neuroprogenitores indiferenciados recién disociados de las neurosferas, por lo tanto, concluimos que el fenotipo bipolar pertenece a células inmaduras adscritas a un linaje astrocitario.

Nuestro **Objetivo 5** ha sido estudiar la función en la neurogénesis de las citoquinas liberadas por la microglía fagocítica. El análisis transcripcional de la microglía fagocítica de los objetivos 2 y 3 reveló la producción de ciertas citoquinas pro-inflamatorias por la microglía fagocítica. Para determinar si estas citoquinas eran las causantes de los distintos fenotipos observados en el ensayo de neurogénesis (Objetivo 4), realizamos un estudio tratando a la microglía primaria con lipolisacáridos bacterianos (LPS) para inducir inflamación y cultivamos las células progenitoras con este medio microglía condicionado. Observamos que los fenotipos resultantes de este tratamiento eran los mismos que al tratar el cultivo con medio condicionado de microglía control, y además, nunca se observó ningún fenotipo bipolar. Estos resultados descartan que el efecto de la microglía fagocítica sobre las neuroesferas esté mediado por citoquinas.

Nuestro **Objetivo 6** ha sido la identificación de los factores concretos que promueven los efectos en la neurogénesis in vitro. Para ello nos centramos en dos de los factores más prometedores que habíamos encontrado en los matrices de expresión génica, VGF y lactato. Para validar la expresión proteica de VGF realizamos inmunofluorescencia en cultivos de microglía fagocítica usando diversos anticuerpos contra diferentes variantes postranscripcionales de VGF. Observamos que tras fagocitar, VGF se expresaba más intensamente por toda la célula de microglía, validando la sobreexpresión observada mediante RT-qPCR. Por otro lado, para la detección de lactato utilizamos ensayos colorimétricos. No observamos diferencias en la producción de lactato entre el medio condicionado de la microglía control y el de la fagocítica, pero sí observamos que la microglía tratada con LPS producía mayor cantidad de lactato. Estos resultados sugieren que el VGF puede ser uno de los mecanismos implicados en el control de la neurogénesis por la microglía fagocítica.

Nuestro **Objetivo 7** ha sido la validación in vivo del programa neuromodulador de la microglía fagocítica obtenidos en los objetivos 2 y 3. Para ello realizamos un análisis de secuenciación de RNA de células individuales (single-cell RNA sequencing) comparando una población enriquecida en microglía fagocítica obtenida del giro dentado (región en la que existe una neurogénesis continua y por lo tanto también apoptosis), con una población de microglía no fagocítica obtenida del CA del hipocampo (región en la que al no haber neurogénesis, tampoco hay apoptosis). Desafortunadamente, esta técnica no logró encontrar diferencias entre las poblaciones de giro dentado y CA, probablemente debido a que la población de giro dentado no está suficientemente enriquecida en microglía fagocítica. Por lo tanto, esto conlleva la necesidad de diseñar futuras estrategias en las que poder demostrar la implicación de la microglía fagocítica en la neurogénesis.

Nuestro **Objetivo 8** ha sido analizar los efectos en la neurogénesis in vivo de los factores secretados por la microglía fagocítica. Para ello infundimos el medio condicionado de microglía control y fagocítica mediante bombas osmóticas en el hipocampo de ratones adultos durante 6 días. Tras este periodo, inyectamos BrdU y sacrificamos a los animales 2h y 28d después. A las 2h observamos no observamos diferencias en la proliferación tras el tratamiento con medio condicionado de microglía fagocítica, ni sobre el número de neuroblastos. Sin embargo, a los 28d observamos un descenso en el porcentaje de neuroblastos más maduros lo que sugiere que los factores secretados por la microglía fagocítica alteran la neurogénesis hipocampal adulta.

Nuestro **Objetivo 9** ha sido caracterizar diferentes tipos de receptores que participan en la fagocitosis microglial con la intención de poder diseñar futuras estrategias para suprimir la expresión de dichos genes y estudiar la neurogénesis en ratones que posean una fagocitosis impedida. Para ello estudiamos la expresión de las subunidades de todos los receptores de glutamato mediante RT-qPCR en microglía aislada por FACS. Encontramos una expresión residual de todas las subunidades en microglía, lo que hace improbable que formen receptores funcionales. Además, también analizamos diversos receptores fagocíticos y purinérgicos mediante RT-qPCR en microglía aislada por FACS de ratones control y tratados con el análogo del neurotrasmisor glutamato, kainato (KA), en los que habíamos demostrado previamente que la fagocitosis microglial estaba dañada. Encontramos que el tratamiento con KA reducía la expresión de todos los receptores fagocíticos mientras incrementaba la expresión de la mayoría de los receptores purinérgicos en la microglía. Estos resultados sugieren que la modulación de la expresión o función de los receptores purinérgicos y de reconocimiento puede alterar la fagocitosis microglial y por lo tanto, podrían ser utilizados como métodos alternativos para poder estudiar el impacto de la fagocitosis microglial sobre la neurogénesis.

En conclusión, nuestros datos demuestran que la microglía fagocítica libera factores que controlan la diferenciación y supervivencia de los neuroprogenitores tanto en cultivo como en la cascada neurogénica hipocampal adulta. Estos resultados sugieren que la fagocitosis microglial es un mecanismo que actúa como el freno de la neurogénesis para mantener la homeostasis de la cascada hipocampal adulta.

2.2. SUMMARY

Neurogenesis, or formation of new neurons, continues throughout adulthood in rodents and humans in two well-defined regions: the subventricular region, which generates interneurons destined for the olfactory bulb; and the subgranular region (SGZ) of the hippocampus, which generates granular neurons that are integrated into the hippocampal circuit and participate in memory and learning processes. There are multiple endogenous factors that regulate the proliferation, survival, differentiation and integration of the new neurons in the adult hippocampus. Among them, our group has recently described the role of microglia, which is responsible for eliminating by phagocytosis the excess of newborn cells (neuroprogenitors, neuroblasts, and neurons) that undergo apoptosis or programmed cell death. Microglia are the resident macrophages of the nervous system, and in addition to

coordinating the inflammatory response they are the professional brain phagocytes. While the inflammatory response is well described, microglial phagocytosis is largely unknown. The microglial branches are highly motile, supporting a constant scanning of brain searching for alterations in homeostasis. In addition, microglia are equipped with receptors for the 'find-me' and 'eat-me' signals produced by apoptotic cells, which exert a positive chemotactic response that results in the engulfment of the apoptotic cell in a phagosome, which subsequently will fuse with lysosomes for its complete degradation. In the adult hippocampus, resting microglia present in the niche is capable of recognizing and eliminating newborn apoptotic cells very effectively, in a process that lasts less than 1.5 hours. Phagocytosis prevents the release of toxic intracellular contents and thus, it is essential to avoid the alteration of the surrounding tissue. In this study, we propose that microglial phagocytosis does conclude with the physical elimination of apoptotic cells, but in addition, **phagocytosis triggers the production of neuromodulatory factors that directly contribute to the correct maintenance of the adult hipocampal neurogenic cascade.**

In order to demonstrate our hypothesis, our **Aim 1** was to develop an in vitro model of phagocytosis that mimicked the highest possible number of in vivo phagocytosis characteristics. We focused especially on the type of cargo, on an early engulfment, and on the presence of key opsonizing molecules. Microglia is able to phagocyte a large variety of cargos, but for this thesis, we focused specifically on the phagocytosis of apoptotic cells. As a physiological target for this study, mouse (NE-4C) and human (SH-SY5Y) cell lines were used to induce apoptosis. Another characteristic of in vivo phagocytosis is that apoptotic cells are rapidly eliminated, so for this study we ensured a short induction of apoptosis, so that microglia would only phagocytose early apoptotic cells and not cells that they were already losing their membrane integrity (secondary necrotic cells). Finally, the presence or absence of opsonizing molecules such as the complement molecule C1q have been described as key to determine the immune response of microglial phagocytosis. We therefore analyzed the role of C1q on microglial phagocytosis of apoptotic cells in vivo before performing the in vitro phagocytic assay. Once all these characteristics were studied in detail, we finally developed an in vitro phagocytosis assay that consisted on feeding murine primary microglia with cell lines (NE-4C or SH-SY5Y) previously treated with staurosporine for the induction of apoptosis. For the following aims, the xenogeneic model (murine microglia, human SH-SY5Y) was used, which allowed us to detect specifically the transcripts of mouse microglia in the subsequent transcriptional analyzes.

21

RESUMEN / SUMMARY

Our **Aim 2** was the study of the transcriptional changes produced in microglia after phagocytosis of apoptotic cells. We used the in vitro model of phagocytosis detailed in the previous paragraph and performed a comparative transcriptional study of naïve vs. phagocytic microglia using gene expression arrays. After applying several statistical filters, those genes that presented significant changes were classified according to their expression profile: up-regulated genes over time, down-regulated, and genes transiently up- or down-regulated. Next, we performed a functional analysis of the expression patterns using three different bioinformatic platforms: DAVID, ClueGO and IPA. All three analyzes revealed the presence of various functions related to neurogenesis that were significantly altered in phagocytic microglia. In addition, the functional analysis also showed changes in numerous signaling pathways such as metabolism, which opened the doors for future studies. Overall, our results suggested the existence of a neuromodulatory program in microglia triggered by phagocytosis.

Our **Aim 3** was the study of the identity of those potentially neuromodulatory molecules produced by phagocytic microglia. For this task we performed a first filtering of the MANGO database genes (The Mammalian Adult Neurogenesis Gene Ontology) which we divided into two categories: autologous genes, which act in the same cell in which are produced; and heterologous genes, which act outside the cell that produced them. Since the modulation of neurogenesis by the phagocytic microglia could only be exerted by heterologous genes, our next step was to filter all the heterologous genes in our array with the help of the GO terms related to the neurogenesis obtained from MANGO heterologous genes. Finally, a manual filtering was performed to select those genes that were extracellularly located in microglia. Several of the selected genes that showed great changes or that had been classically described as neuromodulators were analyzed by RT-qPCR to validate their expression.

Our **Aim 4** was to analyze the effects on in vitro neurogenesis of the molecules produced by phagocytic microglia. We developed a model of in vitro neurogenesis using neurospheres. Neurospheres obtained from mouse postnatal brains were expanded for a week and then dissociated and seeded on glass coverslips in which they were allowed to proliferate for 48h. After this period, the neuroprogenitors were treated with conditioned media from control and phagocytic microglia for 3 and 5 days. After the treatment, we observed the presence of different cellular phenotypes: stellate cells, which expressed high levels of glial cell fibrillary acidic protein (GFAP); ramified cells, which expressed high levels of doublecortin (DCX), and finally bipolar cells, which expressed high levels of nestin and GFAP. In cultures treated with conditioned medium from phagocytic microglia, almost 90% of the cells were bipolar and there

22

were no cells expressing DCX. In contrast, in cultures treated with control microglia conditioned medium, almost 90% of the cells were stellate and about 10% were DCX cells. Due to their morphology and expression of markers, we classified stellate cells as astrocytes and ramified cells as neuroblasts. However, the classification of bipolar cells required further characterization.

Because bipolar cells did not express DCX and showed high levels of nestin and GFAP, we concluded that they could be either astrocytes or neuroprogenitor cells that remained undifferentiated. To study the first scenario, we performed immunofluorescence for S100β, a marker of mature astrocytes, in neuroprogenitors treated with conditioned medium from control or phagocytic microglia. In cultures treated with control microglia conditioned medium, only a small percentage of stellated cells showed S100β labeling, which suggested that most of the culture astrocytes were still immature. We also discovered a small percentage of cells that were only labeled with S100β, and by their morphology we identify them as oligodendrocytes. In cultures treated with conditioned medium of phagocytic microglia, about half of the bipolar cells had weak S100β labeling, which suggest that bipolar cells belong to an astrocytic lineage. To study the possibility that the bipolar cells were neuroprogenitors still to be differentiated, we performed a pluripotency test in which after treating the cells with their corresponding conditioned media, the medium was replaced by the usual media of differentiation of the neurospheres in order to allow bipolar phenotype to differentiate. After 5 and 9d of differentiation, the culture treated with phagocytic microglia medium presented an inverted percentage between bipolar and stellate cells compared to cultures treated with control microglia conditioned medium, again suggesting that this phenotype belonged to an astrocitary lineage. In addition, for a deeper characterization of the different phenotypes, we performed calcium imaging studies of the cultures treated with control and phagocytic microglia. These experiments demonstrated that bipolar cells shared characteristics with both stellar cells and with undifferentiated neuroprogenitors newly dissociated from neurospheres, and therefore, we conclude that the bipolar phenotype belongs to immature cells ascribed to an astrocytic lineage.

Our **Aim 5** was to study the function of the cytokines released by phagocytic microglia on neurogenesis in vitro. The transcriptional analysis of the phagocytic microglia from aims 2 and 3 revealed the production of certain pro-inflammatory cytokines by phagocytic microglia. To determine if these cytokines were responsible for the different phenotypes observed in the neurogenesis assay (Aim 4), we treated primary microglia with LPS to induce inflammation and

cultured the progenitor cells with this conditioned medium. We observed that the phenotypes resulting from this treatment were identical to those resulting from treatment with control microglia conditioned medium, and, in addition, bipolar cells were never observed. These results suggest that the effect of phagocytic microglia on neurospheres is not mediated by cytokines.

Our **Aim 6** was the identification of the specific factors that promote the effects in neurogenesis in vitro. We focused on two of the most promising factors found in the gene arrays, VGF and lactate. To validate the protein expression of VGF, we performed immunofluorescence in phagocytic microglia cultures of different antibodies against different posttranscriptional variants of VGF. We observed that after phagocytosing, VGF was more intensely expressed throughout microglia, validating the overexpression observed by RT-qPCR. On the other hand, for the detection of lactate we used colorimetric tests. We found no differences between the lactate production in conditioned media from control and phagocytic microglia. However, LPS-treated microglia showed a higher production of lactate that control or phagocytic microglia. These results suggest that VGF could be one of the factors involved in the effects of phagocytic microglia on neurogenesis in vitro.

Our **Aim 7** was to validate in vivo the neuromodulatory program of the phagocytic microglia obtained in aims 2 and 3. For this purpose, we performed a single-cell RNA sequencing analysis in order to compare a population enriched in phagocytic microglia obtained from the dentate gyrus (a region in which there is ongoing neurogenesis and therefore also apoptosis), with a population of non-phagocytic microglia obtained from the hippocampal CA (a region in which there is no neurogenesis and therefore, no apoptosis). Unfortunately, this method did not find differences in the expression pattern between the microglial populations of the dentate gyrus and CA, probably because the population of dentate gyrus is not sufficiently enriched in phagocytic microglia. Therefore, future strategies in order to be able to demonstrate the role of phagocytic microglia in neurogenesis must be designed.

Our **Aim 8** was to analyze the effects on in vivo neurogenesis of the factors secreted by phagocytic microglia. For this purpose, we infused the conditioned medium of control and phagocytic microglia in the hippocampus of adult mice for 6 days using osmotic pumps. After this period, we injected BrdU and sacrificed the animals 2h and 28d later. At 2h we did not observe differences in proliferation after treatment with conditioned medium of phagocytic

microglia or on the number of neuroblasts. However, at 28d we observed a decrease in the percentage of more mature neuroblasts, which suggests that the factors secreted by phagocytic microglia alter neurogenesis.

Our **Aim 9** was to characterize different types of receptors that participate in microglial phagocytosis in order to be able to design future strategies to study the impact of phagocytosis on neurogenesis. For this purpose, we analyzed the subunits of all glutamate receptors by RT-qPCR in microglia isolated by FACS. We found a residual expression of all subunits in microglia, which makes it unlikely that they form functional receptors. In addition, we also analyzed various phagocytic and purinergic receptors by RT-qPCR in isolated microglia by FACS of control mice and treated with the neurotransmitter glutamate analog, kainate (KA), in which we had previously shown that microglial phagocytosis was impaired. We found that KA treatment reduced the expression of all phagocytic receptors while increasing the expression of most purinergic receptors in the microglia. These results suggest that modulation of the expression or function of purinergic receptors and recognition can alter microglial phagocytosis and therefore, could be used as alternative methods to study the impact of microglial phagocytosis on neurogenesis.

In conclusion, our data shows that phagocytic microglia releases factors that control the survival and differentiation of neuroprogenitors in vitro and in the adult hippocampal neurogenic niches. These results suggest that microglial phagocytosis is a mechanism that acts as the brake of neurogenesis in order to maintain the homeostasis of the adult hippocampal cascade.

3. INTRODUCTION

3. INTRODUCTION

3.1. INTRODUCTION TO MICROGLIA

The mammalian central nervous system (CNS) comprises several cell types, which include both neurons and glial cells. Of the different types of glia within the CNS, microglia is a unique population due to both their origin and function. Microglia are the main resident macrophages of the CNS, myeloid cells derived from erythromyeloid progenitor cells originating in the yolk sac during mouse embryogenesis (E7.5) (Ginhoux et al., 2010). These progenitors give rise to primitive erythrocytes and macrophages, which colonize the developing CNS to eventually differentiate into microglia (Bertrand et al., 2005; Casano and Peri, 2015; F. Chris Bennett, 2018; Ginhoux and Prinz, 2015).

In both mice and humans, microglia presents a ubiquitous and non-overlapping distribution in all regions of the postnatal CNS, and comprise around a 12% of the total brain cells (Gomez-Nicola and Perry, 2015). Similar to their peripheral macrophage comrades, microglia display a remarkable range in both dynamism and functional behavior, in health as well as in disease. Indeed, microglia possess an extraordinary surveillance capacity of their environment, responding immediately to the most subtle variations of their surroundings, by undergoing modifications in morphology and gene expression (Aguzzi et al., 2013; Kettenmann et al., 2013). This surveillance capacity supports the ability of microglia to exert a large range of functions within the brain parenchyma. The diverse roles of these functions will be summarized in the next section.

3.2. MICROGLIAL FUNCTIONS

Microglia possess a huge capacity to survey the brain parenchyma. Microglial thin, long, and branched processes continuously scan their surroundings to detect different signals released from the environment. Environmental changes sensed by microglia comprise invading microbes, damaged or dead cells associated with neurodegeneration, as well as alterations in the pH of the parenchyma, composition or integrity of the extracellular matrix, or the release of different substances such as inflammatory cytokines and chemokines (Hickman et al., 2013; Nimmerjahn et al., 2005). Thus, microglia are the most sensitive monitors of changes in the brain, which allows them to exert a large variety of functions (Ginhoux et al., 2013; Nayak et

al., 2014). Some of these functions include synapse monitoring/pruning, myelination, vasculogenesis, and neurogenesis during development and adulthood in physiological and pathological conditions.

-**Synapse pruning:** At postnatal stages, microglial cells contribute to the refinement of synaptic circuitries and synapse maturation (Hoshiko et al., 2012; Paolicelli et al., 2011; Schafer et al., 2012; Zhan et al., 2014). During CNS development, there is a massive production of synaptic contacts and mature synaptic networks are formed through an activity-dependent refinement, leading to the elimination of excessive inappropriate synapses and to the functional maturation of the remaining appropriate ones (Hua and Smith, 2004). In the adult CNS, synapse elimination has been proposed to occur via microglia trogocytosis (partial elimination or nibbling) rather than via phagocytosis (Weinhard et al., 2018). However, an excessive removal of synapses might aggravate several pathological diseases such as Alzheimer's disease (AD), schizophrenia, or aging (Vilalta and Brown, 2017).

-**Myelination:** Microglia is also implicated in myelination by the release of several growth factors that control proliferation and survival of oligodendrocytes and their precursors (Pang et al., 2013). In addition, microglia also plays a critical role in remyelination process, since the phagocytosis of myelin debris after demyelination is crucial for a subsequent recruitment and differentiation of oligodendrocyte precursors (Domingues et al., 2016; Kotter et al., 2006). In contrast, microglia might exert a detrimental role in remyelination by the release of pro-inflammatory cytokines during pathological conditions including white matter damage in premature infants and multiple sclerosis in adults (Bennett and Barres, 2017; Pang et al., 2000)

-**Vasculogenesis:** There are increasing evidences suggesting that microglia may play an important role in the formation of new blood vessels or angiogenesis both in health and disease, since the lack of microglia results in a decrease in the vasculature during development (Kubota et al., 2009). In addition, microglia might contribute to abnormalities in vasculature in different neurodegenerative diseases such as AD, Parkinson´s disease (PD), or multiple sclerosis (Zhao et al., 2018). Nevertheless, the mechanisms underlying this communication between microglia and vasculature are still unknown.

-**Neurogenesis:** During embryonic development, microglia has been suggested to limit cell production in the subventricular zone, as it regulates the neuron production in the

prenatal brain directly by phagocytosing neural precursor cells in rats (Cunningham et al., 2013). In addition, during adulthood, microglia remove apoptotic newborn neurons both in the hippocampus (Sierra et al., 2010) and in the subventricular zone (Fourgeaud et al., 2016). In addition, microglia can also contribute to the decline of neurogenesis in different pathological conditions such as aging or neurodegenerative diseases through the release of pro-inflammatory cytokines during the inflammatory response (Sierra et al., 2014b). In this thesis we will focus on the role of microglia in adult hippocampal neurogenesis, which will be introduced in detail in **section 3.3.4**.

Thus, new roles for microglia in the brain are emerging and will be further studied in the future. Most of these functions are related to the two major abilities of microglia: the control of the inflammatory response and phagocytosis, which we will review in detail in the next section.

3.2.1. Immune response

Microglia are the innate immune cells of the brain, and thus, one of the principal function they exert is the regulation of the inflammatory response. The innate immune system represents the first defense of the body from infection by other organisms by launching an inflammatory response. Contrary to the adaptive immune system, the innate system recognizes pathogens unspecifically and might not provide long-lasting immunity to the body (MacLeod and Mansbridge, 2016).

Inflammation is a crucial mechanism triggered in response to stimuli that are recognized as noxious, such as pathogens or excessive cell death. In order to counteract these stimuli, inflammation triggers swelling, redness, heat, pain and fever in the affected tissue, aimed to limit proliferation of pathogens (Lucas et al., 2006).

In the brain, microglia, as the resident immune cells, orchestrate the inflammatory response. In addition, inflammatory mediators can also be produced by neurons, astrocytes and oligodendrocytes (Camara-Lemarroy et al., 2010; Lucas et al., 2006). In the presence of an inflammatory insult, microglia release different inflammatory mediators such as cytokines, chemokines, and proteins of the complement cascade (Nayak et al., 2014). Their function in the organism is to coordinate the response of the immune system and do so they trigger

different responses such as proliferation and differentiation, cell dead, release of other cytokines, etc (Zhang et al., 2009). Six main groups have been described:

Interleukins (ILs) are mainly responsible for regulating the activation of several cells of the immune system and for controlling the differentiation and proliferation of some subpopulations. They also activate the endothelium and increase vascular permeability, facilitating the migration of immunity cells from the bloodstream to the tissue (Brocker et al., 2010).

Tumor necrosis factors (TNFs) are important cytokines during the early stages of the inflammatory response. They are produced by a large variety of cells and play a central role in viral infections, as well as cell proliferation and apoptosis (Sun and Fink, 2007).

Transforming growth factors (TGFs) attract immune cells toward the site. In addition, TGFs play a crucial role in resolution and repair of the injury site (Tisoncik et al., 2012).

Interferons (IFNs) have an important role in the innate response to viruses or other pathogenic microorganisms. They are, therefore, secreted as danger signals: they promote the antiviral activity and the response of immune cells (Fensterl and Sen, 2009).

Colony stimulating factors (CSFs): are growth factors. They stimulate the differentiation and proliferation of stem cells to cells of the immune system, for example (Jeannin et al., 2018). They have functions associated with inflammation and the production of other cytokines.

Chemokines: stimulate the motility of the cells of the immune system, and direct them towards the location of inflammation (chemotaxis) (Hughes and Nibbs, 2018).

According to the effects that they exert upon binding to their receptors, cytokines are generally classified as pro- and anti-inflammatory.

3.2.1.1. Pro-inflammatory cytokines

Pro-inflammatory cytokines facilitate the inflammatory response. Although their main function is to defend the organism, their excessive and chronic release results in detrimental

consequences, which has been demonstrated to contribute to tissue damage in several neurodegenerative diseases such as AD, PD, stroke, multiple sclerosis, or epilepsy, among others (Amor et al., 2010; Vezzani et al., 2011). The detrimental effects of pro-inflammatory cytokines include apoptosis induction in neurons and glial cells, increased blood-brain barrier (BBB) permeability, and enhanced migration of peripheral immune cells into the CNS, which exacerbates neuron damage. In addition, these cytokines trigger the increase of neurotoxic factors such as ROS (reactive oxygen species) or NO (nitric oxide) (Sastre et al., 2006; Smith et al., 2012).

Classically studied pro-inflammatory cytokines include interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor α (TNFα), and interferon gamma (IFN-γ) (Su et al., 2016). Different studies demonstrated that the levels of these classical pro-inflammatory cytokines are elevated in chronic neurodegenerative diseases, significantly aggravating the diseases progression (Rocha et al., 2012; Zheng et al., 2016). In addition, several of these cytokines are able to exert pleiotropic functions, as we will review in **section 3.3.5.**

Therefore, an extended pro-inflammatory cytokine release results in harmful effects during brain pathologies.

3.2.1.2. Anti-inflammatory cytokines

Anti-inflammatory cytokines control the pro-inflammatory cytokine response. Their physiologic role in inflammation and pathologic role in systemic inflammatory states are increasingly recognized. Classically studied anti-inflammatory cytokines include interleukin 4 (IL-4), interleukin 10 (IL-10), and transforming growth factor β (TGFβ) (Su et al., 2016). There are also specific cytokine inhibitors and soluble cytokine receptors that act as anti-inflammatory (Opal and DePalo, 2000). Anti-inflammatory cytokines facilitate the tissue repair and restrain the synthesis of pro-inflammatory cytokines (Loftis et al., 2010). Similar to pro-inflammatory cytokines, several of these cytokines have dual roles, as we will review in **section 3.3.5.**

3.2.2. Phagocytosis

Phagocytosis is the process by which a cell recognizes, engulfs, and degrades large solid particles (>0.5 µm) (Mukherjee et al., 1997). It is an essential element of the innate immune response, which functions as a defense mechanism against pathogens during infection and

clearance mechanism for cellular debris produced during normal brain development and injuries (Fricker et al., 2012; Jones et al., 2013). In the CNS, microglia have been regarded as the major phagocytes. In addition, other cells such as astrocytes (Magnus et al., 2002; Sierra et al., 2010) and neuroblasts (Lu et al., 2011; Sierra et al., 2010) also present the ability to phagocytose and can be recruited when needed, although they are not as efficient as microglia and thus, they are referred as non-professional phagocytes (Magnus et al., 2002; Parnaik et al., 2000).

In addition, microglia can phagocytose different types of cargo during physiological and pathological conditions. Those targets include cargos such as synapses, neural debris, pathogenic proteins (Aβ for example), microbes and apoptotic cells (Diaz-Aparicio et al., 2016; Jana et al., 2008; Meyer-Luehmann et al., 2008; Pomilio et al., 2016; Tahara et al., 2006).

3.2.2.1. Types of cargo

3.2.2.1.1. Synapses and spines

As it has been described above, during nervous system development, there is an overproduction of synapses that eventually need to be eliminated (synaptic pruning) in order to accomplish a correct neuronal network connectivity (Zuchero and Barres, 2015).

Because microglia processes establish both direct and transient connections with neuronal synapses (Tremblay et al., 2010; Wake et al., 2009), microglia have been proposed to execute remodeling of the neural circuitry though the phagocytosis of synaptic components. In addition, there are recent evidences confirming that microglia directly engulf and eliminate synaptic material, however no evidences of phagocytosis of entire synapses have been found (Paolicelli et al., 2011; Stevens et al., 2007; Tremblay et al., 2010). On the contrary, synapse elimination has been proposed to occur via microglia trogocytosis (partial elimination or nibbling) (Weinhard et al., 2018). These data provide a new inside in role for microglia in the control and remodeling of synapses in the healthy developing brain (Nistico et al., 2017).

Several neurodegenerative diseases present aberrant communications between microglia and synapses. In AD mouse models, microglia promotes synapse loss due to an excessive elimination triggered by an increase in synapse targeting by the complement protein C1q (Hong et al., 2016). In ischemia, while microglial processes interact longer with synapses (Wake et al., 2009)(Wake et al., 2009), microglia fails to remove them correctly (Wake et al.,

2009). Synapse loss has also been reported in Huntington's disease, neuropathies, glaucoma and different neurodegenerative diseases (McGonigal et al., 2016; Perry et al., 2010; Williams et al., 2016), however the contribution of microglia to synapse elimination in these diseases is yet to be determined.

3.2.2.1.2. Amyloid β

Several reports suggest that microglia remove amyloid-β (Aβ) deposits through phagocytosis. Aβ is a 36-43 amino acid peptide derived from the amyloid precursor protein (APP) after cleavage (Hamley, 2012). The accumulation of Aβ in the brain renders a major pathological hallmark in AD, which is suggested to trigger a detrimental neurodegenerative set of events that ultimately leads to cognitive impairment (Hardy et al., 2014). Several studies suggest that AD´s progression is due to a failure in the clearance of Aβ rather than an overproduction of the peptide (Mawuenyega et al., 2010).

Microglia are found surrounding amyloid plaques in mouse models of amyloidosis as well as in AD patients (Daria et al., 2017); however, their role in plaque clearance is still under debate. Several studies proposed microglia to mediate Aβ clearance (Krabbe et al., 2013; Liu et al., 2010). Certainly, microglial phagocytosis of Aβ in vitro has been reported in several studies (Liu et al., 2012) (Hellwig et al., 2015); nevertheless, a complete degradation of the peptide has failed to be proven (Grathwohl et al., 2009; Krabbe et al., 2013; Spangenberg and Green, 2017). In addition, opposite results have been observed regarding the capacity of microglia to limit plaque growth. In some studies no changes in plaque size was observed after microglia depletion in AD mouse models (Grathwohl et al., 2009; Prokop et al., 2015); however there are more recent results suggesting that upon microglia depletion, plaques grow in size (Zhao et al., 2017). Since microglial phagocytosis of Aβ has not been detected in vivo yet, microglial function in Aβ clearance is still to be unraveled.

3.2.2.1.3. Axonal and myelin debris

During brain injury, axonal debris represents a barrier for axonal outgrowth and therefore must be eliminated in order to allow a correct repair. The removal of this debris has been proposed to be executed by microglial phagocytosis; however, few studies do actually report phagocytosed myelin debris within microglia (Rawji et al., 2018). Different in vitro studies show beneficial roles of microglial phagocytosis of axonal debris both in rat explants with sectioned neurites (Jin and Yamashita, 2016; Tanaka et al., 2009) or in axonal

degeneration co-culture models (Hosmane et al., 2012), in which axonal debris was eliminated by microglia, allowing axonal regeneration. In addition, ex vivo live multiphoton imaging has shown a decrease motility, surveillance, and phagocytic activity in microglia from demyelinated lesions in aging mice, suggesting that a microglial impairment could contribute to a decline in remyelination efficiency in aged mice (Rawji et al., 2018).

In addition, microglia have also shown positive consequences in the clearance of myelin debris (Rawji and Yong, 2013). In physiological conditions, microglia is responsible for eliminating myelin fragments that increasingly split from myelin sheaths with age (Safaiyan et al., 2016). The highly lipidic nature of myelin represents a challenge to microglia to digest and eventually in aging, myelin forms lysosomal inclusions in microglia filled with insoluble materials (Safaiyan et al., 2016), which contributes to microglial impairment. Moreover, in a CX3CR1 KO model, microglial inefficient clearance of myelin debris results in the impairment of remyelinating processes (Lampron et al., 2015). Thus, correct debris elimination is crucial, especially in demyelinating diseases such as MS or EAE or in spinal and nerve lesions. In these CNS autoimmunity models microglia has been proposed as the major orchestrator of myelin removal via the production of interferon beta (IFNβ), which enhances myelin debris uptake by microglia (Kocur et al., 2015). Thus, a correct elimination of myelin debris is essential for an efficient remyelination of demyelinating sites.

3.2.2.1.4. Apoptotic cells

In this Thesis project, we will focus on the apoptotic cells and their phagocytosis, and we will further detail this mechanism in the next sections.

Apoptosis, or programmed cell death, is a widespread phenomenon that occurs regularly in the brain in both physiological (during development or homeostasis) and pathological conditions (Henson, 2005). Whether apoptotic cells come from physiological or pathological consequences, efficient clearance of these cells by microglia must occur.

Apoptosis is particularly crucial in long-lived mammals (Danial and Korsmeyer, 2004) as it plays a critical function in physiological conditions during both development and adulthood (Hassan et al., 2014). It is a mechanism to remove any unnecessary or undesired cell in an extremely regulated process. Apoptotic pathways can be triggered by either intracellular or extracellular signals, leading to the initiation of the intrinsic (or mitochondrial) or the extrinsic

(or death receptor) apoptotic pathways, respectively. Intracellular signals that induce apoptosis include DNA damage and growth factor and cytokine deprivation (Zaman et al., 2014), whereas extracellular signals include stimuli produced by cytotoxic immune cells after cell damage or infection in order to promote death (Zaman et al., 2014). Both pathways present common downstream executioner caspases (3, 6 and 7), which eventually trigger the characteristic cellular changes of apoptosis (Taylor et al., 2008). These changes include activation of caspases, which cleave cellular components required for normal cellular function, such as cytoskeletal and nuclear proteins. As a result of caspase activity, cells shrink and condense their cytoplasm; they also undergo nuclear fragmentation (karyorrhexis) and chromatin condensation (pyknosis). Eventually, the cytoskeleton breaks up and causes membrane blebs that separate from the cell, taking a portion of cytoplasm with them, forming apoptotic bodies (Eidet et al., 2014; Krysko et al., 2008; Poon et al., 2014). One important feature of apoptosis is that the integrity of the plasma membrane is preserved until the final stages of the apoptotic process (Ren and Savill, 1998). This feature of apoptosis is highly relevant since it allows the clearance of dead cells without any contact of toxic intracellular contents of the cells with the surrounding tissue. Failure to sustain efficient clearance results in secondary necrosis in which the membrane integrity is lost and intracellular toxic contents are released to the surrounding, which can lead to exacerbated inflammation and autoimmune diseases (Nagata et al., 2010). For these reasons both the dying cell and the phagocyte communicate and engage each other to ensure successful removal of apoptotic cells. In the next section, we will further detail the communication between apoptotic cells and microglia in the process of phagocytosis.

3.2.2.2. Phagocytosis of apoptotic cells

As stated above, the rapid elimination of the apoptotic cells is crucial to maintain tissue homeostasis. Thus, the process to eliminate dead cells must be complex and highly regulated as we will detail in this section.

The process of phagocytosis comprises three steps ('find-me', 'eat-me' and 'digest-me') **(Figure 1)**. First, apoptotic cells release 'find-me' signals which act as chemoattractants for microglia (Peter et al., 2010). Microglia then specifically engage the apoptotic cells, via 'eat-me' signals on apoptotic cells and receptors for 'eat-me' signals on microglia (Gardai et al., 2006). Microglia then physically engulf apoptotic cells through signaling induced by engulfment receptors and cytoskeletal reorganization of the microglia membrane

(Arandjelovic and Ravichandran, 2015; Sierra et al., 2013). Lastly, the corpse contents are degraded within microglia through phagolysosomal processing (Kinchen et al., 2008; Kinchen and Ravichandran, 2008; Park et al., 2011). Each step is highly regulated by a plethora of molecules, specially ligands and receptors from the apoptotic cell and microglia respectively, which ensure a correct functioning of the phagocytic process **(Figure 2)**.
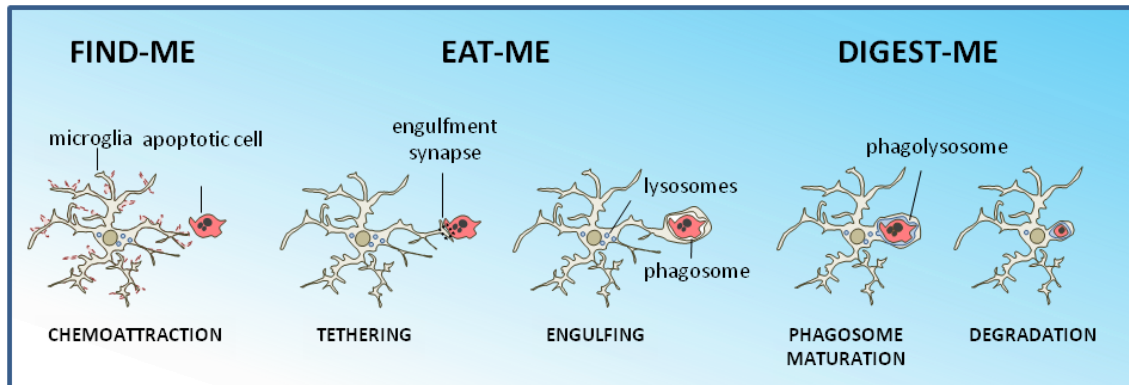
Figure 1. Phagocytosis of apoptotic cells. Phagocytosis comprises three different phases: 'find-me', the stage in which microglial processes find the phagocytic target; 'eat-me', the stage where a direct microglia-target contact is established via a receptor-ligand interaction and recognition and engulfment take place; and 'digest-me', the stage in which the internalized apoptotic cells are degraded within microglia through phagolysosomal processing.

3.2.2.2.1. 'Find-me' stage

Cells undergoing apoptosis release molecules termed 'find-me' signals to attract microglia towards them. These include the lipids lysophosphatidylcholine (LPC) and sphingosine-1-phosphate (S1P), the chemokine fractalkine, and extracellular nucleotides (ATP, UTP) (Arandjelovic and Ravichandran, 2015; Elliott et al., 2009; Gude et al., 2008; Lauber et al., 2003; Sierra et al., 2013; Truman et al., 2008).

LPC is released from apoptotic cells and binds to G2A receptor on microglia, which promotes the recruitment of phagocytes to apoptotic cells (Peter et al., 2008; Xu et al., 2016). In apoptotic cells, phospholipase A2 (iPLA2) mediates the conversion of phosphatidylcholine into LPC (Lauber et al., 2003).

S1P is produced from sphingosine by sphingosine kinase. Apoptotic cells release S1P, which binds to S1P receptors expressed on phagocytes, resulting in the migration of phagocytes toward apoptotic cells (Gude et al., 2008; O'Sullivan et al., 2018). Although the

presence of S1PR on microglia has been demonstrated (O'Sullivan et al., 2018), the role of S1P/S1PR signaling on microglia as a 'find-me' signal has not been studied yet.

Fractalkine (CX3CL1) protein also plays a role as a 'find-me' signal (Truman et al., 2008). Fractalkine is produced as a membrane anchor protein, which needs to be proteolitically cleaved in order to be released from apoptotic cells. The resulting soluble fragment of the protein binds to the microglial-specific CX3C motif chemokine receptor 1 (CX3CR1) and promotes the migration of the phagocyte (Eyo et al., 2016; Jung et al., 2000; Mizutani et al., 2012).

Finally, apoptotic cells release **nucleotides** such as ATP and UTP, which bind to purinergic receptors on phagocytes, facilitating the recruitment of microglia to apoptotic cells (Elliott et al., 2009). Pannexin 1 channels allow nucleotide release from apoptotic cells in a caspase-3-dependent manner (Chekeni et al., 2010). Nucleotides are in turn recognized by the plethora of purinergic receptors present in microglia (Domercq et al., 2013)

After microglia has approached to the apoptotic cell, the second stage of phagocytosis is initiated, so called 'eat-me' stage, in which physical contact between microglia a target cell occurs in order to perform recognition and tethering of the apoptotic cell (Sierra et al., 2013).

3.2.2.2.2. 'Eat-me' stage

After finding the apoptotic cell, microglia must recognize the apoptotic cell in order to engulf the correct target (Sierra et al., 2013). Microglia displays a plethora of surface receptors that bind to the 'eat-me' signals expressed by apoptotic cells. Similarly, apoptotic cells express a variety of ligands in their membrane that are recognized as 'eat-me' signals by phagocytes.

3.2.2.2.2.1. Apoptotic 'eat-me' ligands

Apoptotic cells display 'eat-me' signals on their surface to reflect that they should be engulfed by microglia. Although different 'eat-me' signals have been suggested, the most studied 'eat-me' signal is the expression of **phosphatidylserine (PS)** on the cell surface. PS is a phospholipid localized on the inner membrane leaflet of the plasma membrane in healthy cells and undergoes externalization to the cell surface upon apoptotic stimuli (Fadok et al., 1992). Due to activation of the Xk-related protein 8 (Xkr8), a phosphatidylserine scramblase, PS is irreversibly exposed on apoptotic cells (Suzuki et al., 2016). In addition, asymmetry of the

membrane is maintained via flip-flop of aminophospholipids from the outer leaflet to the inner leaflet of the membrane through the ATP11C ATPase. In cells undergoing apoptosis, it is inactivated by caspase-3-mediated cleavage, permitting phosphatidylserine externalization (Segawa et al., 2014).

Calreticulin (CRT) is another potential 'eat-me' signal. It is usually confined to the endoplasmic reticulum (ER), and both ER stress and apoptosis signaling can result in its exposure on the surface of non-neuronal cells, where it can present a dual role as an `eat-me´ signal or opsonin (Gardai et al., 2005). On neurons, surface CRT binds to microglial LRP (low-density lipoprotein receptor related protein) and triggers their phagocytosis in vitro (Fricker et al., 2012). During cell death induced by ER stress, CRT is translocated from the ER to the Golgi and SNARE (Soluble NSF Attachment Protein Receptor)-mediated exocytosis (Panaretakis et al., 2009). CRT on the apoptotic cell surface is sensed by LRP on microglia to promote engulfment (Gardai et al., 2005). Recently, CRT has been described to associate to PS, which leads to apoptotic cell phagocytosis in vitro (Wijeyesakere et al., 2016).

3.2.2.2.2.2. Phagocyte 'eat-me' receptors

Recognition and engulfment are mediated by phagocytic receptors localized in the surface of the phagocytes and are able to bind different `eat-me´ ligands and opsonins (see next section) in the apoptotic cell.

One of the receptors that mediates apoptotic engulfment is **Triggering receptor expressed on myeloid cells-2 (TREM2)**, an immunoglobulin-like receptor expressed on macrophages, dendritic cells, and microglia (Neumann and Takahashi, 2007). TREM2 associates with adapter protein named DNAX-activation protein of 12 kD (DAP12) (Paloneva et al., 2002), and disruption in the function of these two molecules leads to an impairment in the uptake of apoptotic cells by microglia in vitro (Hsieh et al., 2009). Apolipoprotein E has been suggested as the ligand that directly binds TREM2, and could also present a double role as opsonin by connecting PS with LRP in vitro (Atagi et al., 2015).

Another receptor related to apoptotic cell recognition is **GPR34 (G protein-coupled receptor 34)**, a P2Y family member receptor (Sugo et al., 2006). GPR34 is activated by lysophosphatidylserine (LysoPS), a deacylated form of phosphatidylserine produced by phospholipases (PLA) (Makide et al., 2014). LysoPS is known to induce several cellular

responses both in vitro and in vivo such as increasing degranulation of mast cells and neurite growth; inhibiting T cell proliferation; promoting fibroblast migration; and increasing apoptotic uptake by macrophages (Makide et al., 2014). GPR34 is highly expressed in microglia (Bedard et al., 2007; Butovsky et al., 2014; Hickman et al., 2013), however its relevance in microglial function is beginning to be unraveled. GPR34 deficient microglia presents a decreased capacity for clearance in vitro, strongly suggesting that GPR34 plays an important role in phagocytosis (Preissler et al., 2015).

Another important phagocytic receptor is **Mer tyrosine kinase (MerTK)**, which is the most extensively studied TAM (Tyro3, Axl, and Mer) receptor and is known to play a role in apoptotic cell engulfment both in vivo and in vitro (Scott et al., 2001). Microglial MerTK binds to the opsonins growth arrest-specific protein 6 (GAS6) or protein S, which in addition bind to PS on dead cells in vitro (Wu et al., 2005). Galectin-3 (Gal-3) has also been implicated in MerTK mediated apoptotic cell removal in vitro (Caberoy et al., 2012). Recently, the TAM receptor tyrosine kinases MerTK and Axl were shown to function as phagocytic receptors under different environments. MerTK is mainly involved in the maintenance of the immune balance in vitro, whereas the expression of Axl is promoted upon pro-inflammatory stimuli in order to inhibit immune responses in vivo (Zagorska et al., 2014). MerTK has been involved in regulation of phagocytosis via its receptor ligands GAS6 and protein S (Fourgeaud et al., 2016).

Similarly, **vitronectin receptor (VNR)** mediates phagocytosis of apoptotic cells by binding to the opsonin MFG-E8 (milk fat globule EGF factor 8) both in vitro and in vivo (Hanayama et al., 2002). The activation of VNR triggers the reshaping of microglial cytoskeleton and promotes phagocytosis. Crosstalk between VNR and MERTK signaling pathways has been observed in cell lines, which leads to a synergistic increase in phagocytosis in vitro (Wu et al., 2005).

Moreover, the **complement receptor 3 (CR3)**, which binds to different molecules of the complement cascade such as C1q or C3b (Linnartz et al., 2012), is also well established to enable cellular adhesion and phagocytosis of pathogen and apoptotic cells on microglia in vitro (Trouw et al., 2008). However, depending on the level of expression and the possibly different signaling partners, their participation in phagocytosis varies among cell types. CR3 was shown to induce an anti-inflammatory response in microglia, contributing to the maintenance of immune tolerance in vitro (Ehirchiou et al., 2007; Fraser et al., 2010; Veldhoen et al., 2007).

3.2.2.2.2.3. Opsonins and bridge molecules

Opsonins are soluble proteins that target cells in order to facilitate their phagocytosis. The most described classic opsonins are complement components C1q and C3b. In addition, different molecules that act as a bridge between PS in the apoptotic cells and phagocytic receptors are also considered as opsonins.

One of these bridge molecules is **MFG-E8 (milk fat globule EGF factor 8)**. Produced by macrophages and dendritic cells, MFG-E8 binds to PS on apoptotic cells leading to their phagocytosis both in vitro and in vivo in macrophages (Akakura et al., 2004; Hanayama et al., 2002) and in microglia in vitro (Fuller and Van Eldik, 2008). **Gas6 and protein S** present similar domains and both recognize PS on apoptotic cells to promote the internalization of the cargo in vitro (Anderson et al., 2003; Ishimoto et al., 2000).

Another potential bridging molecule is **C1q, a complement component**. C1q exerts crucial functions in the innate immune system. It recognizes the component of the macromolecular complex (C1), which in turn induces the classical complement pathway. In the immune system, upon binding to apoptotic cell membrane, C1q promotes a proteolytic cleavage of C3, resulting in opsonin C3b deposition of the surface of target cells and consequent phagocytosis by microglia expressing complement receptors (Bialas and Stevens, 2013). Activation of C1 is usually triggered by binding of C1q to Fc portions of antibodies in immune complexes; however, C1 can also be activated in the absence of antibodies (Fraser and Tenner, 2008). In addition to phagocytosis of apoptotic cells, C1q also participates in phagocytosis of synapses. In the developing visual thalamus, both C1q and C3 tag a subset of immature synapses that are engulfed by microglia through the C3-CR3 signaling pathway (Schafer et al., 2012; Stevens et al., 2007). In addition, C1q is emerging as an important molecule in the clearance of apoptotic cells, since it has been shown to bind dead cells in vitro (Korb and Ahearn, 1997; Nauta et al., 2002; Trouw et al., 2008). C1q directly opsonizes target cells by binding to PS on the apoptotic cell surface, which contributes to cell clearance (Fraser et al., 2009; Ogden et al., 2001; Paidassi et al., 2008). Similar to synapse opsonization, C1q triggers C3b deposition and consequently enhances phagocytosis of apoptotic cells through CR3 and/or receptors for C1q (Ogden and Elkon, 2006; Takizawa et al., 1996). In addition, C1q can play opsonin role when binding to PS, CRT or a desialylated surface, or it can directly bind to the phagocytic receptor CR3 (CD11b) on microglia (Linnartz et al., 2012) or multiple epidermal growth factor-like domains protein 10 (MEGF10) on astrocytes (Iram et al., 2016).

3.2.2.2.3. 'Digest-me' stage

After recognition and engulfment, the process of apoptotic cell clearance has not concluded. After internalization, phagosomes undergo a process of maturation in which they fuse sequentially with early and late endosomes, and lysosomes, to form phagolysosomes (Desjardins et al., 1994). Inside phagolysosomes, vacuolar ATPases (vATPases) maintain an acidic environment (pH ≤ 5) in order to ensure the correct functions of the different hydrolases within (Garin et al., 2001; Peri and Nusslein-Volhard, 2008). Several studies have proposed that the signaling cascade triggered after internalization might regulate the phagocytic ability of phagocytes to engulf additional targets (Park et al., 2011; Wu et al., 2000). However, the process of phagosome formation and cargo degradation has been barely addressed in microglia and thus, further research is necessary to unravel the mechanisms of degradation of apoptotic cells by microglia.
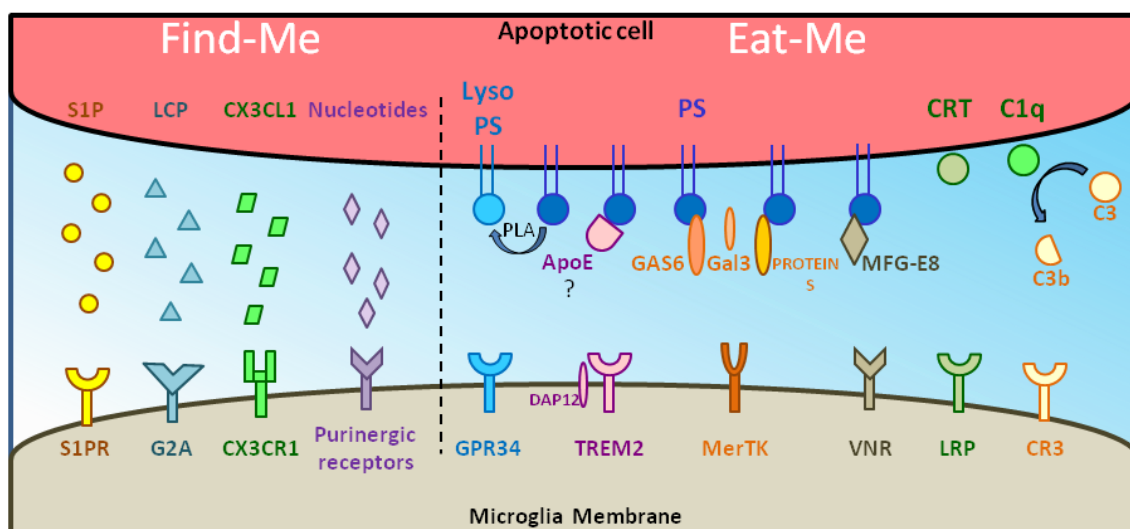
Figure 2. Molecules implicated in the phagocytosis of apoptotic cells. Phagocytosis of apoptotic cells comprises different steps ('find-me', 'eat-me' and 'digest-me'), all of which are highly regulated by a plethora of molecules, specially ligands and receptors from the apoptotic cell and microglia respectively, which ensure a correct functioning of the phagocytic process.

3.2.3.3. Functional consequences of microglial phagocytosis of apoptotic cells

The clearance of apoptotic cells is a crucial process to maintain tissue homeostasis. It is critical that the clearance of the apoptotic cells occurs before they lose the membrane integrity, otherwise, apoptotic cells would evolved into secondary necrotic, which are

characterized by membrane disruption. The spillover of toxic intracellular content might exert the triggering of an inflammatory response or autoimmune diseases (Nagata et al., 2010; Poon et al., 2014) and thus, failure to properly remove apoptotic cells presents deleterious consequences for the brain parenchyma.

The importance of phagocytosis in maintaining tissue homeostasis is highlighted by observing the plethora of redundant phagocytic receptor present in phagocytes, since the overlapping function of different phagocytic receptors could compensate to some extent the expression decrease of any phagocytic receptor in order to ensure the correct mechanism of phagocytosis. In fact, it is well known that inhibitors of individual receptor–ligand pairs only show small and partial effects, often not translated to in vivo, which evidences the critical role of the phagocytic function (Gordon and Pluddemann, 2018).

Importantly, recent data suggests that the uptake of apoptotic cells has more complex benefits than simple removal of noxious substances (Gordon and Pluddemann, 2018). In addition, it has become clearer that phagocytosis of apoptotic cells by phagocytes is not immunologically silent, and several evidences have emerged suggesting that it is indeed anti-inflammatory, at least in vitro (Fadok et al., 1998; Stern et al., 1996; Voll et al., 1997). This anti-inflammatory feature is mainly characterized by the production of TGFβ; however, the mechanism underlying TFGβ release upon phagocytosis of apoptotic cells is yet to be determined (Lucas et al., 2006). Moreover, phagocytosis of myelin has also been described to be anti-inflammatory (Liu et al., 2006). On the contrary, phagocytosis of microbes through toll-like-receptor (TLR) signaling as well as the engulfment of necrotic cells, has been described to be pro-inflammatory in macrophages (Erdman et al., 2009) during infection or as a result of accidental injury (Henson and Bratton, 2013).

The anti-inflammatory response to phagocytosis of apoptotic cells has also been reported in microglia. After phagocytosis, microglial release of TNFα is reduced and TGFβ is increased in vitro compared to microglia challenged with LPS (bacterial lipopolisaccharides) (De Simone et al., 2003; Magnus et al., 2001). The immunosuppressive effect induced by phagocytosis of apoptotic cells have been recently proposed to depend on the presence of the complement protein C1q bound to apoptotic cells. The presence or absence of C1q results in a completely different outcome in the process of phagocytosis, determining the anti- or pro-inflammatory nature of the process respectively (Fraser et al., 2010). However, whether C1q is produced in the brain and mediates the elimination of apoptotic cells by microglia in vivo is yet

to be determined. Moreover, since research focused on understanding the type of inflammatory response triggered by phagocytosis have been mostly performed in vitro, further study is needed to determine the microglial phagocytosis immune response in vivo (Abiega et al., 2016).

In addition to the immunomodulatory nature of phagocytosis, there are also increasing evidences suggesting that microglia are able to produce different trophic factors after apoptotic cell phagocytosis. Phagocytic microglia in culture is capable of producing TGFβ as well as NGF (neuronal growth factor) (De Simone et al., 2003). Similarly, hepatic macrophages produce VEGF (vascular endothelial growth factor) upon phagocytosis (Golpon et al., 2004). These three factors are potent positive (NGF and VEGF) and negative (TGFβ) regulators of hippocampal neurogenesis in vivo (Buckwalter et al., 2006; Cao et al., 2004). Microglia are further able to produce trophic factors *in vitro.* After IL-4 or IFNγ treatment, cultured microglia decrease levels of TNFα and produce insulin-like growth factor1 (IGF-1), which help to maintain critical functions such as neurogenesis or oligodendrogenesis (Butovsky et al., 2006). Thus, trophic factors released from phagocytic microglia may mediate the functional recovery of the damaged tissue during brain diseases (Diaz-Aparicio et al., 2016).

The concept that microglial phagocytosis of apoptotic cells induces the release of different trophic factors could be particularly relevant in the neurogenic niches. For example, in the subgranular zone of the adult hippocampus microglia are in direct contact with all components of the adult hippocampal neurogenic cascade, which allows them to efficiently phagocytose the apoptotic newborn cells (Sierra et al., 2010). In this context, microglial phagocytosis could not only exert its beneficial effects by the physical clearance of the apoptotic cells but, in addition, it may release different neurotrophic factors that could also help to maintain the homeostasis of the cascade. As stated above, there are scarce evidences suggesting that phagocytic microglia produce several factors known to act on different stages of adult neurogenesis (Sierra et al., 2014b). However, it has to be taken into account that most of these observations were obtained in culture and that further research is needed in order to elucidate whether those factors are also secreted and have the same regulatory responses *in vivo*.

Due to the scarce information on this matter, and especially on microglia, the goal of this Thesis project is to perform a systematic analysis of the molecules released after microglial

phagocytosis of apoptotic cells in order to obtain a more complex picture of the inflammatory and trophic factors signature of phagocytic microglia and test their role on adult neurogenesis.

Therefore, in order to obtain a deeper knowledge in the matter, in the next section we will review in detail the process of adult neurogenesis, its different stages and its tight relationship to microglia.

3.3. ADULT HIPPOCAMPAL NEUROGENESIS

Neurogenesis, or the formation of new neurons, is a complex process that extends throughout adulthood in two brain regions of the mammalian brain, the subventricular zone, which gives rise to olfactory bulb interneurons, and the subgranular zone (SGZ) of the hippocampus, which generates granule cells (Ehninger and Kempermann, 2008). This process comprises different steps in which neural stem cell proliferate, differentiate and migrate until they eventually integrate into the existing circuitry and gradually acquire physiological neuronal properties (Kempermann et al., 2004). For this study, we will focus on adult hippocampus, where our group has reported that microglia present in the SGZ efficiently phagocytose the excess of newborn cells that undergo apoptosis (Sierra et al., 2010).

In many species of mammals, hippocampal proliferating neuroprogenitors have been directly or indirectly demonstrated to produce a significant amount of functional new neurons. However, human data on this matter is still scarce and its functional role in humans is currently under discussion. Adult human neurogenesis was first assessed two decades ago when BrdU positive cells were found both in subventricular and subgranular zone (Eriksson et al., 1998). More recently, ^{14}C analyses by Frisen's group assessed that there is a turning over rate of 700 new neurons in each hippocampi per day in middle aged humans, a rate that undergoes a small decline with age (Spalding et al., 2013). Nevertheless, the persistence of hippocampal neurogenesis in the adult human is still controversial. Recently, opposite results have been published regarding this matter. On one hand, (Sorrells et al., 2018) suggest that the number of proliferating progenitors and new neurons in the dentate gyrus is dramatically reduced after the first year and is completely undetectable in the adulthood. On the other hand, (Boldrini et al., 2018) suggest that adult neurogenesis continues in the human dentate gyrus into the eighth decade of life. Thus, there are still many questions unanswered regarding human adult neurogenesis, which might be unraveled in the future with more complete analyses.

Adult hippocampal neurogenesis process encompasses several stages. Neural stem cells (NSCs) reside within the hippocampal neurogenic niche in the SGZ of the dentate gyrus (DG). These cells are normally maintained in quiescence, however, upon activation, they undergo asymmetric division from which an NSC and an amplifying neuroprogenitor (ANP) are produced (Encinas et al., 2011). The highly proliferative nature of the ANPs expands the progenitor cell population and eventually, they differentiate into neuroblasts (Encinas and Sierra, 2012). Afterwards, neuroblasts migrate a short distance to arrive to their definite location into the granular cell layer while differentiating into neurons. During the process of neurogenesis, many newborn cells naturally undergo apoptosis (up to 60% in physiological conditions) during their first life days, while they transit from amplifying neuroprogenitors to neuroblasts, and are rapidly phagocytosed by microglia (Sierra et al., 2010). The surviving cells will mature and integrate into the circuitry. By two months, surviving neurons are completely integrated into the neuronal network and are electrophysiologically indistinguishable from mature neurons (Ge et al., 2008; Piatti et al., 2006; van Praag et al., 2002). Hence, only a small proportion of newborn neurons eventually develop into mature neurons after a month (Encinas and Enikolopov, 2008; Goncalves et al., 2016b; Kempermann et al., 2004; Sierra et al., 2010) **(Figure 3).**
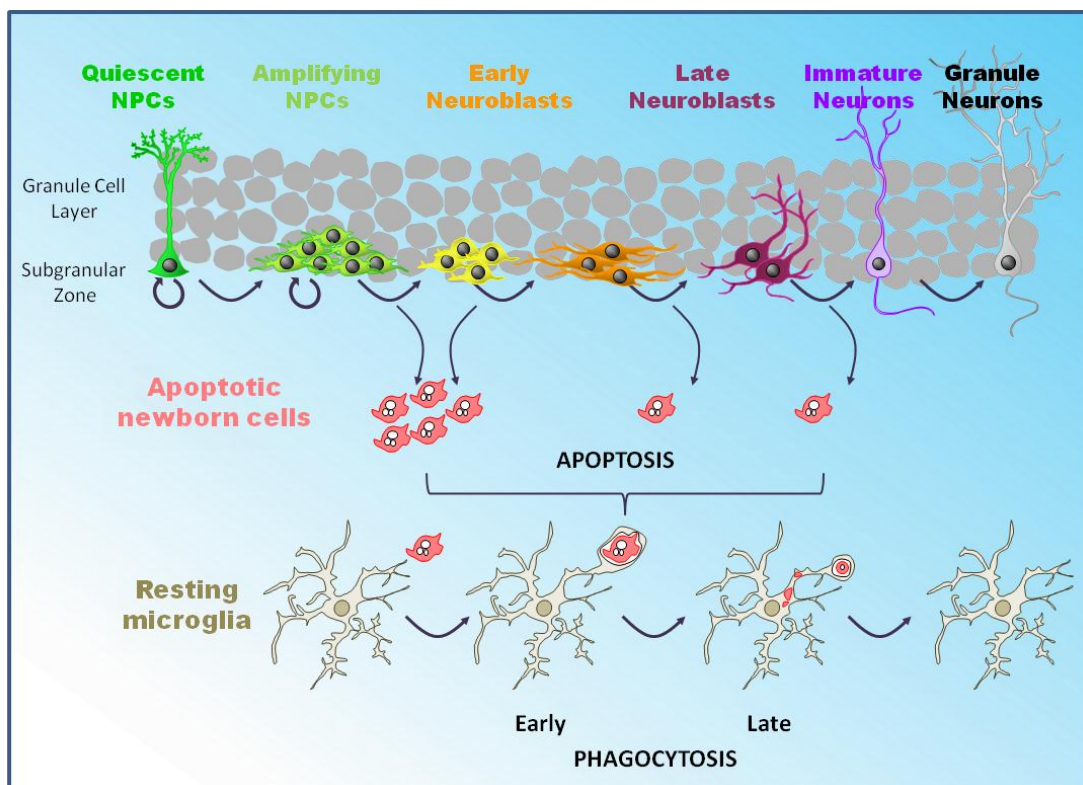
Figure 3. Adult hippocampal neurogenic cascade. *In the adult hippocampal neurogenic cascade rNSC cells proliferate and differentiate, eventually producing newborn neuroblasts. During this process the*

majority of the newborn cells die by apoptosis and are rapidly phagocytosed by microglia. Adapted from (Sierra et al., 2010).

In the next sections we will review the different cells that comprise the adult neurogenic cascade and their characteristics; the functions of the adult neurogenic cascade; the in vitro and the in vivo methods used to study it; and the role of microglia.

3.3.1 Characteristics of the cells of the neurogenic cascade

The different steps of adult neurogenesis occur in parallel with each other within the hippocampal adult neurogenesis, where quiescent NPCs and immature neurons coexist as immediate neighbors (Braun and Jessberger, 2014), and they will be further detailed in this section.

Neural stem cells (NSCs). The neurogenic cascade initiates with NSCs located within the hilus and the granule cell layer, in an area characterized by its loose extracellular matrix: the SGZ (Kempermann et al., 2004). NSCs present a single apical process that spans towards the molecular layer, and for this reason they are also named radial stem cells (rNSCs). In addition, these cells share patterns of protein expression with astrocytes, such as glial fibrillary acidic protein (GFAP), vimentin, and brain lipid binding protein (BLBP); they also present morphological and electrophysiological similarities, such as presence of vascular end-feet and low input resistance. In addition, they express neuroepithelial stem cell lineage markers such as nestin and Sox2 (Kempermann et al., 2004; Seri et al., 2004). At population level they are considered to be primarily quiescent, since NSCs present a very low rate of division (Encinas et al., 2006; Kronenberg et al., 2003). NSCs divide mainly asymmetrically, giving birth to a daughter cell termed amplifying neural progenitor cells (ANP), which differ from NSCs both functional and anatomically.

Amplifying neural progenitor cells (ANPs) are born from NSCs and present short thin processes. They have also been named type 2a cells or transient amplifying progenitors. They lack, or express at low levels, the majority of the typical markers of NSCs mentioned above (GFAP, vimentin, nestin), and they do not express advance neuroblast markers such as prox1, doublecortin (DCX) and PSA-NCAM (polysialated neural cell adhesion protein) either. However, ANPs still maintain BLBP and Sox2 expression (Kempermann et al., 2004). The ANPs act as transient amplifying progenitors (Seri et al., 2004) and divide symmetrically several times (Encinas et al., 2011), before they differentiate into neuroblast. Therefore, in contrast to NSCs,

ANPs have a highly proliferative nature; however, up to 60% die by apoptosis and are phagocytosed by microglia (Sierra et al., 2010). The remaining third of the newborn cells continue their maturation into granule cells.

Neuroblasts. One week after birth, ANPs stop proliferating and become neuroblasts (Kempermann et al., 2004). They begin to express immature neuron markers and also start to extend neurites into the granule cell layer, which will eventually become dendrites. These newly generated cells begin to express DCX and PSA-NCAM (Couillard-Despres et al., 2005). On average, DCX is expressed approximately for 3 weeks (Brandt et al., 2003; Steiner et al., 2004). During this period, the morphology of neuroblast changes into marked polarized cells. The presence and morphology of the apical process of DCX cells have allowed the sequential categorization of neuroblast cells (Plumpe et al., 2006). Six DCX cell categories have been proposed: category A and B comprises cells with no or very short processes parallel to the major axis of the granular cell layer; C and D cells present intermediate length processes and rather an immature morphology; and E and F cells are characterized for displaying a more mature appearance with a thick dendrite that ramifies in the molecular layer (Plumpe et al., 2006). Later on, while DCX expression is lost, cells mature into neurons that integrate into the hippocampal circuitry where they are involved in memory and learning as well as mood control in mice (Goncalves et al., 2016a; Tashiro et al., 2007). With time, the surviving newly generated neurons will be indistinguishable from pre-existent neurons (van Praag et al., 2002).

Astrocytes. In the neurogenic niche of the SGZ, newborn astrocytes are directly generated from NSCs after undergoing several asymmetric divisions (Bonaguidi et al., 2011; Encinas et al., 2011). While the young niche produces mostly neurons, the aging niche produces equal amounts of neurons and astrocytes (Beccari et al., 2017). Little is known about the function of new astrocytes and their contribution to the hippocampus in physiological conditions is still not understood (Goncalves et al., 2016a).

3.3.2. Function of adult hippocampal neurogenesis

Because the hippocampus is strongly associated with learning and memory, many efforts have been made to understand whether adult neurogenesis could play functional roles in cognition. It is unlikely that newborn neurons participate in behavior prior to integrating into the circuitry, generating action potentials or establishing synapses. However, newborn neurons are suggested to exert an important role especially between four-six weeks after

mitosis, because at this point, their excitability and plasticity is increased (Goncalves et al., 2016b). In addition, different hypothesis have emerged from computational modeling suggesting the functions of newborn neurons, such as the codification of temporal information into memory (Aimone et al., 2006; Becker and Wojtowicz, 2007), cognitive plasticity during learning of new tasks (Chambers et al., 2004), and pattern separation (Aimone et al., 2009). Moreover, recent studies confirm several of these functions and therefore, nowadays newly generated neurons are strongly suggested to contribute to hippocampus-dependent learning and memory (Deng et al., 2010). These studies confirmed a role for hippocampal neurogenesis in spatial and object recognition memory (Jessberger et al., 2009), fear conditioning and synaptic plasticity (Saxe et al., 2006) and pattern separation (Nakashiba et al., 2012; Sahay et al., 2011).

In addition, the roles of the newborn neurons in the DG might also differ depending on the exposure to different environmental inputs and cognitive demands during maturation, since integration and connectivity can be experience-dependent during the early maturation periods (Bergami et al., 2015; Piatti et al., 2011; Zhao et al., 2006). Moreover, newborn neurons might also participate in avoiding the interference between memories obtained at different time points (Rangel et al., 2014). However, the mechanism by which newborn neurons could execute these functions are yet to be unraveled. There are several evidences in the literature suggesting that the decrease of neurogenesis results in a reduction in the inhibition of the DG, while an enhancement of neurogenesis allows an increase in the activation of interneurons (Drew et al., 2016; Singer et al., 2011). Therefore, immature neurons might modulate the feedback inhibitory circuits in the DG, which would result in a decrease in the number of granule cells responding to incoming stimuli (Goncalves et al., 2016b). However, further research should be performed in order to understand how immature neurons are associated to inhibitory networks. In addition, in the most recent years, an enhancement in neurogenesis has been associated with the elimination of older memories, by inhibition of mature granule neurons or competitive rewiring of DG outputs (Akers et al., 2014).

Importantly, disruptions in the hippocampal neurogenic cascade have been related to different neurodegenerative diseases such as PD, AD or Huntington's disease (HD) (Goncalves et al., 2016a).

3.3.3. In vitro methods to study neurogenesis

In vitro cultures of adult hippocampal neuroprogenitor cells and their differentiation into granule cell-like neurons present a key tool for understanding the cellular and molecular mechanisms of adult neurogenesis. In vitro experiments of neuroprogenitor cells facilitate the analysis of the cells isolated from any signal produced in the neurogenic niche; however, culture settings must ensure the correct maintenance (proliferation and survival) of the neuroprogenitor cells. Although several procedures have been proposed to successfully extract and culture neuroprogenitor cells, preserving their intrinsic properties is a complex task. Generally, adult neuroprogenitor cells have been studied in vitro using two different culture methods: as neurospheres, non-adherent spherical clusters of cells; and as adherent monolayer cultures. Both models have their individual advantages and disadvantages that will be discussed in this section **(Figure 4)**.
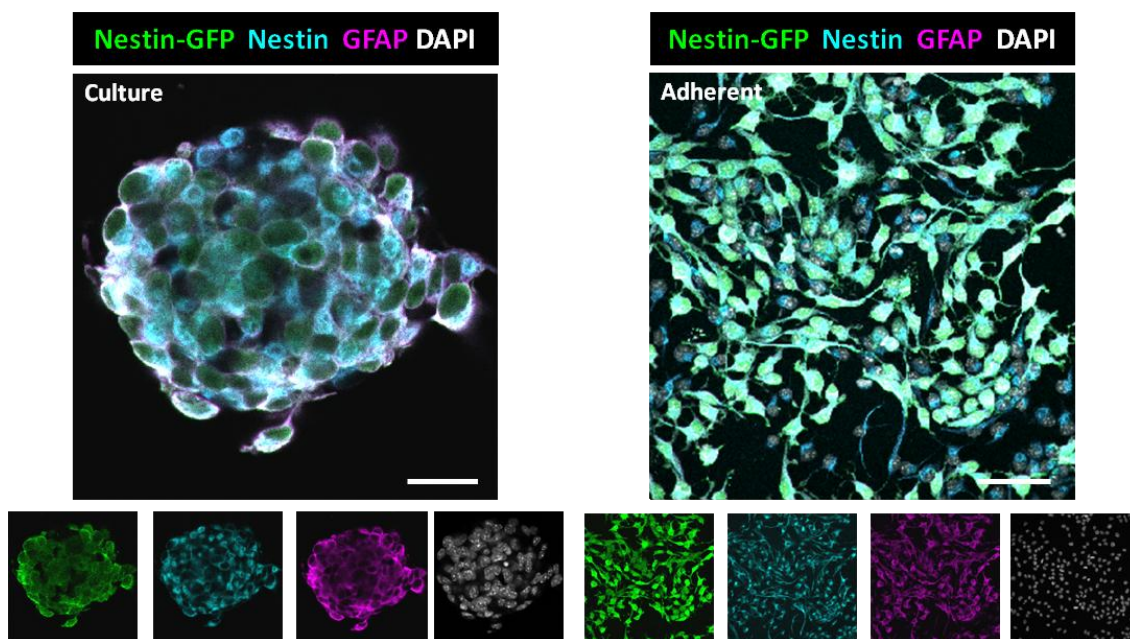
*Figure 4. In vitro methods to study neurogenesis. **Left panel.** Representative confocal z-stack of neural precursor cells from Nestin-GFP mice grown in vitro as neurospheres. **Right panel.** Representative confocal z-stack of neural precursor cells from Nestin-GFP mice grown in vitro as an adherent monolayer. Intrinsic nestin-GFP is labeled in green, nestin in cyan, GFAP in magenta and nuclei were stained with DAPI (white). Scale bars = 20μm*

<u>Neurospheres</u>: Neural stem cells (NSC) in vitro have the ability to proliferate, self-renew, and generate multipotential offspring (Rietze and Reynolds, 2006). NSCs expand giving rise to floating clusters termed neurospheres. This in vitro system allows the analysis of different NSC features such as the proliferative, renewal and differentiation potential (Jensen and Parmar,

51

2006). Because neurospheres are preserved in well-defined culture conditions, the only external signals that they might encounter are those produced by their neighbor cells. In addition, the manipulation of the extrinsic signaling is an easy task that can be achieved by varying different factors of interest in the media or co-culturing with other cells (Campos, 2004). In addition to the many advantages that the neurosphere culture provides, there are also some limitations of their use that need to be considered. Neurosphere cultures might present rather a high variability due to their sensitivity to their environment. Small changes is cell density, number and frequency of passages, or concentration of media factors can result in different proliferative rates or even changes in the properties of the cells within the neurosphere (Arsenijevic et al., 2001; Caldwell et al., 2001; Irvin et al., 2003; Morshead et al., 2002; Tropepe et al., 1999). Due to these variations in culture method, it is often difficult to compare studies from different groups (Hack et al., 2004; Parmar et al., 2002) in order to achieve conclusions on the neuroprogenitor cell properties. Nevertheless, neurospheres have proven useful for neurogenesis-related biological studies (Klein et al., 2005).

Adherent monolayer: Adherent monolayer cultures overcome some of the disadvantages related to neurospheres as they are a less heterogeneous population of precursor cells. Single cells grow as a monolayer and therefore, each cell is equally exposed to culture media growth factor. This setting prevents any spontaneous differentiation and allows uniform proliferation of the cells (Conti and Cattaneo, 2005). Additionally, monolayer cultures facilitate the follow-up of individual cells, in which space between cells allow the analysis of variations in different features such as morphology or cell size.

Nevertheless, it must be highlighted that both neurospheres and monolayer culture represent a reductionist approach since neuroprecursor cells are always surrounded and in close contact to neighbor cells in vivo. Therefore, the advantages and disadvantages of the in vitro cultures must be carefully considered when interpreting the results obtained in this culture conditions.

3.3.4. In vivo methods to study neurogenesis

A complete analysis of neurogenesis in vivo is based on the characterization of the cell types and their tracking through the cascade from the NSC step until the differentiation into neurons. Several strategies have been developed over time in order to perform a systematic

analysis of the different steps of neurogenic cascade in vivo (Kuhn et al., 2016), and we will detailed them in this section.

The most classical strategy utilized to study the different steps of the neurogenic cascade is the tracking of proliferating cells by **thymidine analogues** such as 5-bromo-2´-deoxyuridine (BrdU), 5-chloro-2'-deoxyuridine (CldU), or 5-iodo-2'deoxyuridine (IdU) (Burns and Kuan, 2005; Encinas and Enikolopov, 2008; Kempermann et al., 2004; Vega and Peterson, 2005). Their main advantages are that they can be administered systemically and that the label is retained in the long term, allowing lineage analysis of the newborn cells. The combination of this approach with **cell-specific markers** and **morphology** of the different cells from the cascade, results in a precise tool to study neurogenesis by immunofluorescence. However, one of the limitations that this techniques present is that the analysis of neurogenesis can only be performed in fixed tissue, and therefore, it does not allow the study of electrophysiological properties of the cells of interest.

In order to overcome this limitation, in the recent years the use of **viral vectors** (retroviral and lentiviral vectors) has been validated as an experimental approach to study neurogenesis. **Retroviral vectors** that carry fluorescent transgenes have been used to label dividing cells and their progeny (Enikolopov et al., 2015). The infected cells are permanently labeled, which allows long-term analysis and, in addition, there is no need of fixing the tissue, which is advantageous in order to perform electrophysiological analysis (Laplagne et al., 2006). However, retroviral vectors only infect dividing cells and its efficiency is rather low. On the other hand, **lentiviral vectors** can infect both dividing and non-dividing cells efficiently (Sarno and Robison, 2018) and therefore, they can be used for long-term in vivo tracking of the adult NSC during proliferation, migration and differentiation (Consiglio et al., 2004; Geraerts et al., 2006). In addition, low doses of lentiviral vectors have been used to perform clonal analysis in vivo, allowing a more precise lineage analysis (Bonaguidi et al., 2011).

Another strategy that has been utilized in order to study neurogenesis are **transgenic mice**. The use of **constitutive reporter** mice in which fluorescent reporter genes are under the control of cell type-specific promoters such as nestin, GFAP, or DCX have arisen as useful tools that allows the visualization, characterization and isolation of newborn cells in the adult brain (Dhaliwal and Lagace, 2011; Kuhn et al., 2016). An innovative approach of the recent years has been the development of **inducible transgenic mice**. This technique is based on the targeted expression of the Cre-recombinase fused with a mutated form of the estrogen receptor (e.g.,

CreER[T2]), which presents a high affinity for tamoxifen, an exogenous estrogen (Rotheneichner et al., 2017). After tamoxifen administration, the fusion protein translocates to the nucleus and excises genomic segments comprised between loxP sequences. In many cases, the loxP sites floxed a reporter protein that has a stop codon, which allows the expression of the reporter protein after recombination. One of the most popular promoters in the CreER/lox system to study adult neurogenesis has been nestin (Nestin-CreER[T2]) (Lagace et al., 2007), although recently more CreER/lox systems under different promoters such as GLAST (Glutamate aspartate transporer; specific of astrocytes) (Mori et al., 2006), Sox2 (Sex determining region Y box 2; expressed in NSCs and ANPs) (Favaro et al., 2009) or Tlx (Nuclear receptor subfamily 2 group E; specific of B cells of SVZ) (Liu et al., 2008) have been generated in order to study adult neurogenesis. One of the greatest advantages of this approach is that upon tamoxifen administration, the recombination will be permanent in both the desired cells and their progeny, which will allow the specific visualization and characterization of the cells in a long-term (Dhaliwal and Lagace, 2011). In addition, the tamoxifen system is very useful in loss-of-function studies, since the desired gene will only be excised upon tamoxifen administration, which ensures a normal development of the mice.

3.3.5. The role of microglia in adult hippocampal neurogenesis

Microglia are crucial elements in the adult hippocampal neurogenic niche. The most characterized function of microglia in neurogenesis is inflammation and the detrimental consequences that pro-inflammatory cytokines exert on the adult hippocampal neurogenesis. A persistent neuroinflammation is harmful for the brain. The cytokines released by immune cells provoke behavioral changes in the brain in animals undergoing inflammation, which is termed sickness behavior (Dantzer and Kelley, 2007). The major cytokines, IL-1, TNF-α, and others, act on the hypothalamus to cause alterations in the homeostasis, such as elevated body temperature, increased sleep, and loss of appetite as well as major alterations in lipid and protein metabolism leading to significant weight loss (Tizard, 2008). In addition, inflammation impairs different hippocampal functions such as novel place recognition, spatial learning, and memory formation (Belarbi et al., 2012) and some of these effects have been related to the detrimental consequences of inflammation on neurogenesis. Newborn neuron formation is declined after both systemic or intrahippocampal administration of LPS, likely because it reduces newborn cell survival of (Ekdahl et al., 2003; Monje et al., 2003). In addition, in vitro experiments performed with conditioned media from LPS-treated microglia showed induction in apoptosis of neuroblasts (Monje et al., 2003). However, in addition to

microglia other brain immune cells such as meningeal macrophages, perivascular macrophages, and mast cells (Lopez-Atalaya et al., 2017), are also able to respond to LPS and contribute to the inflammatory response. Therefore, although the negative role of inflammation on neurogenesis is well characterized, the contribution of microglia and the rest of the inflammatory cells in the brain need to be delimited.

In addition to the role of inflammation on neurogenesis, several studies suggest that microglia could produce factors that might modulate proliferation or survival of different cells of the neuronal lineage, which suggests a much less studied beneficial role of microglia. In vitro studies demonstrate that cultured microglia promotes differentiation of precursor cells (Aarum et al., 2003). In addition, microglia conditioned media enhances survival of neurons and neuroblast production (Morgan et al., 2004; Walton et al., 2006). Throughout the literature, a limited number of growth factors, such as FGF-2 (Fibroblast growth factor-2) or IGF-1, have been described to be produced by microglia (Sierra et al., 2014a). These molecules produced by microglia that could potentially affect neurogenesis will be further detailed in **section 3.3.6.** On the other hand, neuroprogenitor cells are known to be influenced by surrounding signals such as different trophic factor. However, direct evidence of neurogenesis regulation by microglia-derived factors is still missing both in vitro and especially in vivo (Sierra et al., 2014b).

3.3.6. Regulation of neurogenesis by phagocytic microglia

As mentioned above, microglia can play an essential role in neurogenesis and its regulation through the release of different trophic factors or the phagocytosis of apoptotic newborn cells. However, little is known about the role of the trophic factors released by phagocytic microglia. One of the goals of the present study is to systematically identify different molecules that were described to be implicated in the regulation of neurogenesis and that have emerged as neuromodulatory factors secreted by microglia in the course of this study. In this section we will review in detail the already described role in neurogenesis of some of those molecules and their potential release by microglia.

3.3.6.1. Neuropeptides

3.3.6.1.1. Cart: Cocaine- and amphetamine-regulated transcript (CART) is a highly conserved neuropeptide across mammalian species (Douglass and Daoud, 1996; Kristensen et al., 1998).

-Expression. CART is largely expressed in stomach and endocrine glands as well as in both CNS and PNS (Rogge et al., 2008; Wierup et al., 2005). In the brain, CART is mainly observed in nerve terminal vesicles, indicating its role in neurosecretory pathway (Rogge et al., 2008). In addition, its high expression in appetite associated regions in the brain suggests a role for CART in energy balance.

-Microglial production. There are very few studies regarding microglia and CART and they mainly address the anorexigenic effects of CART on metabolism and the link between fat-rich diets and microglial inflammation in vivo (Barreto-Vianna et al., 2016; Geloneze et al., 2017), but direct connections between CART and microglia have not been studied.

-Receptors. Although binding to A G protein-couple receptor have been proposed, CART target receptor is still unknown (Lakatos et al., 2005).

-Functions. CART participates in a variety of physiological processes, such as energy balance, reward or neuroprotection (Rogge et al., 2008). This neuropeptide promotes the production of different neurotrophic factors that enhance neural differentiation of stem cells (Zhang et al., 2012). CART is early expressed at P12 and maintains the same expression pattern throughout adulthood, suggesting a role in synapse and spine formation (Abraham et al., 2007). However, further studies are needed to unravel CART function in the development of the central nervous system.

3.3.6.1.2. VGF: VGF (non-acronymic) is a 68kDa polypeptide with a secretory sequence of 22 amino acids at the N-terminal, which promotes translocation to the endoplasmic reticulum (ER) (Jethwa and Ebling, 2008). After proteolytic post-translational processing, a variety of VGF peptides are produced (Bernay et al., 2009; Trani et al., 2002). Bioactive VGF-derived peptides include TLQP-62, TLQP-21, HHPD-41, AQEE-30, AQEE-11, LQEQ-19, and NERP-1 and -2 (Trani et al., 2002; Yamaguchi et al., 2007).

-Expression. VGF mRNA is broadly distributed throughout CNS (Lewis et al., 2015). In adult mice, VGF mRNA is highly expressed in hypothalamus and cerebellum, and in addition it can also be found in olfactory bulbs, hippocampus, cortex, basal ganglia, thalamus, amygdala, midbrain, and the brainstem. In the hippocampus, VGF peptides are mainly located in axons of the mossy fibers and synaptic terminals in the CA3 (Hunsberger et al., 2007).

-Microglial production. In terms of microglia, there are very few studies in which microglia and VGF are linked (Chen et al., 2013; Riedl et al., 2009). Different studies have been performed in order to show that both macrophage and microglia cells express the VGF receptor gC1qR both in vitro and in vivo (Chen et al., 2013); however, whether microglia produce VGF peptides have not been previously studied.

-Receptors. Of all the VGF derived peptides, TLQP-21 has been the most studied. Two receptors have recently been proposed for TLQP-21. First, gC1qR, which is activated in rat macrophages after TLQP-21 binding and in addition, it is also expressed in both brain and spinal cord derived microglia (Chen et al., 2013). Second, the complement receptor C3A receptor-1 (C3AR1), which not only does mediate immune response, but also it has been associated with cancer, neurogenesis and hormone release (Francis et al., 2003; Opstal-van Winden et al., 2012).

-Functions. VGF is implicated in metabolism, and different pathologies such as neurodegeneration (AD and PD) or psychiatric disorders (depression) (Cocco et al., 2010; Foglesong et al., 2016; Razzoli et al., 2012). In addition, VGF peptides enhance adult hippocampal neurogenesis both in vitro and vivo (Thakker-Varia et al., 2014; Thakker-Varia et al., 2007; Yang et al., 2016) and promote neuron survival upon injury in vitro (Severini et al., 2008).

3.3.6.2. Trophic factors

3.3.6.2.1. EGF: EGF (Epidermal Growth Factor) is a polypeptide synthesized as a precursor that is proteolytically cleaved to maturation (Yamada et al., 1997). There are additional growth factors that share structural features with EGF and are also able to bind EGF receptor (EGFR), such as TGFα, Amphiregulin, Epiregulin, Betacellulin, Heparin-binding epidermal growth factor-like growth factor and Epigen (Singh et al., 2006). All these molecules exert combined effects when bind to the receptor in order to achieve a large range of biological responses.

-Expression. EGF is widely express in different tissues. In the brain the highest expression is found in cerebellum, cortex, hippocampus or olfactory bulb among other (Wong and Guillaud, 2004).

-Microglial production. The release of EGF by microglia has been specially studied in cell cultures and ischemic conditions, where it shows a neuroprotective role by enhancing the survival of neurons (Villalonga 2008). In addition, EGF is also released by microglia after ischemia in vivo (Lee et al., 2005).

-Receptors. The epidermal growth factor receptor belongs to ErbB family of receptors, which comprises four receptor tyrosine kinases: EGFR (ErbB-1), HER2/neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4), EGFR immunoreactivity is widely distributed throughout the brain during both development and adulthood (Bodey et al., 2005).

-Functions. EGF promotes proliferation and regulates the fate of neural stem and progenitor cells in the developing and adult CNS. In addition, EGF can promote the migration and proliferation of newborn cells in the SVZ. EGF enhances the differentiation, maturation and survival of a variety of neurons (Wong and Guillaud, 2004).

3.3.6.2.2. FGF-2: Fibroblast growth factor-2 (FGF-2) is a trophic factor that belongs to the superfamily of proteins Fibroblast growth factor (FGFs), which can bind heparin and extracellular heparin sulfate proteoglycans (HSPGs) (Burgess and Maciag, 1989).

-Expression. FGF-2, like some other members of the FGF family, is expressed in the CNS both during development and postnatally (Chadi et al., 1993; Eckenstein, 1994; Emoto et al., 1989). Within the CNS, FGF-2 has been widely found not only in neurons but also in glial cells (Eckenstein et al., 1991). In detail, FGF-2 mRNA has been detected in the olfactory bulb, cortex, hippocampus, striatum, thalamus, substantia nigra, pons, medulla oblongata, motor and sensory nuclei, as well as in the pituitary (Bean et al., 1991; Ernfors et al., 1990; Gomez-Pinilla et al., 1994; Grothe et al., 1991).

-Microglial production. Regarding microglia, microglia is able to both secrete and respond to FGF-2 in vivo (Liu et al., 1998). In addition, the production of FGF-2 by microglia and its possible contribution to the regulation of neuronal development and regeneration has been proposed for almost 30 years (Shimojo et al., 1991).

-Receptors. FGF-2 binds with high-affinity to four different tyrosine kinase receptors (FGFR1-4) (Jaye et al., 1992). FGFR1 and FGFR4 are mainly produced in adult CNS neurons, while oligodendrocytes and astrocytes express large levels of FGFR2 and FGFR3 respectively (Asai et al., 1993; Miyake and Itoh, 1996). FGF-2 displays the highest affinity for FGFR-1 in the CNS (Woodbury and Ikezu, 2014).

-Functions. In the adult CNS, FGF-2 is expressed in the neurogenic niches, SVZ and SGZ, and has been associated with adult neurogenesis regulation because it promotes proliferation and differentiation of adult neural stem and progenitor cells (Rai et al., 2007; Werner et al., 2011). In addition, during development, FGF-2 controls fate, migration and differentiation of neuronal progenitors (Dono et al., 1998).

3.3.6.2.3. GDNF: The glial cell line–derived neurotrophic factor (GDNF) belongs to GDNF family ligands (GFL) which includes different related factors: GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN) (Airaksinen et al., 2006). Each ligand is produced as pre-pro-precursor protein which is cleaved afterwards to produce the bioactive forms of the protein (Lin et al., 1993; Masure et al., 1999).

-Expression. GDNF is mainly produced in hippocampus, cerebellum and cortex (Cortes et al., 2017). Neurons are the main source of GDNF in the brain; however, its expression has been also found in glial cells at much lower rates (Carnicella and Ron, 2009).

-Microglial production. Similar to EGF, microglia also produces GDNF which is enhanced after ischemia in vivo (Lee et al., 2004)

-Receptors. GDNF preferentially binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3, and PSPN to GFRα4, all of which are GFL family receptors (Airaksinen et al., 2006).

-Functions. GDNF acts in the support of CNS and PNS neurons by promoting their survival and differentiation (Fielder et al., 2018). In addition, GDNF is implicated in the process of neuronal networking and synapse plasticity (Hibi et al., 2009). Moreover, GDNF also have a role in progenitor cell differentiation into astrocytes (Boku et al., 2013). Furthermore, GDNF plays neuroprotective functions in the survival of neurons from substantia nigra in PD (Kirik et al., 2004).

3.3.6.2.4. IGF-1: IGF-1 (Insulin Growth Factor-1) is a 70-amino-acid polypeptide hormone which shares structural features with insulin (Laron, 2001).

-Expression. IGF-1 is produced in many tissues including the brain (Sell, 2015; Wrigley et al., 2017). In addition, in developmental brain, mRNA for IGF-1 is expressed in many regions of the adult brain, such as the hippocampus, olfactory bulbs and cerebellum (Bondy and Lee, 1993; Werther et al., 1990). However, adult expression of IGF-1 is mainly limited to the neurogenic niches (Wrigley et al., 2017). In addition, circulating IGF-1 can cross the BBB and influence the brain (Ozdemir et al., 2012).

-Microglial production. Cultured microglia express IGF1 mRNA and produce IGF1 protein at ranges of pg/ml (Suh et al., 2013). In addition, microglia cells have also shown to produce IGF-1 in vivo (Ziv and Schwartz, 2008).

-Receptors. IGF-1 binds to the type 1 IGF receptor, although it also presents affinity for the insulin receptor (Werner and LeRoith, 2014). The IGF-1 receptor is found throughout the body, consistent with its diverse range of actions. In the brain, IGF-1 receptors are expressed in the hippocampus, olfactory bulbs, hypothalamic areas, and in ependymal and epithelial cells of the choroids plexus (Richardson et al., 2017).

-Functions. IGFs is a trophic factor that enhances proliferation of CNS neuroprogenitors (Arsenijevic et al., 2001; Learish et al., 2000). Moreover, IGF-1 affects neuronal maturation at different steps, including neurotransmitter production and electrical activity (Torres-Aleman, 1999). IGF-1 exerts a large variety of actions on neural linage cells, which includes a reduction of the neuroprogenitors cell cycle or even an enhancement in the proliferation of neural cell types (Ye and D'Ercole, 2006). In the adult brain, IGF1 modulates the activity of neural circuitries and the strength of the synapses (Llorens-Martin et al., 2009). In addition, IGF-1 has been proposed as one of the mediators of the effect of physical exercise in adult neurogenesis (Ziv and Schwartz, 2008).

3.3.6.2.5. PDGF-α: Platelet-derived growth factor (PDGF) family comprises four polypeptide chain isoforms that are encoded by different genes (PDGF-A, -B, -C and –D) and form both homo and heterodimers (Heldin, 2012).

-Expression. PDGF is produced by a wide range of cells such as platelets, macrophages, muscle cells, etc. In addition, expression of the four chains is found within the nervous system (Andrae et al., 2008; Ishii et al., 2008; Zheng et al., 2010).

-Microglial production. Microglial have been shown to release PDGF and its production is enhanced after ischemic stroke in vivo (Su et al., 2017).

-Receptors. Two PDGF receptors have been described (PDGFR-α, -β), which bind to different chains (Heldin, 2012). Both PDGFR-α and PDGFR-β are expressed in neurons from cortex, retina or substantia nigra among others (Nait Oumesmar et al., 1997).

-Functions. Role of PDGF and its receptors vascularization is essential for a correct development of CNS (Sil et al., 2018). Besides its well-known role in angiogenesis, PDGF-A plays an essential function in neuronal survival, especially in damaged brain areas (Krupinski et al., 1997). In addition, PDGFs can induce neural precursor cell differentiation into oligodendrocytes, neurons and astrocytes (Chojnacki et al., 2008; Chojnacki and Weiss, 2004).

3.3.6.2.6. VEGF: Vascular endothelial growth factor (VEGF) is a heterodimeric glycoprotein which presents alternative posttranslational modifications, leading to the production of biochemically different proteins (Azam et al., 2010; Cursiefen et al., 2004; Ferrara, 2009).

-Expression. VEGF is produced in a large variety of cell types such as platelets, keratinocytes, smooth muscle cells, etc. In the CNS VEGF is released by astrocytes, microglia and neuronal stem cells (Jin et al., 2016; Nowacka and Obuchowicz, 2012).

-Microglial production. Along with other glial cells, microglia produce VEGF in vivo (Jin et al., 2016; Krause et al., 2014).

-Receptors. VEGF binds three receptor subtypes, VEGFR1, VEGFR2 and VEGFR3; however, in the nervous system, VEGFR2 is the mainly expressed receptor (Nowacka and Obuchowicz, 2012).

-Functions. VEGF promotes proliferation and survival of endothelial cells and stimulates nitric oxidedependent vasodilation. It influences vasculature formation and increases vascular permeability (Azam et al., 2010; Carmeliet and Storkebaum, 2002). In the brain, VEGF participates in angiogenesis both in embryonic and postnatal development (Carmeliet and Storkebaum, 2002). Importantly, VEGF has emerged as a neuronal and glial trophic factor (Brockington et al., 2004). In the nervous system, VEGF participates in the induction of proliferation and migration of neural stem cells and neuroblast, as well as survival and maturation of neurons and axonal outgrowth (Fournier and Duman, 2012; Nowacka and Obuchowicz, 2012). VEGF can also influence a variety of complex processes in adult mice such as learning and memory (Cao et al., 2004).

3.3.6.3. Matrix proteins

3.3.6.3.1. MMP-3: Matrix metalloproteinases (MMPs) are proteolytic enzymes that reshape of the extracellular matrix (ECM) (Seiki, 2002; Stamenkovic, 2003). MMP-3 is able to degrade a broad range of ECM proteins. Apart from this role, MMP-3 also activates different growth factors by cleavage, such as cytokes, chemokines or adhesion molecules (Van Hove et al., 2012).

-Microglial production. Microglia is one of the few brain cells that express MMP-3, and this expression has been shown to be enhanced in AD in vivo (Brkic et al., 2015).

-Expression. In the adult CNS, the level of MMP3 mRNA and protein are almost inexistent (Li et al., 2009) and MMP-3 expression is restricted to neurons and microglia in physiological conditions (Van Hove et al., 2012).

-Functions. MMP-3 has been related to metastatic processes due to its degradatory nature; however, it also exerts multiple physiological roles during development and adulthood. In development, MMP-3 is implicated in migration, apoptosis and axonal growth in the remodeling process of the cerebellum (Vaillant et al., 2003) as well as in the formation and remodeling of synapses (Ethell and Ethell, 2007). In the adult, MMP-3 is associated to neuroprogenitor differentiation and migration (Barkho et al., 2008).

3.3.6.4. Membrane ligands

3.3.6.4.1. Jag1: Jagged1 (Jag1) is a surface ligand that binds to Notch receptor (Zhou et al., 2010).

-Expression. Jag1 is widely expressed in the brain, especially in the cortex, hippocampus and cerebellum (Stump et al., 2002).

-Microglial production. There are very few reports studying Jag1 expression on microglia; however, those studies suggest a very limited Jag1 expression on these cells in vitro (Xing et al., 2013)

-Receptors. A total of four Notch receptors have been described (Notch 1-4) in mammals that trigger their signaling after the direct cell-to-cell binding of different transmembrane ligands (Jagged1 and 2; Delta-like1, 3 and 4) (Zhou et al., 2010).

-Functions. Jag1 promotes the differentiation of neuroprogenitor cells into astrocytes (Grandbarbe et al., 2003; Morrison et al., 2000). In addition, it limits neuron premature differentiation through the inhibition of proneural genes and increase of neuroprogenitor cell survival (Gaiano and Fishell, 2002; Lutolf et al., 2002). Jag1 is also implicated in the regulation of dendrite and axon growth and morphology (Mathieu et al., 2013).

3.3.6.5. Cytokines

3.3.6.5.1. Csf3: Colony stimulating factor 3 (Csf3), also termed granule-colony stimulating factor (G-CSF), is a small glycoprotein formed by several α helices (Wells et al., 1996).

-Microglial production. Along with the rest of the immune system cells, microglia also release Csf3 at least in vitro (Spanos et al., 2015).

-Expression. Csf3 is released by wide range of cells such as bone marrow cells, fibroblasts or macrophages, upon different stimuli (Aloisi et al., 1992; Demetri and Griffin, 1991). Importantly, Csf3 and its receptor (G-CSFR) are expressed in both the SVZ and the DG (Schneider et al., 2005).

-Receptors. G-CSFR is expressed hematopoietic cells and also on neurons, endothelial cells, and glial cells (Boneberg et al., 2000; Schabitz et al., 2003).

-Functions. Csf3 is a neuroprotective cytokine in the brain (Wallner et al., 2015). It has been shown to trigger anti-apoptotic pathways (Komine-Kobayashi et al., 2006) and proliferation in bone marrow stem cells (Jung et al., 2006). In addition, Csf3 promotes the differentiation of neural stem cells both in vitro and in vivo (Schneider et al., 2005). Moreover,

Csf3 exerts an anti-inflammatory role since it reduces levels of TNF-α (tumour necrosis factor α), the inhibition of iNOS (inducible nitric oxide synthase) activity or the decrease of IL-1β (Lu and Xiao, 2006).

3.3.6.5.2. IL-1β: Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that belongs to the IL-1 family.

-**Expression.** In the brain, IL-1β is released mainly by microglia, although astrocytes can also produce this cytokine; and the level of this cytokine are increase upon different stimuli such as injury or disease (Arguello et al., 2009; Pitossi et al., 1997).

-**Microglial production.** The microglial IL-1β release is mainly documented in conditions of neuroinflammation both in vivo and in vitro (Smith et al., 2012).

-**Receptors.** IL-1β binds to type I IL-1 receptor (IL-1RI). This receptor is activated upon the interaction with both cytokines IL-1α and IL-1β. In addition, a third ligand called Interleukin 1 receptor antagonist (IL-1Ra) can also bind IL-1RI, but no signaling cascade is triggered afterwards, acting as an inhibitor (Dinarello, 2011; Weber et al., 2010).

-**Functions.** An overproduction of IL-1β in the hippocampus during neuroinflammatory conditions contributes to decline hippocampal neurogenesis in many neurodegenerative and psychiatric disorders (Ye et al., 2013). In addition, IL-1β prevents neuron differentiation in the hippocampus (Mathieu et al., 2010). In physiological conditions the role of IL-1β is still not well understood and contradictory results have emerged regarding its implication in the induction of proliferation (Koo and Duman, 2008; Spulber et al., 2008). Moreover, there is some evidence suggesting that IL-1β is necessary for hippocampal dependent learning and memory (Goshen et al., 2007). Thus, additional studies are needed in order to understand the role of IL-1β in physiological conditions.

3.3.6.5.3. IL-6: Interleukin-6 (IL-6) is a pleiotropic cytokine that can exert its function through two signaling pathways. In classical signaling, IL-6 binds to the α-receptor IL-6R, which triggers the dimerization with β-receptor gp130, promoting the downstream signaling pathway (Hibi et al., 1990). On the contrary, IL-6R can be cleaved and released as a soluble fraction (sIL-6R) which will bind to IL-6. This complex can trigger the alternative pathway in cells that lack IL-6R but express gp130 (trans-signaling) (Rose-John and Heinrich, 1994).

-**Expression.** IL-6 is produced by different immune cells such as T-, B-cells or macrophages and microglia, especially under neuroinflammatory conditions; and also by fibroblast, adipocytes or even neurons (Rothaug et al., 2016).

-Microglial production. Similar to IL-1β production, IL-6 released from microglia has been mainly studied in the context of inflammation and microglia activation in vivo and in vitro (Smith et al., 2012)

-Receptors. In the brain, IL-6R is expressed in very limited cell types (immune cells mainly), but is not found on oligodendrocytes or astrocytes (Hsu et al., 2015; Lin and Levison, 2009). On the other hand, gp130 is ubiquitously expressed on almost all cell types including glia and neurons (Rothaug et al., 2016).

-Functions. The two signaling pathways result in both positive and negative effects in the brain. Classical signaling mainly plays a regenerative role in the tissue, whereas trans-signaling mediates most of the traditionally studied detrimental consequences of this cytokine. IL-6 can both trigger neuronal degeneration (Campbell et al., 2005) as well as regeneration by promoting oligodendrocyte differentiation and acting as neurotrophic factor (Yang et al., 2012). In addition, the lack of IL-6 signaling has also been implicated in both inflammation and neuroprotection (Klein et al., 1997; Penkowa et al., 1999).

3.3.6.5.4. TGF-β*:* The human transforming growth factor-beta (TGF-β) family includes a large group of secreted proteins: TGF-βs themselves, activins, nodal, bone morphogenetic proteins and growth and differentiation factors (Morikawa et al., 2016). TGF-βs are synthesized as inactivated pre-pro-proteins that upon secretion are proteolytically activated (ten Dijke and Arthur, 2007).

-Expression. In the CNS, TGF-β2 and TGF-β3 are found in the NSCs, mature astrocytes and numerous neuron populations. TGF-β1 is expressed in the meninges and the choroid plexus as in astrocytes and microglia (Wachs et al., 2006). Moreover, after traumatic spinal cord injury, TGF-β1 and TGF-β2 are up-regulated in neurons (Buss et al., 2008).

-Microglial production. Similar to other cytokines, microglia release TGF-β, especially under inflammatory stimuli, lesions or pathology in vitro and in vivo (Wachs et al., 2006).

-Receptors. There are three different TGF-β receptors, TGFBRI, TGFBRII and TGFBRIII. TGF-β can bind to either TGFBRII directly or TGBRIII, which will result in the presentation of TGF-β to TGFBRII. Afterwards, TGFBRII recruits TGFBRI in order to trigger the downstream signaling pathway (Blobe et al., 2001). In the normal CNS, TGF-β receptors are expressed by neurons, astrocytes and microglia (Lane et al., 2007).

-Functions. TGF-β can exert dual functions in the CNS. During hippocampal neurogenesis, TGF-β promotes cell cycle exit of the neuroprogenitor cells and neuronal differentiation (Vogel et al., 2010). Nevertheless, TGF-β also plays negative effects on

neurogenesis, since an overexpression can result in a decreased proliferation of neuroprogenitor cells (Buckwalter et al., 2006; Wachs et al., 2006).

3.3.6.5.5. TNF-α: Tumor necrosis factor alpha (TNF-α) belongs to the large TNF ligand superfamily and is generally produced a precursor transmembrane-associated TNF-α protein that is proteolytically cleaved into a soluble TNF-α homotrimer (Wajant et al., 2003). Both transmembrane and soluble forms are biologically active (Montgomery and Bowers, 2012).

-Expression. TNF-α is synthesized in many cell types, including immune cells and non-immune cells such as endothelial cells, fibroblasts, etc. Within the CNS, microglia, astrocytes and neurons can release TNF-α (Hanisch, 2002; Klintworth et al., 2009; Lau and Yu, 2001).

-Microglial production. In the CNS, microglia is one of the cell sources of TNF-α, and its production is enhanced upon an immunological stimulus in vitro and in vivo (Garden and Moller, 2006; Klintworth et al., 2009).

-Receptors. Two transmembrane glycoprotein receptors have been reported: TNF-receptor 1 (TNF-RI) and TNF-receptor 2 (TNF-RII). Both receptors are differentially expressed and controlled (Montgomery and Bowers, 2012). TNF-RI is constitutively expressed on the majority of the cells, except for erythrocytes. On the other hand, TNF-RII expression is triggered after an inflammatory stimulus on hematopoietic lineage cells (McCoy and Tansey, 2008).

-Functions. Classically, TNF-α binding to TNF-RI has been related to an increase of apoptosis and cytotoxicity, whereas binding to TNF-RII has mostly been associated with survival and proliferation. This dual role of TNF-α is also observed in adult neurogenesis. Upon binding to TNF-RI, hippocampal NSC proliferation is decreased and cell death is enhanced, which results in an inhibition of adult neurogenesis (Cacci et al., 2005). On the contrary, when TNF-α is bound to TNF-RII, it triggers the increase in proliferation and survival of NSCs, leading to a maintenance of neurogenesis (Iosif et al., 2006). Thus, TNF-α can exert both beneficial and detrimental effects in adult hippocampal neurogenesis, which result upon the binding to different receptors.

3.3.6.6. Metabolic products

3.3.6.6.1. Lactate: L-lactate is the monosaccharide compound that result from the fermentation of pyruvate during both normal metabolism and exercise.

-Production. Different cell types produce lactate in basal conditions, such as erythrocytes, hepatocytes or skeletal myocytes (Phypers et al., 2006). In the physiological CNS, astrocytes are the main lactate source (Bonvento et al., 2005; Choi et al., 2012).

-Microglial production. Both microglia and macrophages can release lactate; however, it has only been studied in the context of inflammation associated activation in vitro (Kelly et al., 2015; Tsukada et al., 2014).

-Receptors. Lactate can enter a cell via different monosaccharide transporters in order to be used as fuel (Choi et al., 2012). In addition, in the recent years, a specific receptor for lactate have been describe, the G protein coupled receptor 81 (GPR81), which is especially localized in hippocampus, cortex and cerebellum and it has been mainly reported to be expressed by neurons (Morland et al., 2015).

-Functions. Lactate had classically been considered just a waste product of metabolism, however, it is currently considered an important source of energy for many cells. In addition, lactate does not only play a role in metabolism, but it also exerts hormone functions and it has been related to memory formation and neuroprotection (Proia et al., 2016). Lactate supports synaptic function and promotes long-term potentiation (Cater et al., 2001; Skriver et al., 2014). Moreover, upon glucose deprivation, lactate protects tissue integrity and reestablishes neuron excitability (Ainscow et al., 2002; Cater et al., 2001). In addition, lactate might also support neurogenesis in vivo (Alvarez et al., 2014), however, further research is required.

4. HYPOTHESIS AND OBJECTIVES

4. HYPOTHESIS AND OBJECTIVES

During adult hippocampal neurogenesis, the majority of the newborn cells die by apoptosis and are rapidly and efficiently eliminated by microglia in the process termed phagocytosis (Sierra et al., 2010). In this study, we hypothesize that microglial phagocytosis is not merely the end point of the elimination of apoptotic cells, but has an active role in maintaining homeostasis of the neurogenic cascade by triggering a coordinated neuromodulatory program in microglia, which contributes to the maintenance and correct regulation of the neurogenic niche.

Aim 1. To develop an in vitro model of phagocytosis of apoptotic cells that mimics phagocytosis in vivo. For this purpose we will first study the phagocytosis cargo identity; early engulfment; and presence of key opsonizing molecules in physiological conditions in order to translate this information to an in vitro model of phagocytosis. Afterwards we will set up our in vitro model of phagocytosis in which primary microglia will be fed with different (mouse or human) neuronal lines that have been previously treated with staurosporine to induce apoptosis. Microglial phagocytosis of apoptotic cells will be quantified by immunofluorescence by confocal microscopy.

Aim 2. To study the transcriptional changes induced by phagocytosis of apoptotic cells in microglia in vitro. For this purpose we will perform a full genome wide transcriptomic analysis of naïve and phagocytic microglia using the in vitro model of phagocytosis set up in Aim 1. We will perform a functional analysis of the phagocytic microglia transcriptome by using three different bioinformatic platforms (DAVID, ClueGO, and IPA).

Aim 3. To identify the molecules expressed by phagocytic microglia that could potentially modulate neurogenesis. For this purpose we will use the information obtained from the transcriptomic analysis performed in Aim2. We will perform an initial filtering by differentiating those genes whose protein would exert their function outside the expressing cell (heterologous) and genes that would act directly on the cell expressing them (autologous) in the MANGO database (The Mammalian Adult Neurogenesis Gene Ontology). Then, GO terms related to neurogenesis will be selected for the heterologous MANGO genes. Afterwards, we will search back in the array in order to find the molecules that present the

neurogenic GO terms. Finally, the genes will be filtered only to select those that appeared extracellularly (heterologous genes).

Aim 4. To analyze the effects of the factors secreted by phagocytic microglia on neurogenesis in vitro. For this purpose we will collect conditioned media from naïve and phagocytic microglia using the model from Aim 1 and we will test its effects in vitro on neuroprogenitor cells obtained from disaggregated neurospheres. Neuroprogenitor cells will be treated with naïve or phagocytic microglia conditioned media and the cell marker expression and morphology of the resulting cells will be analyzed by immunofluorescence and confocal microscopy. In addition, the resulting cells will be also subjected to calcium imaging under different stimuli in order to perform a further characterization of these cells.

Aim 5. To discriminate the role of cytokines released by phagocytic microglia on neurogenesis in vitro. For this purpose we will collect the conditioned media of LPS-treated primary microglia and we will compared the cell phenotypes produced by the LPS-treated conditioned media with the ones observed in phagocytic microglial conditioned media in the model of neurogenesis in vitro from Aim 4.

Aim 6. To identify the specific factors secreted by phagocytic microglia that trigger the effects on neurogenesis in vitro. For this purpose we will focus in the two most promising neuromodulatory molecules expressed by phagocytic microglia obtained in Aim 3, VGF and lactate. We will validate their production and secretion by inmunofluoresce and/or ELISA and in order to test whether these molecules could be responsible of the cell phenotypes obtained with microglial conditioned media (Aim 4).

Aim 7. To validate the neuromodulatory program of phagocytic microglia in vivo. For this purpose we will perform single-cell RNA sequencing in order to compare a population enriched in phagocytic microglia isolated from the dentate gyrus, where there is ongoing neurogenesis and therefore apoptosis, with non-phagocytic microglia isolated from the CA region of the hippocampus, where there is no neurogenesis and therefore no apoptosis.

Aim 8. To analyze the effects of the factors secreted by phagocytic microglia on neurogenesis in vivo. For this purpose we will infuse the conditioned media obtained for Aim 4 into the hippocampus of 1 month old mice using osmotic pumps in order to analyze the effects

of the factors secreted by phagocytic microglia. All parameters regarding neurogenesis will be quantified by immunofluorescence in mouse tissue sections imaged by confocal microscopy.

Aim 9. To develop new strategies in order to validate the relevance of phagocytic microglia in neurogenesis in vivo. For this purpose we will characterize several phagocytic and purinergic receptors in a microglia impaired model (via administration of the glutamate agonist kainic acid) in FACS-sorted microglia by RT-qPCR. In addition, glutamatergic receptor subunits will also be characterized in order to unravel the mechanism of communication between microglia and the neurogenic niche.

5. EXPERIMENTAL PROCEDURES

5. EXPERIMENTAL PROCEDURES

5.1. ANIMALS

All experiments were performed in fms-EGFP (MacGreen) mice, except for some of the experiments involving administration of microglial conditioned media, which were performed in Nestin-GFP (Encinas et al., 2006; Mignone et al., 2004) and single-cell RNA sequencing experiments, which were performed in CX3CR1$^{GFP/+}$ mice, respectively. In both fms-EGFP (Sasmono et al., 2003; Sierra et al., 2007) and CX3CR1GFP/+ mice (Jung et al., 2000), all microglia express the fluorescent reporter. All mice used were in a C57BL/6 background. Mice were housed in 12:12h light cycle with ad libitum access to food and water. All procedures followed the European Directive 2010/63/EU and NIH guidelines, and were approved by the Ethics Committees of the University of the Basque Country EHU/UPV (Leioa, Spain; CEBA/205/2011, CEBA/206/2011, CEIAB/82/2011, CEIAB/105/2012).

5.2. CELL CULTURES

5.2.1. NE-4C cell line

NE-4C (American Type Culture Collection), a mouse neural stem cell line derived from the cortex of 9 day old (P9) tumor protein 53 (p53, a tumor suppressor gene) knock-out embryos was used for phagocytic assay experiments. NE-4C cells were grown as an adherent culture in Poly-L-lysine-coated (15µl/ml, Sigma) culture flasks covered with 10-15ml of medium. The medium consisted on Minimum Essential Medium (MEM, Gibco), supplemented with 1% Glutamax, 2,5% Fetal Bovine Serum (FBS) and 1% antibiotic/antimycotic (all from Gibco). When confluency was reached, cells were trypsinized and replated at 1:4.

5.2.2. SH-SY5Y cell line

SH-SY5Y (American Type Culture Collection), a human neuroblastoma cell line derived from the bone marrow of 4 year-old female was used for phagocytic assay experiments. SH-SY5Y cells were grown as an adherent culture in non-coated culture flasks covered with 10-15ml of medium. The medium consisted on Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic/antimycotic (all from Gibco). When confluency was reached, cells were trypsinized and replated at 1:4.

5.2.3. BV2 cell line

BV2 (Interlab Cell Line Collection San Martino-Instituto Scientifico Tumori-Instituto Nazionale per la Ricerca sul Cancro), a cell line derived from raf/myc-immortalized murine neonatal microglia was used to obtain LPS induced condition media. BV2 cells were grown as an adherent culture in non-coated culture flasks covered with 10-15ml of medium. The medium consisted on Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic/antimycotic (all from Gibco). When confluency was reached, cells were trypsinized and replated at 1:4.

5.2.4. Primary Microglia Cultures

Primary microglia cultures were performed as previously described (Abiega et al., 2016; Beccari et al., 2018). Postnatal day 0-1 (P0-P1) fms-EGFP mice pup brains were extracted and the meninges were peeled off. The olfactory bulb and cerebellum were discarded and the rest of the brain was then mechanically homogenized by careful pipetting and enzymatically digested with papain (20U/ml, Sigma), a cysteine protease enzyme, and deoxyribonuclease (DNAse; 150U/µl, Invitrogen) for 15min at 37ºC. The resulting cell suspension was then filtered through a 40µm nylon cell strainer (Fisher) and transferred to a 50 ml Falcon tube quenched by 5 ml of 20% Fetal Bovine Serum (FBS; Gibco) in HBSS. Afterwards, the cell suspension was centrifuged at 200 g for 5 min, the pellet was resuspended in 1ml Dulbecco's Modified Eagle's Medium (DMEM, Gibco) complemented with 10% FBS and 1% Antibiotic/Antimycotic (Gibco), and seeded in Poly L-Lysine-coated (15µl/ml, Sigma) culture flasks with a density of two brains per flask. Medium was changed the day after and then every 3–4 days, always enriched with granulocyte-macrophage colony stimulating factor (5 ng/ml GM-CSF, Sigma), which promotes microglial proliferation. After confluence (at 37°C, 5% CO_2 for approximately 14 days), microglia cells were harvested by shaking at 100-150 rpm, 37°C, 4 h. Isolated cells were counted and plated in a density of 80.000 cell/well on poly-l-lysine-coated glass coverslips in 24-well plates for immunofluoresce purposes or 1.000.000 cell/dish on coated Petri dishes for RT-qPCR and Western Blot. Microglia were allowed to settle for at least 24h before any experiment.

5.2.5. Neurospheres cell culture

Neurospheres cultures were performed as previously described (Babu et al., 2011) with some modifications. Briefly, P0-P1 fms-EGFP pups were decapitated and the brains extracted and placed in cold HBSS. The homogenization process was performed as detailed in **section**

5.2.4, except that no FBS was used in any step in order to avoid undesired neurosphere adhesion and differentiation. Afterwards, the cell suspension was centrifuged at 200 g for 5 min, the pellet was resuspended in 1ml DMEM/F12 with Glutamax (Gibco) supplemented with 1% Penicillin/Streptomycin, 1% B27, EGF (12,5 ng/ml), FGF-2 (5 ng/ml) (Xapelli et al., 2013). Cells were plated on uncoated Petri dishes (P60 aprox. 28 cm^2), each brain was plated in 4 Petri dishes with supplemented DMEM/F12. After six days, neurospheres were then disaggregated into a single cell suspension using NeuroCult chemical dissociation kit following manufacturer's instructions and each Petri dish was plated in two 6-multiwell plates. In order to maintain replicability through the experiments, neurospheres were frozen until their use at -80ºC in 15% DMSO after the first passage.

5.3. IN VITRO PHAGOCYTOSIS ASSAY

Microglia were allowed to rest and settle for at least 24h before phagocytosis experiments. Primary microglia cells were fed for different time points with NE-4C or with SH-SY5Y. Both cell lines were previously labeled with the membrane marker CM-DiI (5µM; 10 min at 37°C, 15 min at 4°C; Invitrogen) and treated with staurosporine (1µM for NE-4C or 1,5uM for SH-SY5Y, 4h; Sigma) to induce apoptosis. Apoptotic cells were added to the microglial cultures in a proportion 10:1 approximately. Apoptotic cells were visualized and quantified by trypan blue in a Neubauer chamber. Because cell membrane integrity is still maintained in early induced apoptotic cells, cells not labeled with trypan blue were considered apoptotic. The media of naïve and phagocytic (24h) microglia was immediately stored at -80ºC until its use as conditioned media for neuroprogenitor cells.

For the physiological phagocytosis assay, apoptosis of SH-SY5Y was induced with 3µM STP (4h) and only the floating dead cell fraction was collected from the supernatant and added to the primary microglia cultures in a proportion of 1:1 approximately. As in 10:1 paradigm, apoptotic cells were quantified by trypan blue staining in a Neubauer chamber and non-labeled cells were considered apoptotic.

In some experiments, control and phagocytic microglia were treated with LPS. Three different LPS paradigms were used. In the low LPS concentration paradigm, media was removed and fresh medium with 150 ng/ml LPS or vehicle (PBS) was added for 18 hours to primary microglia (Fraser et al., 2010). In the high LPS concentration paradigm, media was removed and fresh medium with 1 µg/ml LPS or vehicle (PBS) was added for 24h to primary or

BV2 cells (Monje et al., 2003). In order to control for LPS presence in the phagocytic media, a third paradigm was performed in which primary microglia was treated with 1 µg/ml LPS or vehicle (PBS) for 6h, then media was changed into fresh media for another 18h. All supernatants were collected and stored at -80ºC until its use as conditioned media for neuroprogenitor cells, and all of them were filter-sterilized prior to adding to the neuroprogenitor cell culture.

5.4. DETECTION OF DIFFERENT MOLECULES IN THE MICROGLIAL CONDITIONED MEDIA

5.4.1. VGF protein detection

VGF protein levels in microglia conditioned media were detected by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kit following manufacturer's instructions (Cloud-Clone). Briefly, plates were pre-coated with an antibody specific to VGF. Standards and samples were added to a microplate and detected with a biotin-conjugated antibody specific to VGF. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each well and incubated. After TMB substrate solution was added, only wells containing VGF, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450nm. The concentration of VGF in the samples was then determined by comparing the optical density of the samples to the standard curve. Cortex and serum were used as positive and negative controls respectively. Different sample dilutions were tested prior to the experiment.

5.4.2. Lactate levels detection

Lactate levels in microglia conditioned media were detected by L-lactate assay kit (Colorimetric) (Abcam) following manufacturer's instructions. Briefly, protein was removed from the samples using the deproteinizing sample preparation kit – TCA (trichloroacetic acid; Abcam). Next, standards and samples were added to a flat-bottom well plate. Then wells were incubated for 30 mins with the reaction mix containing lactate assay buffer, lactate substrate mix, and lactate enzyme mix. Finally, the color change was measured spectrophotometrically at a wavelength of 450nm

5.5. NEUROPROGENITOR PROLIFERATION AND DIFFERENTIATION

Neurospheres of passage 1 were thawed and expanded for a week prior to the experiment in proliferative conditions (two passages were performed in total).The day of the experiment passage 3 neurospheres were dissociated, cells were counted and plated at a 80,000 cell/well density on poly-L-lysine coated glass coverslips in 24-well plates in supplemented (Penycillin/streptomycin, B27, EGF, and FGF2) DMEM/F12. Neuroprogenitors were allowed to proliferate for 48h (Babu et al., 2011) and then were treated with conditioned media from control or phagocytic (24h) microglia. The experimental group DMEM was also added as control because it is the media in which microglia is grown. Neuroprogenitors were then fixed with 4% PFA for 10 mins at 3d, and 5d of differentiation.

For pluripotency experiments, neuroprogenitors treated for 3d with conditioned media (control, 24h phagocytosis of DMEM) were transferred back to DMEM/F12 (without trophic factors) medium and were allowed to differentiate for 5d and 9d.

For late survival and differentiation assay, after the 48h of proliferation, neuroprogenitors were allowed to differentiate in DMEM/F12 (no trophic factors) for 10d and then were treated with conditioned media from control or phagocytic (24h) microglia or DMEM for another 3 and 5d.

5.6. CALCIUM IMAGING

Intracellular calcium imaging experiments were performed as described before (Alberdi et al., 2013). CM-treated neuroprogenitors were incubated and loaded with 5 µM Fura-2 AM (Invitrogen) for 30 min at 37°C and then washed in HBSS containing 20 mM HEPES, pH 7.4; 10 mM glucose; and 2 mM $CaCl_2$ for 10 min at room temperature. The perfusion chamber was assembled on the platform of a Zeiss (Oberkochen) inverted epifluorescence microscope (Axiovert 35) equipped with a 150-W xenon lamp Polychrome IV (TILL Photonics, Martinsried, Germany) and a Plan Neofluar 40× oil immersion objective (Zeiss). Neuroprogenitor cells were treated with 50mM KCl, 10µM AMPA, 1mM ATP and 100µM Histamine sequentially. Cells were allowed to recover their baseline prior to adding the next compound. Cells were visualized with a digital black/white CCD camera (ORCA; Hamamatsu Photonics Iberica, Barcelona, Spain). Intracellular calcium signaling responses were calculated as the proportion of different cell phenotype responding to the different stimuli. The baseline was calculated as the mean of

the first 60sec of recording for each cell. Only peaks that increase or decrease 3 times the SEM of the baseline were considered as response.

5.7. FACS SORTING

Microglia cells were isolated from brains as described previously (Abiega et al., 2016; Sierra et al., 2007). The corresponding tissues from fms-EGFP mice were dissected and placed in enzymatic solution (116 mM NaCl, 5.4 mM KCl, 26 mM NaHCO3, 1 mM NaH2PO4, 1.5 mM CaCl2,1mM MgSO4 , 0.5 mM EDTA, 25 mM glucose, 1 mM L-cysteine) with papain (20 U/ml) and DNAse I (150 U/μl, Invitrogen) for digestion at 37ºC for 15 min. The homogenization process was also helped by careful pipetting. After homogenization, tissue clogs were removed by filtering the cell suspension through a 40μm nylon strainer to a 50 ml Falcon tube quenched by 5 ml of 20% heat inactivated FBS in HBSS. For further enrichment of microglia, myelin was removed by using Percoll gradients. For this purpose, cells were centrifuged at 200 g for 5 min and resuspended in a 20% Solution of Isotonic Percoll (20% SIP; in HBSS), obtained from a previous stock of SIP (9 parts Percoll per 1 part PBS 10X). Then, each sample was layered with HBSS poured very slowly by fire-polished pipettes. Afterwards, gradients were centrifuged for 20 min at 200 g with minimum acceleration and no brake so the interphase was not disrupted. Then the interphase was removed, cells were washed in HBSS by centrifuging at 200 g for 5 min and pellet was resuspended in 500μl of sorting buffer (25 mM HEPES, 5 mM EDTA, 1% BSA, in HBSS). Microglia cell sorting was performed by FACS Jazz (BD), in which the population of green fluorescent cells was selected, collected in Lysis Buffer (Qiagen) containing 0.7% β-mercaptoethanol and stored at −80ºC until processing.

5.8. ADMINISTRATION OF MICROGLIA CONDITIONED MEDIA IN VIVO

Conditioned media from control and phagocytic (Ph24h) microglia was administrated via osmotic pumps for 6d to 2mo fms-EGFP mice. Briefly, osmotic pump (Model 2001; flow rate 1μl/h; Alzet) and infusion catheter tubes (Alzet) were filled with the conditioned media (200μl), and connected together. Pumps were incubated overnight at 37ºC in PBS before the surgery. Mice were anesthetized with ketamine/xylazine (10/1 mg/kg) and received a single dose of the analgesic buprenorphine (1 mg/kg) subcutaneously. The infusion cannulae were inserted at anteroposterior −1.7 mm (AP), laterolateral (LL) −1.6 mm, and −1.9 mm dorsoventral (DV) from Bregma. Afterwards, a surface of dental cement was created from the cannulae to the screw to avoid any unwanted removal of the cannulae. Osmotic pumps were

inserted inside the skin of the mice. After 6d, mice were intraperitoneally injected with BrdU (150mg/kg, single injection), and transcardically perfused 2h later to assess proliferation.

For differentiations experiments, CM containing osmotic pumps were inserted for 6d to 2mo fms-EGFP or Nestin-GFP negative mice. Pumps were removed at 6d and afterwards, a single injection of BrdU (150mg/kg) was administered, and mice were sacrificed 28d later.

5.9. KAINATE ADMINISTRATION

Intrahippocampal administration of kainate (KA) was performed as previously described (Abiega et al., 2016). Briefly, 2 mo fms-EGFP mice were anesthetized with ketamine/xylazine (10/1 mg/kg) and buprenorphine (1 mg/kg) was administered subcutaneously as analgesic. A 0.6 mm hole was drilled and a glass microcapillary was inserted at AP -1.7 mm, LL -1.6 and DV -1.9 mm in order to administer 50 nl of either saline of KA (20 mM) into the right hippocampus using a microinjector (Nanoject II, Drummond Scientific, Broomal, PA, US). Mice were sacrifice at 24h after the injection for FACS sorting.

5.10. GENE EXPRESSION ARRAYS

Gene arrays analysis was performed following the recommendations of the MIAME (Minimum Information About a Microarray Experiment) consortium (Brazma et al., 2001).

Cell samples were lysed and kept at -80C until processing. Total RNA was isolated using PureLink RNA Mini kit (AMBION), following manufacturer´s instructions. RNA amount was quantified in a UV/VIS NanoDrop 1000 spectrophotometer (Thermofisher), and its integrity was analyzed with Lab-chip technology in an Agilent 2100 Bioanalyzer in combination with Agilent RNA 6000 Nano Chips. Eukaryote Total RNA Nano Assay was used as type of test. In all samples, RIN>9.3, and 28S/18S>1.3 values were obtained **(Table 1)**. Sample labeling, hybridization and scanning Gene expression profiling was performed at the Gene Expression Unit of Genomics Core Facility (SGIKer) of the University of the Basque Country UPV/EHU.

Sample	ng/ml	A260/A280	A260/A230	RIN	28S/18S
Control micro1	55.85	2.06	1	9.5	1.6
Control micro2	60.47	1.96	1.06	9.7	1.6
Control micro3	57.63	2.04	0.67	9.7	1.3
SH1 Ph3h	117.95	2.06	1.72	9.7	1.8
SH2 Ph3h	106.95	2.11	1.86	9.6	1.6
SH3 Ph3h	121.71	2.11	1.68	9.7	1.8
SH1 Ph24h	92.11	2.03	1.52	9.6	1.8
SH2 Ph24h	80.9	2.1	1.35	9.6	1.7
SH3 Ph24h	85.47	2.06	1.78	9.5	1.8

Table 1. Characteristic of samples used for gene arrays. Concentration, A260/A280 ratio and A260/230 ratio of naïve and phagocytic (3h, 24h) microglia samples used for gene arrays were measured by Nanodrop 2000, and RNA integrity (RIN and 28, 18 ribosomal subunits) were measured in a Agilent 2100 Bioanalyzer.

One-color microarray-based gene expression analysis was performed following the One-Color (p/n5190-2305) protocol from Agilent Technologies (Low Input Quick Amp Labeling kit) for the labeling of the samples. First, 50 ng of total RNA were retrotranscribed with the AffinityScript Reverse enzyme Transcriptase (AffinityScript RT), a thermostable modified enzyme derived from Moloney murine leukemia virus retrotranscriptase (Moloney Murine Leukemia Virus (MMLV) reverse transcriptase), using promoter-coupled Oligo dT primers Of T7. The double-stranded cDNA synthesized by AffinityScript RT was transcribed in vitro by the T7 RNA pol in the presence of Cy3-CTP to generate labeled and amplified cRNA. The labeled samples were purified with columns of RNeasy Mini kit (Qiagen). Subsequently the labeled samples were quantified in the Nanodrop ND-1000 to determine the performance of the specific activity of the fluorochromes after labeling. All the hybridized samples met the following minimum requirements: Yield> 0.825 µg per reaction and Cyanine 3 specific activity> 6 pmol / µg.

For the hybridization, 600 ng of labeled cRNA were fragmented and co-hybridized to SurePrint G3 Mouse GE 8x60K Microarray Design ID: 028005. Each array/slide contained 8

identical subarrays of more than 60.000 60-mer oligonucleotides of high resolution and performance. It contained probes for 55.681 sequences or transcripts (biological features or non-control features). Several of these biological probes were replicated 10 times for the calculations and quality control measurements (QCMetrics) of the microarrays. It also contained probes for internal positive controls (spike-ins), which were added to the RNA sample before labeling and were used for evaluation and verification of the microarray processing. Manual washing method was performed following Agilent's recommendations to prevent ozone-related problems.

Slides were scanned on a G2565CA Microarray DNA Scanner from Agilent Technologies with a resolution of 3 μm and a Tiff image size of 20bit, using the Scan software version 8.5.1 with default settings (Scan profile AgilentG3_GX_1color). The scanned TIFF images were processed and the fluorescence of the probes quantified using The Agilent Feature Extraction Software 10.7.3.1 (Agilent Technologies). Feature Extraction (FE) protocol for data extraction: GE1_107_Sep09, Design File: 28005_D_F_20140728. Software extracts information of the raw fluorescence signal (mean signal) for the fluorochrome or channel (Cy3: green Channel) from the spot containing the probes (positive and negative controls and no controls or biological feature) and the background, obtained from the negative controls (which contains sequences for which no hybridization is expected, non-specific binding indicators).

Default parameters (The Agilent Feature Extraction Software 10.7.3.1) for one-color gene expression microarrays were used for flagging of non-uniform features, population outliers for replicated probes, and features with no significant intensities in Cy3 channel. Agilent Feature Extraction (AFE) raw data was processed with software GeneSpring GX 13.0 (Agilent Technologies). Probes not present in any sample were filtered out. A list of the filtered 36.665 probes was used in the statistical analysis.

5.10.1. Statistical analysis

To analyze the differential expression between naïve (t=0) and phagocytic (t=3h and t=24h) microglia groups over time the statistical analysis the maSigPro package of R/Bioconductor was used (R version 3.0.3, Bioconductor release version 2.13, maSigPro version 1.34.1). This method is based on a general regression approximation for the modeling and adjustment of the parameters required according to the type of analysis. The parameter

"Time" is considered as a continuous variable, and creates a regression model of the gene response.

The analysis was performed in three steps. First genes that exhibited changes in expression over time were selected based on a p-corrected Benjamini-Hochberg (FDR) value. Secondly, for each of the genes that presented a significant change in their expression over time a regression was applied to determine their model in order to identify patterns or models of change based on time variables, obtaining 20,800 probes. Finally, the probes were selected according to their fit to the regression model. Next, the number of genes with a very high differential pattern was reduced by applying a more restrictive criterion (R-squared (rsq)> 0.9), obtaining 13,146. The rsq>0.7 list was used for the identification of neurogenesis related genes and the rsq>0.9 list was used for the study of transcriptional profile of phagocytic microglia.

5.11. FUNCTIONAL ANALYSIS OF PHAGOCYTIC MICROGLIAL TRANSCRIPTOME

5.11.1. Database for Annotation, Visualization and Integrated Discovery (DAVID)

The Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) v6.8 provides a comprehensive set of functional annotation tools to understand biological meaning behind large list of genes. DAVID was used to generate a gene-GO term enrichment analysis that identified enriched biological themes and to highlight the most relevant GO terms associated with the array gene list. The array gene list of rsq> 0.9 was used for this gene profile analysis. The analysis of each expression pattern was performed separately and only terms with an adjusted p-value (Benjamini-Hochberg) > 0.05 were considered significant.

5.11.2. ClueGO

ClueGO was used to generate protein pathways and to constitute the network of pathways based on the Gene Ontology and KEGG database (Bindea et al., 2013; Bindea et al., 2009). ClueGO is a plugin of Cytoscape (http://www.cytoscape.org/) that visualizes the non-redundant biological terms for large clusters of genes in a functionally grouped network. A ClueGO network is created with kappa statistics and reflects the relationships between the

terms based on the similarity of their associated genes (Mlecnik et al., 2018). Gene ontology (GO) analysis of mouse array data were performed with ClueGO v1.4 (Bindea et al., 2009) using the following parameters: enrichment/depletion two-sided hypergeometric statistical test; correction method: Benjamini-Hochberg; GO term range levels: 3–8; minimal number of genes for term selection: 10; minimal percentage of genes for term selection: 10%; κ-score threshold: 0.8; general term selection method: smallest p value; group method: κ; minimal number of subgroups included in a group: 3; minimal percentage of shared genes between subgroups: 50%.

5.11.3. Weighted gene co-expression network analysis (WGCNA)

Weighted gene co-expression network analysis (WGCNA) is a method to study biological networks that allows identifying modules (clusters) of highly co-expressed genes (Langfelder and Horvath, 2008, 2012). Filtering was performed on the raw datasets of the array. A cut-off was determined by the 95 quantile of the controls and at least four samples needed to have an expression higher than this cut-off before gene expression was determined. The proteins interactions, pathways, upstream regulatory analysis, and functional networks were generated with the Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., www.qiagenbio-informatics.com/prducts/ingenuity-pathway-analysis). Then, different molecular networks were generated according to biological or molecular functions, which include canonical pathways, upstream regulatory analysis, and disease-based functional networks.

5.12. RNA ISOLATION, RETROTRANSCRIPTION AND qPCR

5.12.1. Tissue or cultured cells RNA isolation and retrotranscription

The corresponding tissue (right hippocampus for KA injected mice; retina or P8 hippocampi for positive PCR controls) was rapidly isolated immediately under tribromoethanol overdose, and stored at -80°C. Tissue was disrupted with a roto-stator homogenizer with Lysis Buffer (Qiagen) containing 0.7% β-mercaptoethanol and stored at -80°C until processed. Cultured cells (> 500,000 cells) were lysate and stored at-80°C until processed. Total RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen), following manufacturer's instructions, including a DNAse treatment step to eliminate genomic DNA residues. RNA was quantified in a Nanodrop 2000, and 1.5μg were retrotranscribed using random hexamers (Invitrogen) and Superscript III Reverse Transcriptase kit (Invitrogen), following manufacturer's instructions in a Veriti Thermal Cycler (Applied Biosystems).

5.12.2. FACS-sorted cells RNA isolation and retrotranscription

RNA from FACS-sorted microglia (< 500,000 cells) was isolated by RNeasy Plus micro kit (Qiagen) according to the manufacturer instructions, and the RNA was retrotranscribed using an iScript Advanced cDNA Synthesis Kit (Biorad) following manufacturer instructions in a Veriti Thermal Cycler (Applied Biosystems).

5.12.3. RT-qPCR

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) was performed following MIQE guidelines (Minimal Information for Publication of Quantitative Real Time Experiments (Bustin et al., 2009). Three replica of 1.5µl of a 1:3 dilution of cDNA were amplified using Power SybrGreen (Biorad) for tissue or cell culture experiments or SsoFast EvaGreen Supermix (Biorad) for FACS-sorted microglia experiments in a CFX96 Touch Real-Time PCR Detection System (Biorad). The amplification protocol for both enzymes was 3 min 95°C, and 40 cycles of 10 s at 95°C, 30 s at 60°C.

5.12.4. Primers

Primers were designed to amplify exon–exon junctions using PrimerBlast (NIH) to avoid amplification of contaminating genomic DNA, and their specificity was assessed using melting curves and electrophoresis in 2% agarose gels. Primer sequences are listed in **Table 2**. For each set of primers, the amplification efficiency was calculated using the software LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009) or standard curve of 1:2 consecutive dilutions, and was used to calculate the relative amount using the following formula:

$$\Delta\Delta Ct = (1 + eff.target\ gene)\exp(Ct\ sample - Ct\ control)/(1 + eff.reference\ gene)\exp(Ct\ sample - Ct\ control)$$

Up to three independent reference genes were compared: L27A, which encodes a ribosomal protein of the 60S subunit (Sierra et al., 2007); OAZ-1, which encodes ornithine decarboxylase antizyme, a rate-limiting enzyme in the biosynthesis of polyamines and recently validated as reference gene in rat and human (Kwon et al., 2009); and HPRT, which encodes hypoxanthine guanine phosphoribosyl transferase (van de Moosdijk and van Amerongen, 2016). The expression of L27A, OAZ-1 and HPRT remained constant independently of time and treatments (data not shown), validating their use as reference genes. In all experiments, the pattern of mRNA expression was similar using the assigned couple of reference genes, and in

each experiment the reference gene that rendered lower intragroup variability was used for statistical analysis.

	Gene	Gene Bank	Amplicon size	Sequence 5'-3'
Reference genes	OAZ1	NM_008753	51	Fwd AGCGAGAGTTCTAGGGTTGCC Rev CCCCGGACCCAGGTTACTAC
	L27A	BC086939	101	Fwd TGTTGGAGGTGCCTGTGTTCT Rev CATGCAGACAAGGAAGGATGC
	HPRT	NM_013556.2	150	Fwd ACAGGCCAGACTTTGTTGGA Rev ACTTGCGCTCATCTTAGGCT
Metabotropic receptors	GRM1	NM_016976.3	150	Fwd AAACCCGAGAGGAATGTCCG Rev GCCGTTAGAATTGGCGTTCC
	GRM2	NM_001160353.1	143	Fwd TGTCAAGTTTGATGCCCCCT Rev AGCCTACCTTCTGGTAGCGA
	GRM3	NM_181850.2	73	Fwd TTCACAGCTCCATTCAACCCA Rev CCATCCCGTCTCCGTAAGTG
	GRM4	NM_001013385.1	107	Fwd CACCAAGCCTGAACGAGTGG Rev CGTAGCTGATCTGGGGGATCTTG
	GMR5	NM_001081414.2 NM_001143834.1	123	Fwd ACAACCTCTACAGTGGTGCG Rev GGAGCTTAGGGTTTCCCCAG
	GRM7	NM_177328.3	141	Fwd CTCCAGGGCTGTCGTGATTT Rev TGCTGATGCAGTGGGTTGAT
	GRM8	NM_008174.2	142	Fwd ACCCATATTCACCAAGCCCG Rev GTCACTTAGCTCTGGGGCTG

AMPA ionotropic glutamate receptors	Gria1	NM_001113325.2 NM_008165.4 NM_001252403.1	111	Fwd TACATTGAGCAACGCAAGCC Rev GTTTACGGGACCTCTCAGGG
	Gria2	NM_001083806.1 NM_013540.2 NM_001039195.1	148	Fwd GGGGACAAGGCGTGGAAATA Rev CCAATCTTCCGGGGTCCATT
	Gria3	NM_016886.4 NM_001281929.1	107	Fwd TGACAGCTCATCCTCCGAGA Rev AGCGCTCATTTCCTTCCAGT
	Gria4	NM_019691.4 NM_001113180.1 NM_001113181.1	135	Fwd AAGCACGTCAAAGGCTACCA Rev TTTCGTCACCATGGGCGTAT
KAINATE ionotropic glutamate receptors	Grik1	NM_146072.4 NM_010348.3	76	Fwd CTCGCTTGCCTAGGAGTCAG Rev GGTGGGGGTTATACCACTCG
	Grik2	NM_001111268.1 NM_010349.2	102	Fwd CTTCTCTTCCCTCAGCCGTG Rev TGCAAGCGAATGAGACCAGT
	Grik3	NM_001081097.2	131	Fwd GTTCCTAGTGTGCGCCTTCT Rev ATCGAAAGGCGTGCTCTTCA
	Grik5	NM_008168.2	135	Fwd TGATAGTCGCCTTCGCCAAT Rev TCCCATTGATCTGCTCTCGG
NMDA ionotropic glutamate receptor	Grin1	NM_008169.2 NM_001177656.1 NM_001177657.1	114	Fwd GACACAAGGATGCCCGTAGG Rev TCTTTTTAGGGTCGGGCTCTG

	Grin2a	NM_008170.2	148	Fwd CGCATCCATGGCTTGGTGTTT
				Rev TGGATGTCGGATCCTTGTCAG
	Grin2b	NM_008171.3	112	Fwd CATGGGTGTCTGTTCTGGCA
				Rev GGGGGAGTTCATCACGGATT
	Grin3a	NM_001276355.1	146	Fwd TGGTACAAGGGGTTTCAGCG
		NM_001033351.1		Rev GTGCAGGGGATTCTGACTCT
	Grin3b	NM_130455.2	140	Fwd TGTTTGTCCTGCTGTGCCT
				Rev GTGGATCTTCTGGCTCGTGTGAA
Recognition receptors	Trem2	NM_031254.3	110	Fwd CTGATCACAGCCCTGTCCCAA
		NM_001272078.1		Rev CGTCTCCCCCAGTGCTTCAA
	CR3	NM_001082960.1	100	Fwd AATTGAGGGCACGCAGACA
		NM_008401.2		Rev GCCCAGCAAGGGACCATTAG
	MerKT	NM_008587.1	131	Fwd AAGGTCCCCGTCTGTCCTAA
				Rev GCGGGGAGGGGATTACTTTG
	GPR34	NM_011823.4	151	Fwd CTTCAGGAAAGCTTCAACTC
				Rev GTAACTATCAGGAGGAGAGC
Purinergic receptors	P2X4	NM_011026	52	Fwd TAAGTATGTGGAAGACTACGAGCAGG
				Rev TCACTGGTCCGTCTCTCCG
	P2X7	NM_011027	51	Fwd ACTATACCACGAGAAACATCTTGCC
		NM_001038845		Rev GAAAGGTACAAGAGCCGTTCATAGTT
		NM_001038839		
	P2Y6	NM_183168	82	Fwd ACAGACTCTCCGAGCATAGGAAA
				Rev GGCGGCAAGCCTGGA
	P2Y12	NM_027571	88	Fwd GCAGAACCAGGACCATGGAT
				Rev CTGACGCACAGGGTGCTG

C1q subunits	C1qA	NM_007572.2	97	Fwd CACGGAGGCAGGGACAC
				Rev GGCAGACATCTTCAGCCACT
	C1qB	NM_009777.2	77	Fwd ATTCCATACACAGGAAGCCCC
				Rev GCAGTAACAGGTGTGTCCAGA
	C1qC	NM_007574.2	147	Fwd GCTGCTGCTGTTTCTTCTGG
				Rev GGGATTCCTGGCTCTCCCT
Peptides & hormones	Vgf	NM_001039385.1	74	Fwd CACCGGCTGTCTCTGGC
				Rev AAGGAAGCAGAAGAGGACGG
	Cartpt	NM_013732.7	106	Fwd GCGCTATGTTGCAGATCGAAG
				Rev GCGTCACACATGGGGACTTG
	SCG2	NM_001310680.1	90	Fwd CCTACCTGAGAAGGAATTTGCAG
				Rev CTGGCTCCACCAACCCATTT
Trophic factors	BDNF	NM_007540.4	104	Fwd TACCTGGATGCCGCAAACAT
		NM_001048141.1		Rev GCTGTGACCCACTCGCTAAT
		NM_001048142.1		
		NM_001048139.1		
	GDNF	NM_010275.2	145	Fwd CGCTGACCAGTGACTCCAA
				Rev TGCCGATTCCTCTCTCTTCG
	FGF2	NM_008006.2	113	Fwd CGGCTGCTGGCTTCTAAGTG
				Rev AGTGCCACATACCAACTGGAG
	EGF	NM_010113.3	136	Fwd GGACTGAGTTGCCCTGACTC
				Rev CAATATGCATGCACACGCCA
	IGF-1	NM_010512.4	122	Fwd TTACTTCAACAAGCCCACAGG
		NM_184052.3		Rev GTGGGGCACAGTACATCTCC

		NM_001111274.1		
		NM_001111275.1		
		NM_001111276.1		
	PDGFa	NM_008808.3	94	Fwd TACCCCGGGAGTTGATCGAG Rev TCAGCCCCTACGGAGTCTATC
	NT-4	NM_198190.1	130	Fwd GGTTGCCCCCTCCCTCT Rev TTGCACACCTGTCAACAGCA
	NGF	NM_013609.2	112	Fwd ATCTCCCGGGCAGCTTTTTG Rev CACAGGCCAAAACTCCACCAT
	VEGFa	NM_001025250.3	88	Fwd GGCCTCCGAAACCATGAACT Rev CTGGGACCACTTGGCATGG
Matrix protein	Mmp3	NM_010809.2	88	Fwd ACCCAGTCTACAAGTCCTCCA Rev GGAGTTCCATAGAGGGACTGA
Surface ligands	Jag1	NM_013822.5	119	Fwd TTCAGGGCGATCTTGCATCA Rev CACACCAGACCTTGGAGCAG
	Dll3	NM_007866.2	82	Fwd TGGATGCCTTTTACCTGGGC Rev GGAGCCAGAGGTTTCACTACA
	Dll4	NM_019454.3	113	Fwd GGTTACACAGTGAGAAGCCAGA Rev GGCAATCACACACTCGTTCC
Cytokines	Csf3	NM_009971.1	70	Fwd GCAGCCCAGATCACCCAGAAT Rev TGCAGGGCCATTAGCTTCAT
	IL1β	NM_000576.2	72	Fwd AGATGAAGTGCTCCTTCCAGG Rev GGTCGGAGATTCGTAGCTGG
	IL6	NM_000600.3	107	Fwd GAAAGCAGCAAAGAGGCACTG Rev TTCACCAGGCAAGTCTCCTCAT

	TNFα	NM_000594.3	142	Fwd TGCACTTTGGAGTGATCGGC
				Rev GCTTGAGGGTTTGCTACAACA
	TGFβ	NM_000660.5	112	Fwd TCCTGGCGATACCTCAGCAA
				Rev CAATTTCCCCTCCACGGCTC

Table 2. Primer sequences. *Primer sequences for mRNA expression analysis by RT-qPCR. The table indicates the gene name, the GeneBank code, the amplicon size, and the primer sequence*

5.13. SINGLE CELL RNA- SEQUENCING

CA and DG were dissected from 1mo CX3CR1 mice by carefully cutting with a needle through the hippocampal fissure under a dissecting scope. The enthorrinal cortex was also dissected out from CA. Samples were enzymatically homogenized and FACS-sorted as describes in **Section 5.7**. Cell populations isolated by flow cytometry were diluted to a final concentration range of 250–400 cells per µl and were loaded onto C1 system (Fluidigm) to carefully isolate single cells into individual reaction chambers in its integrated fluidic circuit (IFC). The optically clear IFC allowed to automatically stain captured cells and examine them by microscopy for viability. After staining, cells were automatically lysed and their RNA was retrotranscribed with oligo (dT) primers and amplified on a C_1 Single-cell Auto Prep System according to the mRNA-seq protocol of the manufacturer (Fluidigm). The amount of cDNA generated from single cells was quantified with a Quant-iT PicoGreen dsDNA Assay Kit (PN P11496; Life Technologies), and quality was checked with High Sensitivity DNA Reagents (PN 5067-4626) according to the manufacturer's instructions (Agilent Technologies). Only single cells with high-quality cDNA were processed for RNA-seq. For the RNA-seq, single-cell multiplexed libraries were prepared utilizing a Nextera_XT Kit (PN FC-131-1096; Illumina) with dual indices (PN FC-131-1002; Illumina), which were sequenced as 51-bp single-end reads on the Illumina HiSeq 2000 platform.

5.14. IMMUNOFLUORESCENCE

5.14.1. Primary microglia cultures

Primary microglial cultures were fixed for 10 min in 4% PFA and then transferred to PBS. Fluorescent immunostaining was carried out following standard procedures (Abiega et al., 2016; Beccari et al., 2018). Coverslips with primary microglial cultures were blocked in 0.1% Triton X-100, 0.5% BSA in PBS for 30 min at RT. The cells were then incubated with primary antibodies in permeabilization solution (0.2% Triton X 100, 0.5% BSA in PBS) for 1 h at RT,

rinsed in PBS and incubated in the secondary antibodies containing DAPI (5 mg/ml) in the permeabilization solution for 1 h at RT. After washing with PBS, primary cultures were mounted on glass slides with DakoCytomation Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA).

For VGF antibody immunostaining of primary microglia, all the procedure was performed without Triton X-100. Coverslips were blocked in 0.1% BSA for 1h at RT, following the incubation of primary antibodies in the same blocking solution overnight at 4°C. Afterwards, coverslips were rinsed in PBS and incubated in the secondary antibodies containing DAPI for 2h at RT.

5.14.2. SH-SY5Y cell line

For fluorouridine labeling, SH-SY5Y were treated with 2mM 5'-Fluorouridine (Sigma) for 30min. Afterwards, cells were fixed in 4% PFA with 0.5% Triton X 100. The immunofluorescence was performed as described in **section 5.14.1** and anti-BrdU primary antibody was used to detect fluorouridine.

5.14.3. Neuroprogenitors cell cultures

Neuroprogenitors cell cultures were fixed for 10 min in 4% PFA and then transferred to PBS. Coverslips containing the cells were blocked in blocking solution (0.5% Triton-X100, 3% BSA in PBS) for 1 hr at room temperature, and then incubated overnight with the primary antibodies diluted in the permeabilization solution (0.2% Triton-X100, 3% BSA in PBS) at 4°C. After overnight incubation, coverslips were allowed to thaw and were thoroughly rinsed in PBS. Next, the coverslips were incubated with fluorochrome-conjugated secondary antibodies and DAPI (5mg/ml; Sigma) diluted in the permeabilization solution for 2h at RT. After washing with PBS, the coverslips were mounted on glass slides with DakoCytomation Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA).

5.14.4. Brain tissue sections

Mice were transcardially perfused with 30ml of PBS followed by 30ml of 4% PFA. The brains postfixed with the same fixative for 3h at RT, then washed in PBS and kept at 4°C. Six series of 50μm-thick coronal sections of mouse brains were cut using a Leica VT 1200S vibrating blade microtome (Leica Microsystems GmbH, Wetzlar, Germany). Fluorescent

immunostaining was carried out following standard procedures (Beccari et al., 2018; Sierra et al., 2010). Free-floating vibratome sections were blocked in permeabilization solution (0.3% Triton-X100, 0.5% BSA in PBS; all from Sigma) for 3 hr at RT, and then incubated overnight with the primary antibodies diluted in the permeabilization solution at 4°C. For C1q immunostaining an incubation of two overnights was required. For BrdU (bromo-deoxyuridine) labeling an antigen retrieval procedure was performed by incubating in 2M HCl for 30min at 37°C and then washing with 0.1M sodium tetraborate for 10min at RT prior to the blockade of the sections. After overnight incubation with primary antibodies, brain sections were thoroughly washed with 0.3% triton in PBS. Next, the sections were incubated with fluorochrome-conjugated secondary antibodies and DAPI (5mg/ml; Sigma) diluted in the permeabilization solution for 3h at RT. After washing with PBS, the sections were mounted on glass slides with DakoCytomation Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA).

5.14.5. Antibodies

The following antibodies were used **(Table 3)**:

	Primary Abs	Source
Primary microglia	chicken anti-GFP (1:1000)	Aves Labs
	guinea Pig anti-BQ17 (1:400)	Courtesy of Gian-Luca Ferri and Christina Cocco
	sheep anti-BQ42 (1:400)	
	rabbit anti-BQ22 (1:400)	
	rabbit anti-BQ28 (1:400) rabbit anti-BQ11 (1:1000)	
SH-SY5Y culture	rat anti-BrdU (1:400)	Biorad
Neuroprogenitor cell culture	chicken anti-nestin (1:1000)	Aves Labs
	rabbit anti-GFAP (1:1000)	Dako
	goat anti-Dcx (1:500)	Santa Cruz
	rabbit anti-S100β (1:750)	Dako
In vivo	chicken anti-GFP (1:1000)	Aves Labs
	rat anti-C1q (1:100)	AbD Serotech

	rat anti-BrdU (1:400)	Biorad
	rabbit anti-GFAP (1:1000)	Dako
	goat anti-Dcx (1:1000)	Santa Cruz

Table 3. Antibodies. Primary and secondary antibodies used for immunofluorescent staining.

5.15. IMAGE ANALYSIS AND QUANTIFICATION

5.15.1. Phagocytosis analysis

Apoptotic cells were defined based on their nuclear morphology after DAPI staining as cells in which the chromatin structure (euchromatin and heterochromatin) was lost and appeared condensed and/or fragmented (pyknosis/karyorrhexis) (Abiega et al., 2016). In addition, phagocytosis was defined as the formation of an enclosed, three-dimensional pouch of microglial processes surrounding an apoptotic cell.

In microglia primary cultures, over 4-5 random z-stacks were collected per coverslip using a Leica SP8 laser scanning microscope under a 40X oil-immersion objective and a z-step of 0.7μm. The percentage of phagocytic microglia was defined as cells with pouches containing apoptotic NE-4C/SH-SY5Y nuclei and/or CM-DiI particles (Beccari et al., 2018).

For mouse tissue sections for C1q experiment, the DG in 3-4 sections was fully scanned under the microscope to find all apoptotic cells using a 40X oil-immersion objective with a zoom of 2,5. Colocalization of C1q with microglia cells was determined by orthogonal projections.

5.15.2. Neuroprogenitor cell analysis in vitro

In neuroprogenitor cell cultures, over 4-5 random z-stacks were collected per coverslip using an Olympus Fluoview or a Leica SP8 laser scanning microscope under a 40X oil-immersion objective, a z-step of 0.7μm, a zoom of 0,75 and a resolution of 1024x1024 pixels. The effect of microglia-derived conditioned media on neuroprogenitor cells was analyzed considering both their morphology and the expression of cell-specific markers. Percentages of the different morphologies present in the population were obtained as well as the percentages of the different cell markers (Nestin, GFAP, DCX, S100β) per morphology.

5.15.3. Neurogenesis analysis in vivo

For the neurogenesis analysis in vivo, the three tissue sections closest to the injection site were analyzed. 5 z-stacks were collected per section using a Leica SP8 laser scanning microscope under a 40X oil-immersion objective, a z-step of 1 μm, a zoom of 1 and a resolution of 512x512 pixels. Proliferation was assessed by BrdU cell quantification; neural stem cells were identified by the expression of the markers Nestin and GFAP and their radial morphology for cell quantification; neuroblast were assessed by DCX$^+$ cell quantification and morphology in order to classify them in AB, CD or EF neuroblasts; neurons were assessed by NeuN$^+$ cell quantification; and astrocytes identified as GFAP$^+$/Nestin$^-$ cells with stellated morphology. The proliferation of either of these populations was assessed by their mentioned staining combined with BrdU. Numbers of cells were estimated in the volume of the DG of the z-stack, which was determined by multiplying the thickness of the z-stack (12 μm) by the area of the DG at the center of the stack using the software ImageJ (Fiji).

5.16. STATISTICAL ANALYSIS

SigmaPlot (San Jose, CA, USA) was used for statistical analysis. For the analysis of neurocandidates and cytokine mRNA expression, a logarithmic transformation was performed to comply with ANOVA assumptions (normality and homocedasticity). In all cases, Holm-Sidak method was used as a posthoc test to determine the significance between groups in each factor. Only $p < 0.05$ is reported to be significant. Data is shown as mean ± SEM (standard error of the mean).

6. RESULTS

6. RESULTS

6.1. SETTING UP AN IN VITRO MODEL OF PHAGOCYTOSIS

In order to study the relationship between microglial phagocytosis and neurogenesis in vivo, we focused in the adult hippocampal neurogenic cascade, where the neural precursors of the subgranular zone (SGZ) of the dentate gyrus (DG) proliferate to give rise to granular neurons that incorporate in the hippocampal circuitry. Importantly, many of the newborn cells naturally undergo apoptosis and are rapidly and efficiently phagocytosed by resident, unchallenged microglia (Sierra et al., 2010). As neurogenesis rapidly declines with age, so does newborn cell apoptosis and phagocytosis (Sierra et al., 2010). Therefore, the hippocampus of young mice (1 month old) is a great model to study both phenomena, phagocytosis and neurogenesis, in physiological conditions in vivo.

In addition, to study the interaction between phagocytosis and neurogenesis in vitro we developed an in vitro model of phagocytosis of apoptotic cells that mimicked as many features as possible of phagocytosis in vivo. Here we will focus on three cardinal features: phagocytosis cargo identity; early engulfment; and presence of key opsonizing molecules.

The first key issue is the identity of the phagocytosed cargo. Microglia is in charge of the phagocytosis of multiple brain-specific types of cargo, such as axonal and myelin debris, Aβ-deposits, invading microorganisms, apoptotic cells, etc (Sierra et al., 2013). For this study, we will specifically focus on microglial phagocytosis of apoptotic cells. Traditionally, one of the most utilized models to study microglial phagocytosis in vitro has been the use of latex beads as phagocytic targets; however, these artificial targets do not release the characteristic 'find-me' molecules of the apoptotic cells to ensure the attraction of microglia (Diaz-Aparicio et al., 2016). In addition, latex beads do not express 'eat-me' ligands and while they are commonly opsonized by a serum coating their mechanism of recognition is different from physiological targets. Similarly, synthetic latex beads cannot be degraded, and therefore their engulfment will result in incomplete phagocytosis. Thus, since latex beads cannot mimic the different steps of phagocytosis, we performed our in vitro phagocytic assay using a physiological relevant target, ie., apoptotic cells. We used the neuroprogenitor NE-4C cell line, in which we induced apoptosis prior to feeding them to microglia. We used this mouse neuronal cell line to characterize phagocytosis in mouse primary microglial cultures. However, to study

transcriptional changes induced by phagocytosis we later on switched to use the human neuronal cell line SH-SY5Y, which allowed discrimination of species-specific transcripts and thus, to specifically evaluate changes in mouse phagocytic microglia .

A second key factor is the early engulfment. In vivo, phagocytosis is characterized by its rapid and efficient performance. As soon as a cell undergoes apoptosis, microglia completely engulf and degrade the apoptotic cell, as shown by the fact that over 90% of the apoptotic cells are already engaged in phagocytosis in the adult hippocampus (Abiega et al., 2016; Sierra et al., 2010). As a result, the estimated clearance time in vivo is around 90min (Sierra et al., 2010). Therefore in our in vitro model we ensured that only primary apoptotic cells, but not cells undergoing primary or secondary necrosis, were fed to microglia.

Finally, a key opsonizing molecule that facilitates and modulates engulfment is the complement protein C1q. The complement protein C1q plays several key functions in the innate immune system, and in particular as the key recognition protein of the C1 macromolecular complex which initiates the classical complement pathway (Fraser and Tenner, 2008). In addition a large body of evidence now suggests that C1q is an important factor in the clearance of apoptotic cells (Trouw et al., 2008). In particular, the presence or absence of C1q results in a completely different outcome in the process of phagocytosis, determining the anti- or pro- inflammatory nature of the process respectively (Fraser et al., 2010). Consequently, we deepened into the presence of C1q in the CNS and its relationship with apoptotic cells prior to deciding whether the complement protein would be part of our phagocytic system in vitro.

In this section, we will first analyze the presence of C1q and its relationship with apoptosis and phagocytosis in vivo. Then, we will start the setup of our in vitro model of phagocytosis by characterizing the apoptosis induction of a mouse neuronal cell line. Finally, we will conclude our phagocytic model setup by feeding the apoptotic neuronal cell line to primary microglia in order to describe phagocytosis dynamics.

6.1.1. Characterization of C1q expresion in the hippocampus

6.1.1.1. Phagocytosed apoptotic cells are related to C1q

To test whether C1q played a role in phagocytosis in vivo, we analyzed by immunofluoresce and confocal imaging the presence of C1q in the hippocampus of 1mo fms-

EGFP[+] mice **(Figure 5A)**, in which microglia express the green fluorescent protein. We quantified a total of 159 apoptotic cells along the DG, pooled from 3 different mice. Apoptotic cells, likely to be newborn cells, located in the neurogenic niche (SGZ) were phagocytosed by ramified, unchallenged microglia (93.0% of the apoptotic cells), as shown by the formation of a three-dimensional phagocytic pouch surrounding the apoptotic cell, as already described (Sierra et al., 2010). C1q was expressed in the SGZ and showed a dotted pattern. To assess the relationship between C1q labeling and apoptosis, we quantified the expression of C1q in apoptotic cells **(Figure 5B)** and found that C1q labeling was associated with 84.9% of the phagocytosed apoptotic cells. To distinguish whether the C1q signal belonged to the cytoplasm of the apoptotic cell or the microglial pouch, we analyzed orthogonal projections of confocal z-stacks. Orthogonal projections showed C1q labeling within the microglial cytoplasm **(Figure 5C)**, strongly suggesting that C1q was part of the microglial pouch, rather than the apoptotic cell cytoplasm. Moreover, we found that out of 8.1% of apoptotic cells without C1q staining in the phagocytic pouch, up to 69.3% belonged to a C1q[+] microglia, as C1q staining was found either in the microglial soma and/or branches **(Figure 5B)**. The remaining 30.7% of the C1q[-] pouches were formed by microglia that we cannot exclude as C1q[+] because part of the microglial cell was located outside the Z-stack. On the other hand, none of the non-phagocytosed apoptotic cells presented any C1q staining. Altogether, this data suggested a clear relationship between C1q and phagocytosis.

6.1.1.2. C1q protein is mainly expressed in microglia in the hippocampus

C1q labeling was found inside the microglial pouches surrounding apoptotic cells, but nonetheless the staining was not exclusive of the phagocytic pouches **(Figure 5A, C)**. Therefore, we analyzed C1q expression in DG microglia and we found that 100% of the analyzed microglia from DG presented C1q staining especially in the soma –surrounding the nucleus-, although it could also be found throughout their processes **(Figure 5D)**. We only consider complete cells for the quantification of microglia, therefore, microglial branches that had their soma outside the Z-stack or microglial somas with branches outside the Z-stack were not included. In addition, very few scattered C1q dots were found in non-microglial cells.

To test whether microglia expression of C1q solely occurred in the presence of apoptotic cells, we also analyzed its expression in the Cornu Ammonis (CA), a nearby region of the hippocampus where there is no neurogenesis and therefore no apoptosis nor phagocytosis (Sierra et al., 2010). We analyzed two CA, CA1 and CA3, and found that microglia throughout

CA also expressed C1q protein either in their soma (perinuclearly) and/or inside the microglial processes, distributed as a dotted pattern along the branches **(Figure 5E)**. Similarly to DG, almost no presence of C1q was found in non-microglial cells. Therefore, in the adult brain, microglia are the main cell type expressing the complement protein C1q.

Thus, although we cannot exclude that the C1q plays an important role in non-phagocytic microglia (from CA), the association of C1q to the phagocytic pouches strongly suggests that C1q is involved in the process of phagocytosis.

6.1.1.3. Microglia is the major source of C1q in the hippocampus

C1q was almost exclusively found inside microglia, however, the immunofluorescence data could not disregard that C1q was originated in apoptotic cells and incorporated in microglia during their engulfment and degradation. To further confirm the microglial origin of C1q, we compared the C1q mRNA expression in microglia versus non-microglial cells. The two cell populations were acutely purified from the hippocampus of fms-EGFP mice by fluorescent-activated cell sorting (FACS) **(Figure 5F)**. Microglia were selected as GFP$^+$ cells, whereas other non-microglial cell types were characterized as GFP$^-$ (See description of Fig 5F for gating strategy). Next, the mRNA expression of the three subunits conforming C1q protein (C1qA, C1qB and C1qC) was assessed by RT-qPCR **(Figure 5G)**. We found that the mRNA of each subunit was almost exclusively restricted to microglia, with an enrichment of 98.1 ± 0.5 % for C1qA, 97.5% ± 1.4 for C1qB, and 84.1 ± 7.5 % for C1qC. Therefore, the translation of this mRNA into protein likely renders microglia as the major source of C1q in the hippocampus.

Consequently, since we found a close interaction between C1q and microglia in the CNS and the presence or absence of the protein would have dramatic differences in the downstream experiments, which would determine the immunomodulatory nature of the process (Fraser et al., 2010), we decided that the complement protein would be part of our in vitro phagocytosis model by using non-inactivated serum in the cell culture medium.
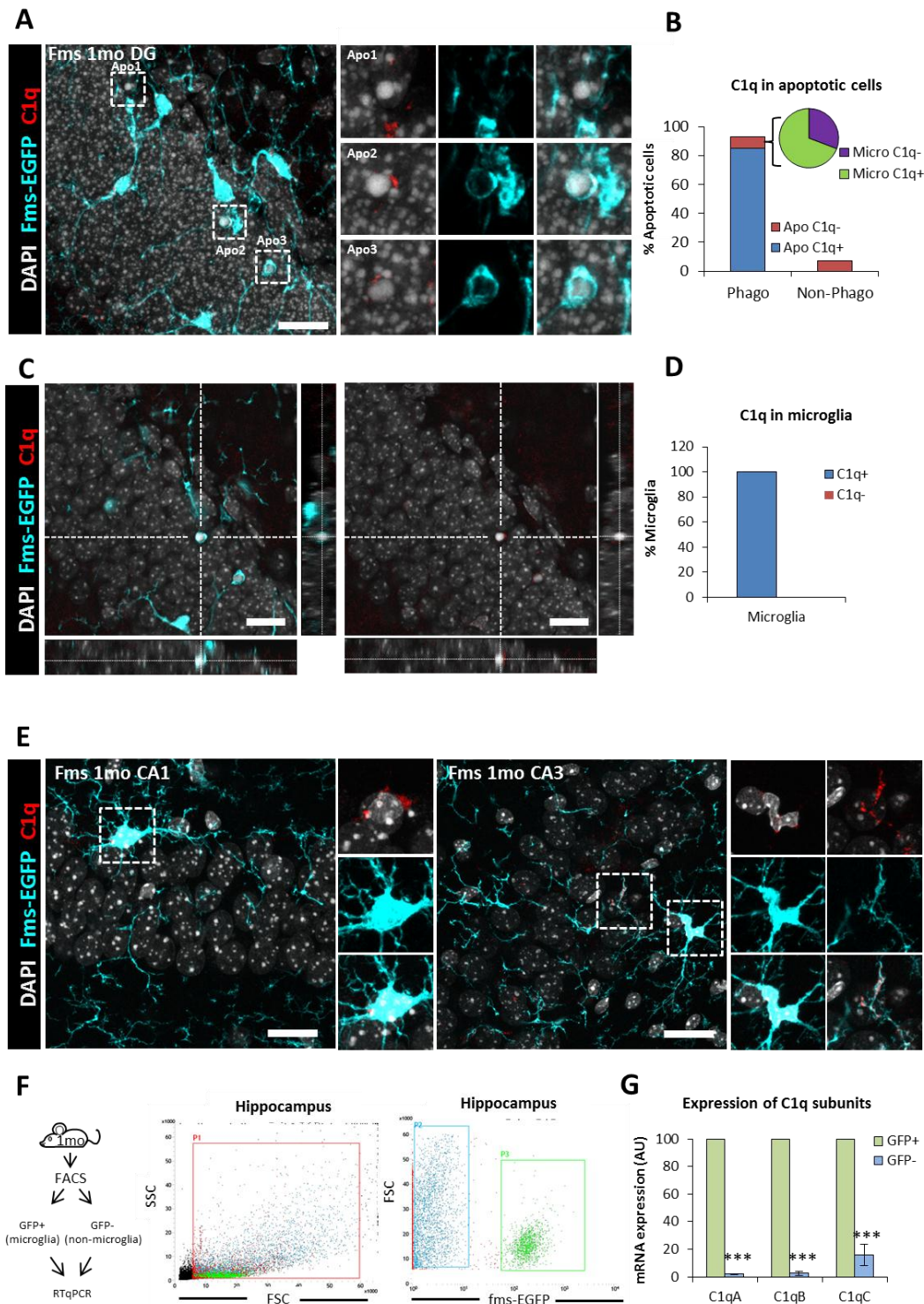
Figure 5. Characterization of the presence of C1q protein in the mouse adult brain. [A] *Representative confocal z-stack of C1q immunofluorescence in the mouse hippocampal DG in physiological conditions at 1 month. Microglia were labeled with fms EGFP+ (cyan), C1q (red) and apoptotic nuclei were detected by pyknosis/karyorrhexis (white, DAPI). C1q presented a dotted pattern in the cytoplasm of microglia cells, both in the soma and the processes. Phagocytic microglia showed C1q also in the pouches that surrounded apoptotic cells. High magnification details show each apoptotic cell individually. Apoptotic cell 1 was not phagocytosed and did not present C1q staining. Apoptotic cells 2 and 3 were engulfed by*

*microglia and C1q was present in the phagocytic pouches. Scale bars = 20µm (C, G), z = 16,5µm (DG). **[B]** Percentage of phagocytosed and non-phagocytosed apoptotic cells with and without C1q labeling. The majority of the phagocytosed apoptotic cells had C1q in their surrounding microglia pouch. A small percentage of the phagocytic pouches did not present C1q in the pouch per se, but the majority of the microglia forming those pouches did have C1q in their soma and/or branches. Few phagocytosed apoptotic cells did not express C1q neither in their pouch nor in the microglia engulfing them. A total of 159 apoptotic cells were quantified along the DG of 3 different mice (2 sections per mouse) by confocal microscopy. **[C]** Orthogonal projection of a confocal z-stack of C1q immunofluorescence in the mouse hippocampal DG in physiological conditions at 1month. Microglia were labeled with fms EGFP+ (cyan), C1q (red) and apoptotic nuclei were detected by pyknosis/karyorrhexis (white, DAPI). **[D]** Percentage of microglia with C1q labeling in the soma, branches or pouch. A total of 289 microglia cells were quantified along the DG of 3 different mice (2 sections per mouse) by confocal microscopy. **[E]** Representative confocal z-stack of the presence of C1q in the CA1 and CA3 of 1mo fms-EGFP mice in physiological conditions. Microglia were labeled with fms EGFP+ (cyan), C1q (red), and cell nuclei were labeled with DNA marker DAPI (White). C1q presented a dotted pattern inside microglia cells, both in the soma and the processes. High magnification details show each microglia cell individually. C1q staining colocalized with the soma and/or branches of microglia. Scale bars = 20µm, z = 7.5 µm (CA1), 9 µm (CA3). **[F]** Experimental design used to isolate microglia (GFP+) vs non-microglial cells (GFP-) from 1 mo fms-EGFP mice. Flow cytometry analysis of the expression of C1q subunits in hippocampal cells. First, debris was excluded using the P1 gate in FSC versus SSC (left panel). Next, gates for GFP$^+$ microglia cells (P3) and GFP$^-$ non-microglial cells (P2) were defined based on the distribution of the fms-EGFP$^+$ cells in EGFP vs FSC (right panel). **[G]** Expression of C1q subunits in microglia (GFP+) vs non-microglial cells (GFP-) by RTqPCR in FACS-sorted cells from fms-EGFP mice hippocampi (n = 3, each from 8 pooled hippocampi). OAZ1 was selected as a reference gene. Bars represent mean ± SEM. *** indicates p < 0.001 by Student´s t test.*

6.1.2. Characterization of apoptosis in a mouse neuroprogenitor cell line (NE-4C)

After determining the use of non-inactivated serum in the culture medium, we continued with the setup of our phagocytic assay by characterizing apoptosis induction in the neuroprogenitor mouse cell line NE-4C. Apoptosis induction was performed by treatment with staurosporine (STP), a protein kinase inhibitor and a well-known apoptotic inductor (Lawrie et al., 1997). Two different techniques were used for the quantification of apoptotic cells in order to contrast and compare results: flow-cytometry and fluorescence microscopy.

For the analysis of cell death using flow-cytometry, we cultured NE-4C cells with 1µM STP along a time course of 2h, 4h, and 6h. Then, cells were stained with propidium iodide (PI), a DNA intercalating agent that is not extruded by cells with a disrupted cytoplasmic membrane, a hallmark of necrosis (Krishan, 1975), and they were also labeled with Annexin V, which binds to phosphatidylserine when it becomes exposed on the outside leaflet of the membrane, hallmark of apoptosis (Wlodkowic et al., 2012). Five different populations were observed in the cytometer depending on the PI/Annexin labeling (**Figure 6A**):

1. Cells with no labeling (PI$^-$, Annexin$^-$) were defined as live since they possessed an intact membrane and no exposure of phosphatidylserine (PS).

2. Cells with an intact membrane (PI$^-$) but exposure of PS (Annexin$^+$) were considered primary apoptotic cells.

3. On the other hand, Annexin$^+$ combined with high levels of PI$^+$ (loss of cell integrity) characterized secondary necrotic cells.

4. An Annexin$^+$ / low PI$^+$ cell population was also found, and these cells were identified as primary apoptotic cells that were evolving into secondary necrotic cells.

5. Loss of membrane integrity without PS exposure (PI$^+$, Annexin$^-$) determined necrotic cells.

The quantification of these data (**Figure 6B**) showed a decrease in the percentage of live cells over time (67.0% ± 4.6% at 4h; 54.2% ± 3.2% at 6h), while the percentage of primary apoptotic cells significantly increased (27.4% ± 10.4% at 4h; 40.4% ± 8.2% at 6h). Necrotic cells never reached more than the 2.5 ± 1.1 % of the total number of cells (**Figure 6C**).

For the analysis of cell death using fluorescence microscopy, we again induced NE-4C apoptosis with 1µM STP for different time points and labeled the cells with propidium iodide (PI, 5µg/ml) during the last hour of STP treatment to minimize its toxicity. Apoptosis was determined by aberrant nuclear morphology, as visualized with the DNA dye DAPI, defined as pyknotic (condensed nuclei) and karyorrhectic (fragmented nuclei) cells (Eidet et al., 2014; Krysko et al., 2008). The results were consistent to those obtained by cytometry: apoptosis significantly increased over time reaching 35.0 ± 7.4% of apoptotic cells after 4h and 69.5 ± 7.0% after 6h **(Figure 6D, E)**. Based on the collective data obtained by flow cytometry and fluorescence microscopy, we selected a 4h STP treatment that was enough to induce early apoptosis in vitro which, as mentioned above, mimics the physiological conditions where apoptotic cells do not evolve into necrotic due to the rapid efficiency of phagocytosis.

6.1.3. Setting up the microglial phagocytosis assay

After characterizing NE-4C apoptosis and determining the use of non-inactivated serum in the culture medium, we continued with the setup of the phagocytosis assay. Primary microglia obtained from P0-P2 fms-EGFP mice were fed with NE-4C, which were previously labeled with CM-DiI (membrane marker; 5µM, 25mins) and treated with staurosporine (1µM, 4h) for the induction of apoptosis. Microglia were allowed to phagocytose over a time course of 1, 3, 5, and 15h and phagocytosis was assessed by the presence of phagocytic pouches, i.e., apoptotic cell fragments completely surrounded by microglia **(Figure 6F, G)**. For this analysis only fully closed pouches with CM-DiI and/or DAPI inclusions were quantified. When cultured with apoptotic NE-4C cells, the quantification showed that, as early as 1h, 66.2 ± 0.8% of microglia were phagocytic, a percentage that kept increasing over time reaching 95.6 ± 1.1% of phagocytosis at 15h **(Figure 6H)**. A further analysis of the different engulfed particles (DAPI, CM-DiI or DAPI and CM-DiI) revealed that the proportion of each inclusion type varied throughout the time course. At early time points, more and larger DAPI and CM-DiI particles were observed, whereas at later time points, small and numerous CM-DiI inclusions were detected, indicative of their degradation **(Figure 6I)**. There was a small decrease in microglia cell numbers along the time course **(Fig ure6J)**, which was in agreement with a small increase in microglial apoptosis. Nonetheless, microglial apoptosis never reached more than the 9.6 ± 1.7% of the total number of cells **(Figure 6K)**. In order to exclude the possibility that some STP residue could remain when feeding microglia with apoptotic NE-4C, we washed the apoptotic NE-4C with PBS several times prior to adding them to the assay. However, microglia died in the same proportion as before, suggesting that virtual STP residue was not inducing their apoptosis **(data not shown)**. We also speculated that microglia undergo apoptosis because they were overfed with a large amount of apoptotic cells; however, as we will see in **Section 4.4**, lower and more physiological amounts of apoptotic cells also resulted in microglial apoptosis. While in physiological conditions in the adult neurogenic DG niche we never observed any apoptotic microglia {Abiega, 2016 #9102}, the possibility that apoptotic cell engulfment triggers microglial death is an interesting hypothesis that deserves further testing, although it is out of the scope of this thesis.
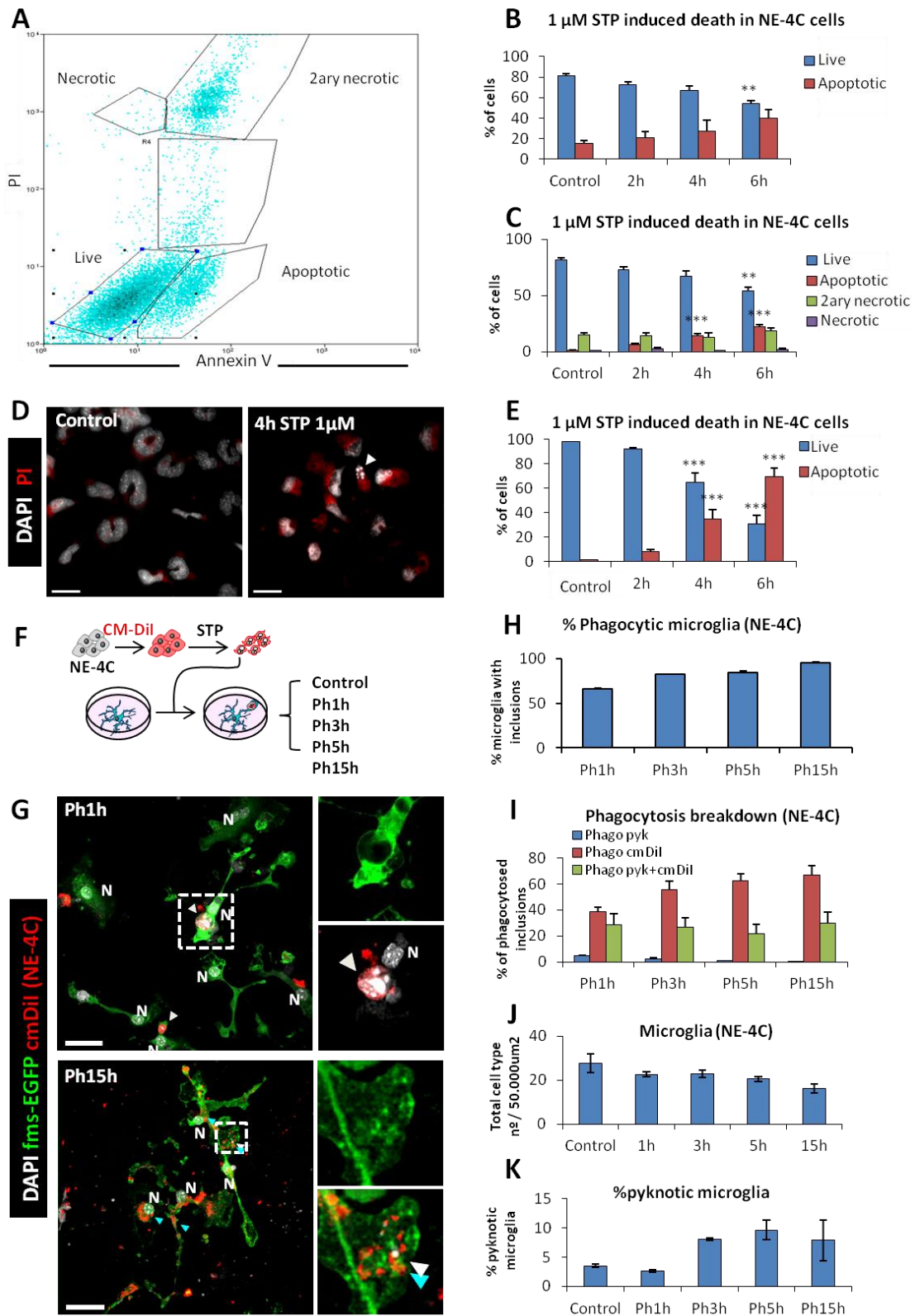
Figure 6. Characterization of the apoptosis of a mouse neuroprogenitor cell line (NE-4C) and set up of microglial phagocytosis assay. *[A] Flow cytometry analysis of the induction of apoptosis of NE-4C cell line by 1µM STP. Gating strategy for different cell populations according to their distribution in Annexin V (green fluorescence) versus PI (red fluorescence) chart. The populations live (Annexin⁻/PI⁻), apoptotic*

*(Annexin$^+$/PI$^-$), secondary necrotic (Annexin$^+$/PI$^+$) and necrotic (Annexin$^-$/PI$^+$) were identified based on Annexin V/PI labeling. **[B, C]** Quantification of the different cell populations identified by flow cytometry (N=3 independent experiments). **[D]** Representative epifluorescence images of NE-4C cells treated with STP for 4h. Images were obtained with an oil-immersion 40x objective. Nuclei were labeled with DAPI (blue) and cell death was detected by pyknosis/karyorrhexis (white, DAPI) and/or PI (red). Scale bar=30μm. **[E]** Quantification of live and dead (both apoptotic and secondary necrotic cells) NE-4C cells after STP treatment (N=3 independent experiments). Bars represent mean ± SEM. **[F]** Experimental design of the phagocytosis assay. **[G]** Representative confocal microscopy images of primary microglia (GFP, green) fed with NE-4C, which were previously labeled with CM-DiI (red) and treated with STP (1μM, 4h) for the induction of apoptosis (pyknosis/karyorrhexis, DAPI, white). N, microglial nucleus; white arrowheads, phagocytosed apoptotic NE-4C cells; blue arrowheads, phagocytosed CM-DiI; scale bars, 30μm. z=6.3 μm. **[H]** Quantification of the percentage of microglia with CM-DiI and/or DAPI inclusions along a time course. Only fully closed pouches with particles within were identified as phagocytosis. **[I]** Breakdown of the percentage of each inclusion type (DAPI, CM-DiI or DAPI and CM-DiI). **[J]** Quantification of average microglia density in control and different phagocytic time points. **[K]** Quantification of the percentage of pyknotic microglia. N=3 independent experiments. Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA was significant at p < 0.05).*

6.1.4. Microglial phagocytosis assay in vitro with a human neuronal apoptotic cell line (SH-SY5Y)

As stated above, the use of mouse origin cell line (NE-4C) might not be ideal to perform further transcriptional analysis of phagocytic microglia due to the technical unfeasibility to design cell-specific probes or primers when the apoptotic cells derive from the same species as microglia. At the end of the phagocytic process, the apoptotic cells would completely degrade within microglial lysosomes, resulting in almost inexistent mRNA from apoptotic cells. However, should any residual mRNA from apoptotic cells be left, the use of the mouse origin NE-4C cells would not permit to differentiate between the changes from microglia and residual apoptotic cells mRNA in transcriptional studies since designed probes and primers for transcriptional purposes would recognize genes of mouse origin, regardless of the cell of origin. Therefore, after characterizing microglial phagocytosis of **allogeneic** apoptotic cells (mouse NE-4C cells) we switched to a **xenogeneic** model (human SH-SY5Y cells) that would allow the use of species-specific probes and primers, and to specifically assess transcriptional changes in phagocytic microglia.

As in the NE-4C experiments, we started by characterizing SH-SY5Y apoptosis. Two different STP concentrations, 1µM and 1.5µM, were used for the induction of apoptosis at different time points. When applying 1µM **(Figure 7A, B)**, no SH-SY5Y apoptosis occurred over time, whereas 1.5µM STP showed a similar apoptosis pattern as in mouse cells: 41.4 ± 5.3% of apoptosis was induced after 4h and 67.0 ± 5.4% after 6h **(Figure 7C, D)**. In order to make downstream results more comparable, we chose 4h of STP treatment, as with mouse NE4C cells. For phagocytosis assays, primary microglia obtained from P0-P2 fms-EGFP mice were fed for different time points (1, 3, 5, 15 and 24h) with SH-SY5Y cells that were previously labeled with CM-DiI and treated with STP (1.5uM, 4h) for the induction of apoptosis **(Figure 7E, F)**. When cultured with apoptotic human cells, we found that as early as 1h, 30.2% ± 5.8% of microglia were phagocytic, a percentage that kept increasing until 15h (81.9% ± 3.7%), and was maintained until 24h (83.8% ± 3.6%) **(Figure 7G)**. A breakdown of the engulfed inclusion type (DAPI, CM-DiI or DAPI and CM-DiI) showed that the proportion of particle type varied throughout the time course, similar to what occur during phagocytosis of NE-4C cells. Early time points showed more and larger DAPI and CM-DiI particles, whereas at later time point, as apoptotic cells degrade within microglia, small and numerous CM-DiI inclusions were detected **(Figure 7H)**. A small decrease in microglia density along the time course was found **(Figure 7I)**, which was in accordance with a small increase in microglial apoptosis, similar to phagocytosis of NE-4C cells. Microglial apoptosis never reached more than the 12.6 ± 0.9 % of the total **(Figure 7J)**. Therefore, although the phagocytosis of human apoptotic cells was slightly delayed in comparison to the mouse cell line, probably due to the need of alternative recognition mechanisms to phagocytose human apoptotic cells, SH-SY5Y also proved to be a valid target to study microglial phagocytosis in vitro.
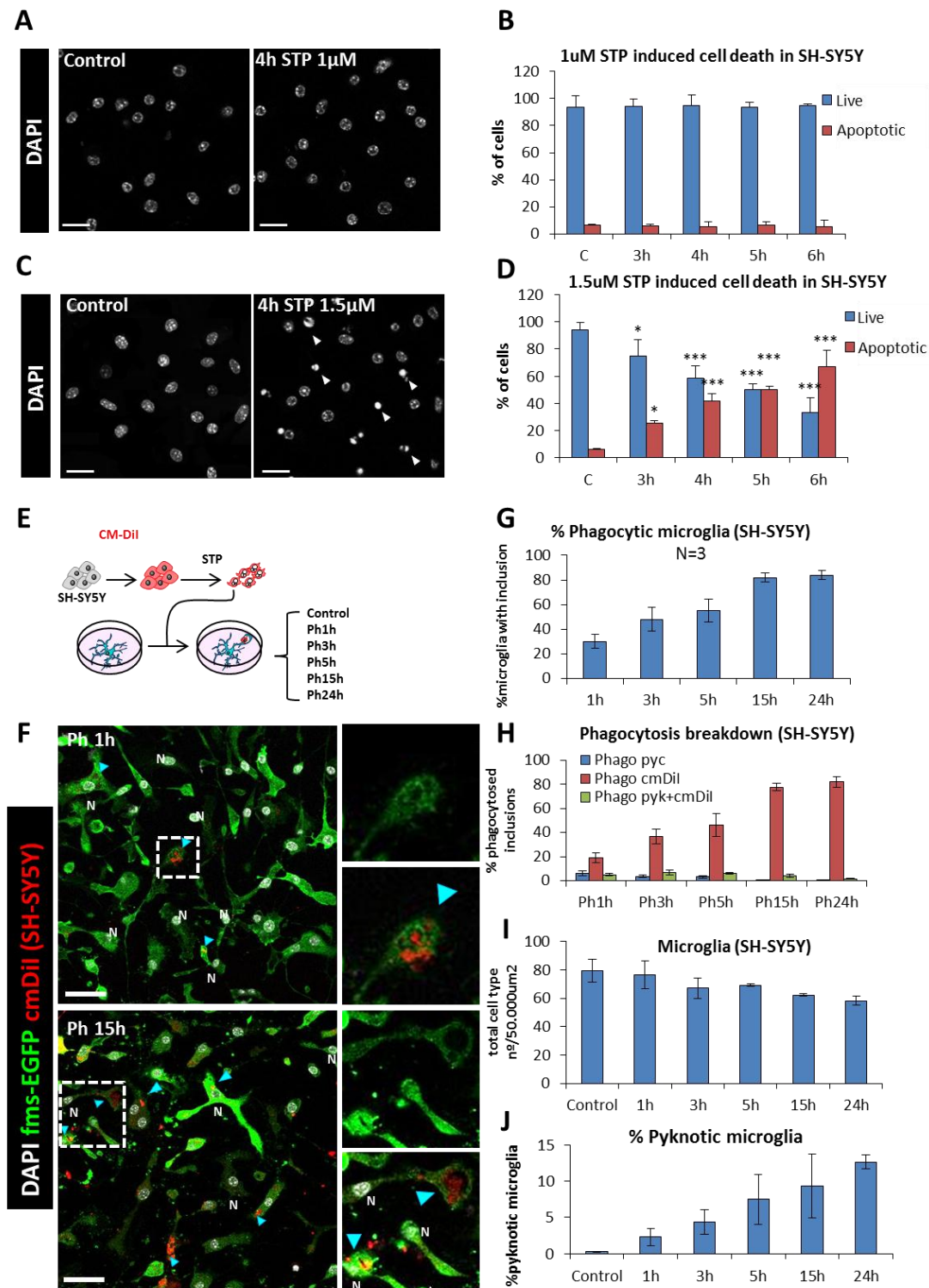
Figure 7. Phagocytosis assay with a human neural cell line (SH-SY5Y). *[A] Representative confocal images of SH-SY5Y cell treated with 1 µM STP for 4h. Images were obtained with an oil-immersion 40x objective. Nuclei were labeled with DAPI (white) and cell death was detected by pyknosis/karyorrhexis (white, DAPI). Scale bar=30µm. [B] Quantification of live and dead SH-SY5Y cells after 1 µM STP treatment. [C] Representative confocal images of SH-SY5Y cell treated with 1.5 µM STP for 4h. Nuclei*

110

were labeled with DAPI (white) and cell dead was detected by pyknosis/karyorrhexis (white, DAPI). Scale bar=30µm. [D] Quantification of live and dead SH-SY5Y cells after 1.5 µM STP treatment. [E] Experimental design of the phagocytosis assay. [F] Representative confocal microscopy images of primary microglia (GFP, green) fed with NE-4C, which were previously labeled with CM-DiI (red) and treated with STP for the induction of apoptosis (pyknosis/karyorrhexis, DAPI, white). N, microglial nucleus; white arrowheads, phagocytosed apoptotic SY-SY5Y cells; blue arrowheads, phagocytosed CM-DiI; scale bars, 30µm. z=6.3 µm. [G] Quantification of the percentage of microglia with CM-DiI and/or DAPI inclusions along a time course. Only fully closed pouches with particles within were identified as phagocytosis. [H] Breakdown of the percentage of each inclusion type (DAPI, CM-DiI or DAPI and CM-DiI). [I] Quantification of average microglia density in control and different phagocytic time points. [J] Quantification of the percentage of pyknotic microglia (N=3 independent experiments). Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA was significant at p < 0.05).

6.2. PHAGOCYTOSIS OF APOPTOTIC CELLS TRIGGERS TRANSCRIPTIONAL CHANGES IN CULTURED MICROGLIA

6.2.1. Specific detection of phagocytic microglia transcripts

Using the xenogeneic phagocytic assay model, we performed a full genome-wide transcriptomic analysis of the phagocytic microglia using gene expression arrays comparing naïve vs phagocytic microglia. In order to evaluate changes in microglia that could be triggered at different phagocytic times, both early (3h) and late (24h) phagocytosis time points were chosen for the arrays **(Figure 8A).**

We used the SurePrint G3 Mouse GE 8x60K Microarray (Agilent), which possessed a complete coverage of known RefSeq for mouse coding transcripts, and is `mouse-specific' as per manufacturer´s specifications. In addition, we double-checked every mouse probe sequence of the array against the whole human transcriptome by BLAST (Basic Local Aligment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to find those probes that could virtually detect residual mRNA from human apoptotic cells. We found that 4% of the mouse array probes had a high homology (MegaBlast, homology ≥ 95%) with the human transcriptome **(data not shown)**, which urged us to further study the possible presence of apoptotic mRNA in the phagocytic microglia samples. For this purpose we compared the RNA of control and phagocytic microglia as well as apoptotic SH-SY5Y cells that were allowed to degrade for 24h by an Agilent 2100 Bioanalyzer, a microfluidics-based platform for sizing,

quantification, and quality control of DNA and RNA. We examined the integrity of the ribosomal subunits 18S and 28S, which would determine the degradation state of the RNA of the different samples **(Figure 8B)**. We found that control microglia showed two clear peaks in the electropherogram that represented 18S and 28S rRNAs. On the contrary, apoptotic SH-SY5Y cells that were allowed to degenerate for 24h presented a strong degradation profile, in which no 18S or 28S were found. Phagocytic microglia displayed a very similar profile than control microglia and no degradation profiles were found, probably due to the ability of microglia to digest apoptotic cell mRNA. This data suggested that no RNA from apoptotic SH-SY5Y could be found in phagocytic microglia.

In addition, we also studied whether SH-SY5Y apoptotic cells could still have the capacity of synthesizing new mRNA. To study transcription during apoptosis, we used 5'-Fluorouridine (FU), a uridine analog that integrates at transcription sites **(Figure 8C)**. We found that live SH-SY5Y cells displayed a large FU signal in their nuclei, which evidenced the high transcriptional activation of live cells. On the contrary, apoptotic SH-SY5Y cells did not exhibit any FU signal, which indicated, as expected, that apoptotic cells did not have the capacity of synthesizing new mRNAs.

Altogether, the RNA profiling and analysis of transcription in apoptotic cells strongly suggests that although the gene arrays used could virtually detect up to 4% of mRNAs from apoptotic cells, the lack of residual RNA from apoptotic cells would result solely in the detection of microglial-specific transcriptional changes after phagocytosis. In addition, the results obtained from the arrays were later validated by RT-qPCR using mouse-specific primers.

6.2.2. Whole genome transcriptome of phagocytic microglia reveals four main patterns of gene expression

Hence, we performed a full genome-wide transcriptomic analysis of the phagocytic microglia using gene expression arrays comparing naïve vs phagocytic microglia (3h and 24h). The clustering of the samples (control microglia, Ph3h, and Ph24h) in each experiment was analyzed by Hierarchical Clustering (HCL) and Principal Component Analysis (PCA). Both clustering methods showed that the three experimental groups had strong differences in their expression profile **(Figure 8D, E)**.

To analyze which particular genes were different among the three experimental groups, we searched for array probes with significant changes over time. The analysis was performed in three steps (Conesa et al., 2006). First, probes that exhibited changes in expression over time were selected based on a p-corrected Benjamini-Hochberg value. Second, for each probes with a significant change in its expression over time a polynomial regression model was applied to identify the pattern. Finally, probes with significant expression changes over time were selected according to the fitness to the regression model. We obtained 20,800 significantly regulated probes using a conventional fitness criterion for R squared (rsq) > 0.7, and 13,146 probes using a more restrictive criterion of rsq > 0.9. This restrictive gene list was used to identify the transcriptional profile of phagocytic microglia.

Further screening of the probes was performed by selecting those with a fold change (FC) higher than 1.5 or lower than -1.5, resulting in 10,000 probes with significant changes in expression over time within the experimental groups. Since several probes corresponded to the same gene and some probes recognized sequences of yet unknown genes, eventually we obtained 6,585 genes that presented significant changes over time **(Figure 8F)**. The genes were classified according to four main expression patterns: UP (up-regulation both at 3h and 24h) **(Figure 8G)**, DOWN (down-regulation in both time points) **(Figure 8H)**, Transient UP (up-regulation at 3h and down-regulation at 24h) **(Figure 8I)** and Transient DOWN (down-regulation at 3h and up-regulation at 24h) **(Figure 8J)**. Genes in the expression pattern UP showed the largest average FC changes (14.2 ± 2.7 for 3h, 20.8 ± 3.1 for 24h) among all the patterns, some of them even reaching 8,000FC at 24h. The rest of the patterns presented more modest changes on average.

In the next section, we will describe the functional analysis of these patters using three different bioinformatics tools: DAVID, ClueGO and WGCNA and IPA. With these tools, we will analyze in detail changes in both functions and pathways.
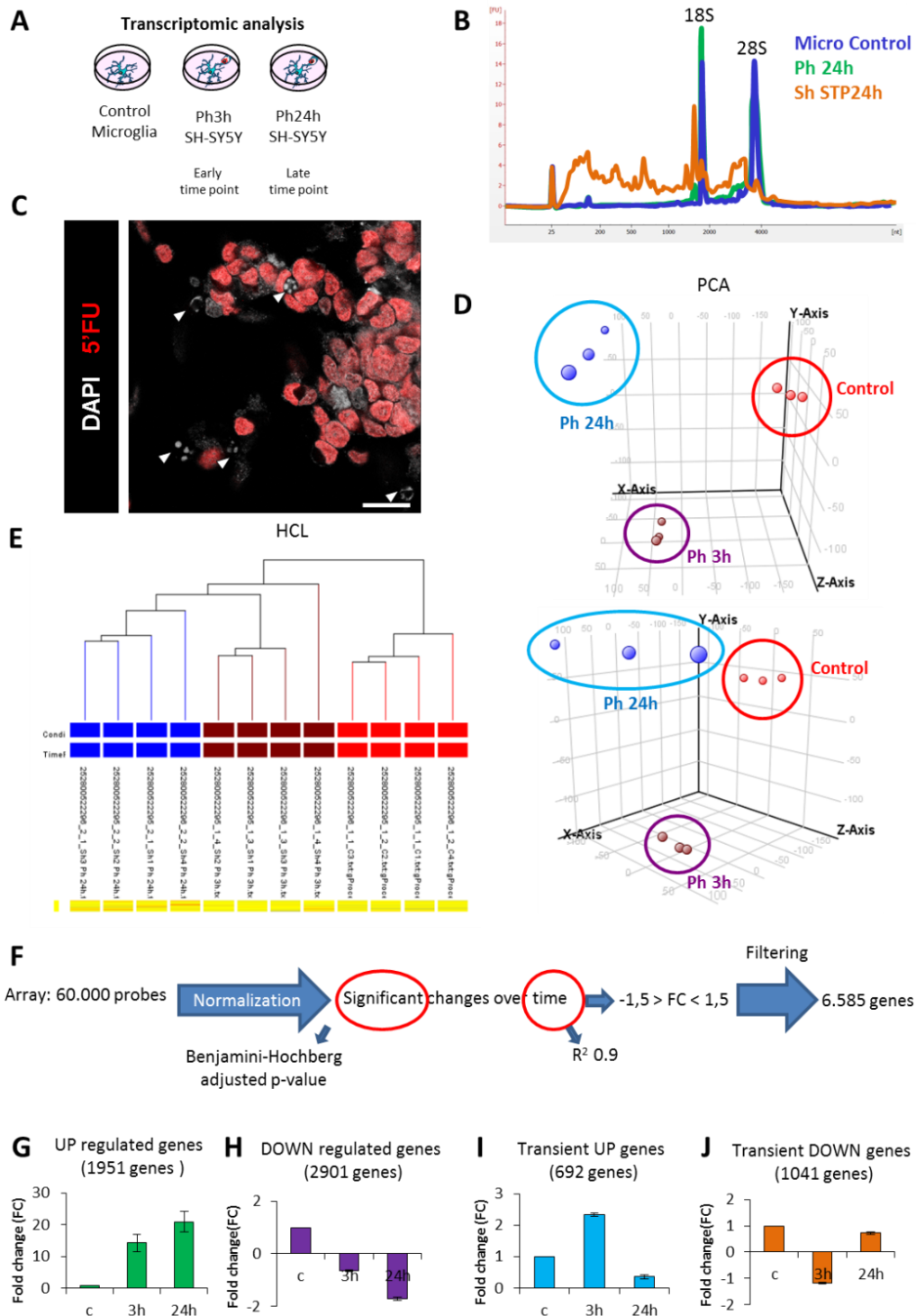
Figure 8. Transcriptional profile of phagocytic microglia. *[A] Experimental design of the gene expression arrays. [B] Electropherogram obtained by a Bioanalyzer comparing the RNA profile of control and phagocytic microglia as well as apoptotic SH-SY5Y that were allowed to degrade for 24h. [C] Representative confocal images of active transcription sites of SH-SY5Y cells after FU administration. Nuclei were labeled with DAPI (white), cell death was detected by pyknosis/karyorrhexis (white, DAPI, arrowheads), and transcription sites were detected by FU (red). Scale bar=20μm. [D] Principal Component Analysis (PCA) of the different replica of the samples: Control microglia, Ph3h, and Ph24h. [E]*

*Hierarchical Clustering (HCL) of the different replica of the samples Control microglia (blue), Ph3h (brown), and Ph24h (red). **[F]** Representation of the strategy followed to screen genes from the gene array. **[G]** FC mean of the 1,951 genes classified under the UP regulation pattern. **[H]** FC mean of the 2,901 genes classified under the DOWN regulation pattern. **[I]** FC mean of the 692 genes classified under the Transient UP regulation pattern. **[J]** FC mean of the 1,041 genes classified under the Transient DOWN regulation pattern. N=3 independent experiments. Bars represent mean ± SEM.*

6.3. The transcriptional profile of phagocytic microglia reveals major general changes such as metabolism, cytoskeleton and transcription, as well as several potential modulators of neurogenesis

6.3.1. Functional analysis of the phagocytic microglia transcriptome by DAVID

Next, we performed a functional analysis of the phagocytic microglia transcriptome using the DAVID software (Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/) in the four main expression patterns separately. This software analyzes the Gene Ontology (GO) of groups of genes related to biological processes rather than individual genes in order to find significant changes in different cellular pathways. The analysis revealed many biological functions that were significantly enriched in phagocytic microglia. For a better understanding of these biological functions and after discarding redundant functions, we grouped them in 16 major pathways: 'neurogenesis', 'cell cycle', 'differentiation', 'migration', 'development', 'angiogenesis', 'death', 'immune system', 'behavior', 'cytoskeleton', 'metabolism', 'transcription', 'DNA modifications', 'RNA modifications', 'signaling', 'phosphorylation', and 'others' **(Figure 9A)**. The majority of these pathways presented a generalized up-regulatory pattern in their functions. Only pathways related to DNA and RNA modification showed a generalized down-regulatory pattern in their functions. The categories 'cell cycle', 'immune system', and 'others' exhibited a more mixed expression patterns with up, down, and transient-up regulations.

Taking these data into account, we focused on the major pathways that are grouped under the up-regulatory pattern. We found that the transcription profile revealed several up-regulatory changes in functions related to metabolism, specially glycolisis (GO:0006096) and
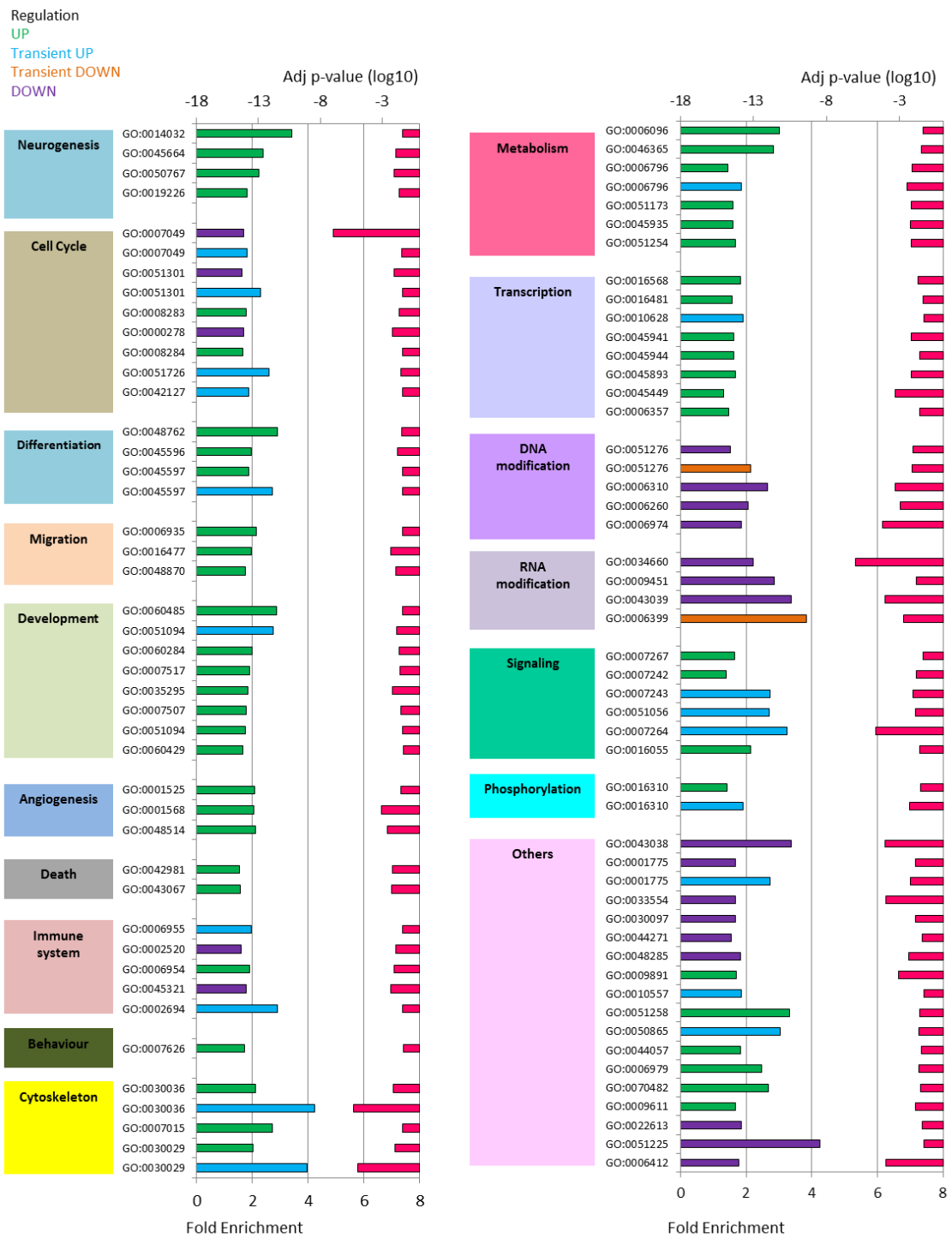
115

catabolism of different macromolecules **(Figure 9A)**. In addition, the transcription profile analysis also showed up-regulatory changes in many functions related to transcription. Moreover, the phagocytic transcription profile analysis also revealed that all the functions grouped under 'neurogenesis' term appeared significantly up-regulated. Only enriched functions with P value <0.05 were considered. Many of these changes in biological functions were also observed by ClueGO and IPA functional analysis and will be further detailed in **sections 6.3.2, 6.3.3 and 6.3.4**.
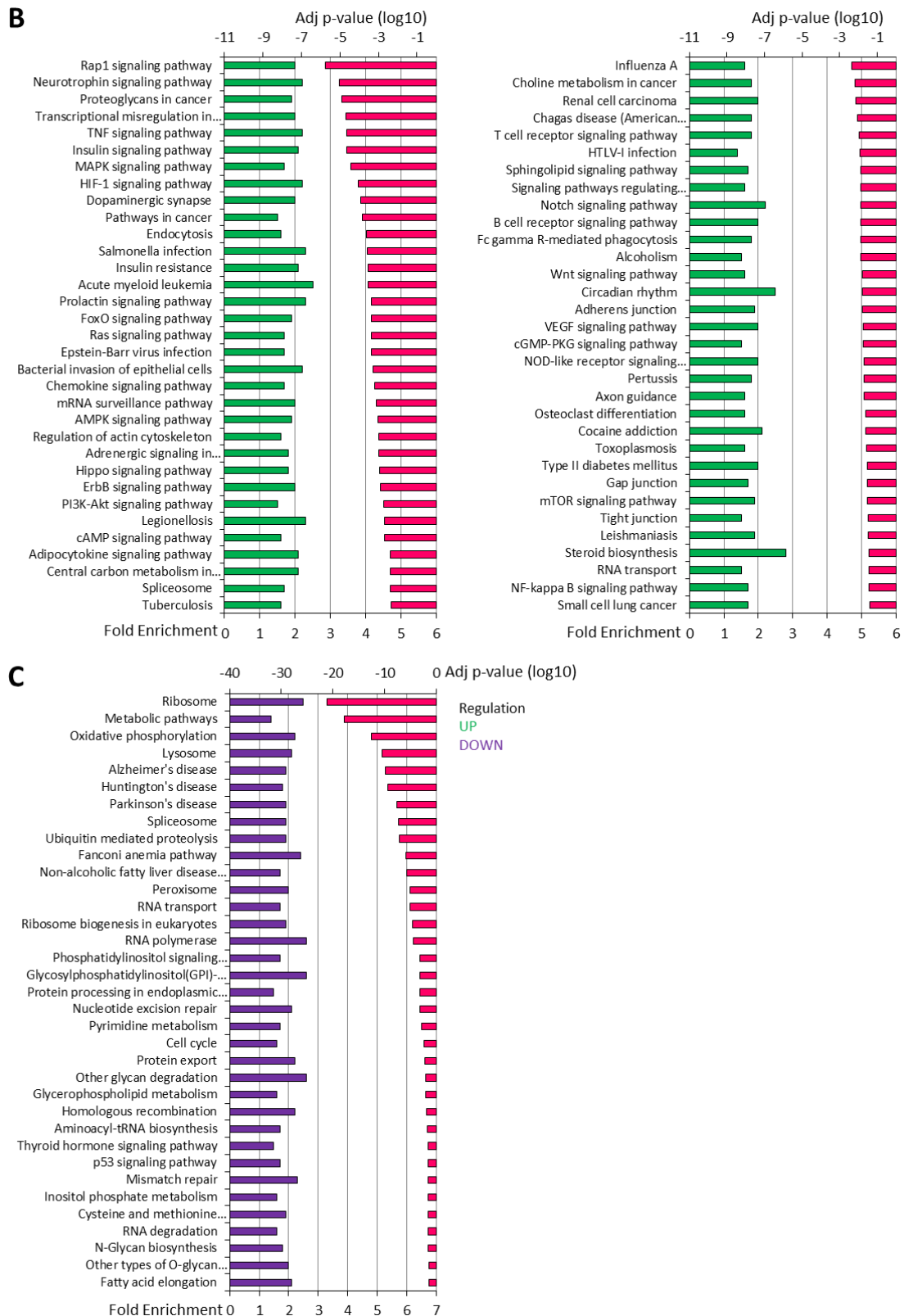
In addition to study changes in biological functions, we also analyzed which pathways were enriched in the regulatory patterns. We specially focused on the UP and DOWN patterns in the pathways found using DAVID **(Figure 9B)**. First, we found a variety of ubiquitous classical signaling pathways (MAPK (mitogen-activated protein kinase), Ras, AMPK (AMP-activated protein kinase), PI3K-Akt (phosphatidylinositol3 kinase), cAMP, etc.). In addition, the analysis of the UP-regulated pattern also revealed several significantly enriched pathways related to neurogenesis such as 'neurotrophin signaling pathway', 'TNF (tumor necrosis factor) signaling pathway', 'Dopaminergic synapse', 'Notch signaling pathway', 'Wnt (Wingless) signaling pathway', 'VEGF (vascular endothelial growth factor) signaling pathway' and 'Axon guidance'. Besides, we shall also highlight the significant enrichment in Hif1 signaling pathway, since, as we will further explain in **section 6.3.4**, this transcription factor is able to regulate metabolism as well as trigger the release of several trophic factors. On the other hand, the analysis of the DOWN-regulated pattern showed more miscellanea results **(Figure 9C)**. The analysis revealed a general down regulation in several organelles such as ribosomes, lysosomes, and peroxisomes. Importantly, it also showed a shutdown of the oxidative phosphorylation and metabolic pathways, a result that will also be found in IPA analysis **(section 6.3.3)** and further explained in **section 6.3.4**.

Therefore, both GO terms and pathway analysis by DAVID software revealed several neurogenesis-related functions in phagocytic microglia suggesting that phagocytic microglia might be involved in the maintenance and correct regulation of the neurogenic niche.

A

Figure 9. Functional analysis of phagocytic microglia by DAVID. [A] *Functional analysis of phagocytic microglia using DAVID software. Left axis represents the fold enrichment of each biological function and right axis represents the adjusted p-value of each GO term. The regulatory patterns of each function are represented under the following color code: UP (green), DOWN (purple), Transient-UP (blue), and*

Transient-DOWN (orange). Key for the GO terms: GO:0014032, neural crest cell development; GO:0045664, regulation of neuron differentiation; GO:0050767, regulation of neurogenesis; GO:0019226, transmission of nerve impulse; GO:0007049, cell cycle; GO:0051301, cell division; GO:0008283, cell proliferation; GO:0000278, mitotic cell cycle; GO:0008284, positive regulation of cell proliferation; GO:0051726, regulation of cell cycle; GO:0042127, regulation of cell proliferation; GO:0048762, mesenchymal cell differentiation; GO:0045596, negative regulation of cell differentiation; GO:0045597, positive regulation of cell differentiation; GO:0006935, chemotaxis; GO:0016477, cell migration; GO:0048870, cell motility; GO:0060485, mesenchyme development; GO:0051094, positive regulation of developmental process; GO:0060284, regulation of cell development; GO:0007517, muscle organ development; GO:0035295, tube development; GO:0007507, heart development; GO:0051094, positive regulation of developmental process; GO:0060429, epithelium development; GO:0001525, angiogenesis; GO:0001568, blood vessel development; GO:0048514, blood vessel morphogenesis; GO:0042981, regulation of apoptosis; GO:0043067, regulation of programmed cell death; GO:0006955, immune response; GO:0002520, immune system development; GO:0006954, inflammatory response; GO:0045321, leukocyte activation; GO:0002694, regulation of leukocyte activation; GO:0007626, locomotory behavior; GO:0030036, actin cytoskeleton organization; GO:0007015, actin filament organization; GO:0030029, actin filament-based process; GO:0006096, glycolysis; GO:0046365, monosaccharide catabolic process; GO:0006796, phosphate metabolic process; GO:0006796, phosphate metabolic process; GO:0051173, positive regulation of nitrogen compound metabolic process; GO:0045935, positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; GO:0051254, positive regulation of RNA metabolic process; GO:0016568, chromatin modification; GO:0016481, negative regulation of transcription; GO:0010628, positive regulation of gene expression; GO:0045941, positive regulation of transcription; GO:0045944, positive regulation of transcription from RNA polymerase II promoter; GO:0045893, positive regulation of transcription, DNA-dependent; GO:0045449, regulation of transcription; GO:0006357, regulation of transcription from RNA polymerase II promoter; GO:0051276, chromosome organization; GO:0006310, DNA recombination; GO:0006260, DNA replication; GO:0006974, response to DNA damage stimulus; GO:0034660, ncRNA metabolic process; GO:0009451, RNA modification; GO:0043039, tRNA aminoacylation; GO:0006399 tRNA metabolic process; GO:0007267, cell-cell signaling; GO:0007242, intracellular signaling cascade; GO:0007243, protein kinase cascade; GO:0051056, regulation of small GTPase mediated signal transduction; GO:0007264, small GTPase mediated signal transduction; GO:0016055, Wnt receptor signaling pathway; GO:0016310, phosphorylation; GO:0043038, amino acid activation; GO:0001775, cell activation; GO:0001775, cell activation; GO:0033554, cellular response to stress; GO:0030097, hemopoiesis; GO:0044271, nitrogen compound biosynthetic process; GO:0048285, organelle fission; GO:0009891, positive regulation of biosynthetic process; GO:0010557, positive regulation of macromolecule biosynthetic process; GO:0051258, protein polymerization; GO:0050865, regulation of cell activation; GO:0044057, regulation of system process; GO:0006979, response to oxidative stress; GO:0070482, response to oxygen levels; GO:0009611, response to wounding; GO:0022613,

ribonucleoprotein complex biogenesis; GO:0051225, spindle assembly; GO:0006412, translation. [B] Pathway analysis of the UP-regulated pattern of phagocytic microglia using DAVID software. Left axis represents the fold enrichment of each biological function and right axis represents the adjusted p-value of each GO term. Only statistically significant changes are shown. [C] Pathway analysis of the DOWN-regulated pattern of phagocytic microglia using DAVID software. Left axis represents the fold enrichment of each biological function and right axis represents the adjusted p-value of each GO term. Only statistically significant changes are shown.

6.3.2. Functional analysis of the phagocytic microglia transcriptome by ClueGO

To obtain further information from the array data, we also performed a functional analysis of the phagocytic microglia transcriptome with the ClueGO network (http://apps.cytoscape.org/apps/cluego), which allows investigating interrelationships among pathways. Gene-annotation enrichment analysis based on ClueGO, a computational tool that integrates GO terms as well as Kyoto encyclopedia of genes and genomes (KEGG)/ BioCarta pathways (Bindea et al., 2009), also revealed a number of functional biological pathways associated with each of the four main expression patterns of the phagocytic microglia obtained from the array.

The network graph showed several enriched GO terms for each expression pattern. Related terms were automatically grouped under the highest enriched term and, in turn, groups were connected based on shared genes (kappa score or k score; k score was fixed at 0.8, which means that only groups that share 80% of the genes or more will be connected) **(Figure 10)**. Similarly to those results obtained from the DAVID analysis, ClueGO also showed downregulation in pathways related to DNA and chromosomes. In addition, different functions associated with metabolism also exhibited an upregulation. Importantly, for many upregulated genes, ClueGO revealed specific terms like 'generation of neurons', 'neuron differentiation' or 'neuron development' that were grouped under the term 'Neurogenesis' and had a direct interrelation with 'Neuron projection development' group. Altogether, both DAVID and ClueGO analyses suggest that phagocytosis triggers a neurogenic-modulator program in microglia.
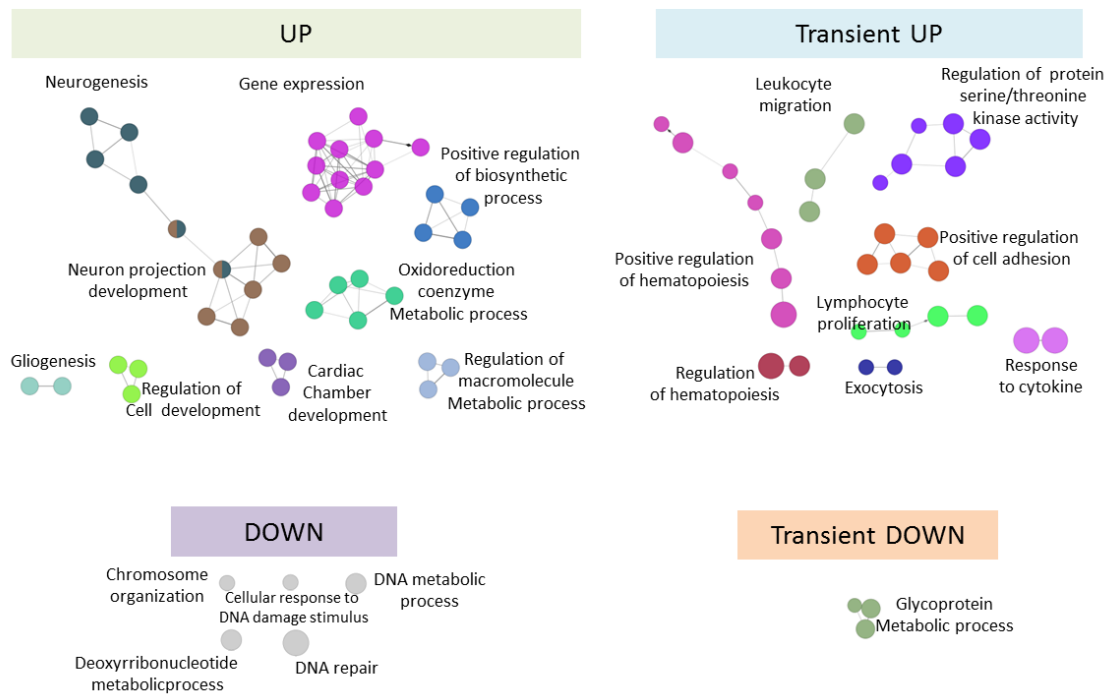
Figure 10. Functional analysis of phagocytic microglia by ClueGO. Functional analysis of phagocytic microglia using ClueGO software. Charts showing the interactions among the significantly different functions for the four main expression patterns. Biological pathways are visualized as colored nodes linked to related groups based on their kappa score level. The node size reflects the enrichment significance of the term and functionally related groups are linked. Non-grouped terms are shown in grey.

6.3.3. Functional analysis of the phagocytic microglia transcriptome by WGCNA and IPA analysis

In order to independently analyze the transcriptomic profile of phagocytic microglia, we established a collaboration with Erik Boddeke (University Medical Center of Groningen, The Netherlands).

First, we performed a weighted gene co-expression network analysis (WGCNA), a method to study biological networks that allows defining gene modules (clusters). Filtering was performed on the raw datasets of the array. A cut-off was determined by the 95 quantile of the controls and at least four samples needed to have an expression higher than this cut-off before gene expression was determined. With these conditions, 34,617 probes were selected for further classification. We found four main modules that showed significant changes and carried biological information **(Figure 11)**. The yellow module presented an up-regulatory pattern throughout the phagocytosis time course **(Figure 11A)**, whereas on the contrary,
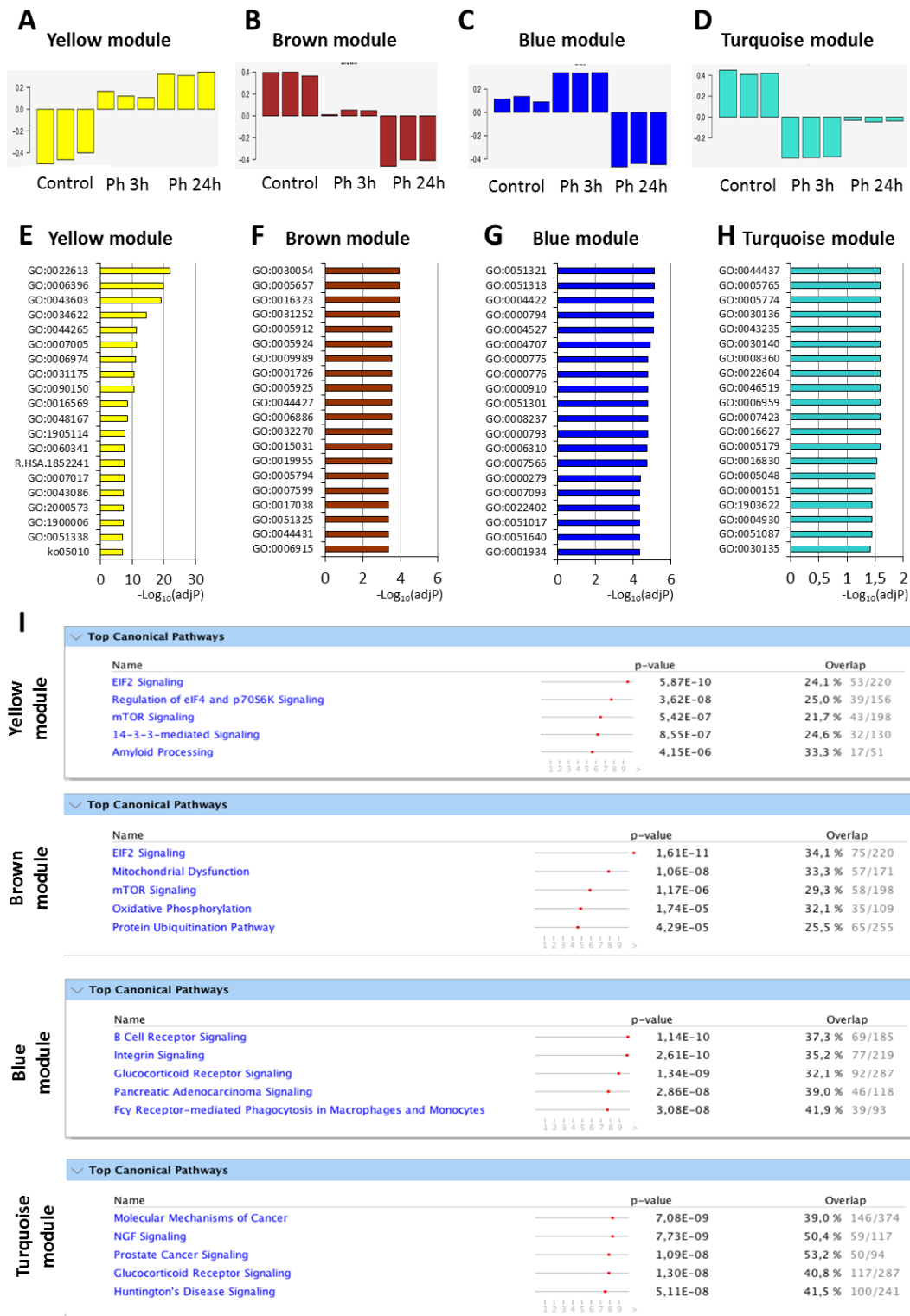
brown module was defined as down-regulatory pattern along the time course **(Figure 11B)**. There were also blue and turquoise modules, which presented a pattern that switched at 3h of phagocytosis, up regulation at 3h and down regulation at 24h for the blue module **(Figure 11C)** and down regulation at 24h and up regulation at 24h for the turquoise module **(Figure 11D)**. The four modules found by WGCNA analysis, highly resembled the results obtained by R-squared (rsq)> 0.9 criterion analysis where we had found the four main expression patterns **(Figure 8 G-J)**.

In order to give some insight into the signature of the modules of the WGCNA, we next performed an Ingenuity Pathway Analysis (IPA) as a functional analysis. Similar to the results obtained with DAVID **(section 3.1)**, the Yellow module (up-regulatory profile) also showed strongly significant GOs related to neurogenesis, such as 'positive regulation of dendrite development' (GO:1900006), 'regulation of synaptic plasticity' (GO:0048167), and 'neuron projection development' (GO:0031175) among others **(Figure 11E)**. In addition, Yellow module also displayed some functions associated with metabolism ('negative regulation of catalytic activity', GO:0043086) of chromatin modification ('covalent chromatin modification', GO:0016569). On the other hand, the Brown module (down-regulatory profile) showed a miscellany of functions, some of them related to junctions ('cell substrate adherens junction', GO:0005924 or 'cell matrix junction', GO:0009989) or transport ('intracellular protein transport', GO:0006886) **(Figure 11F)**. The Blue module (transient up-regulatory profile) displayed a large number of significantly changed GO associated with cell cycle, such as 'G1 phase' (GO:0051318), 'mitotic cell cycle checkpoint' (GO:0007093) or 'cytokinesis' (GO:0000910) among others **(Figure 11G)**. Finally, the Turquoise module (transient down-regulatory profile showed some significant changes in functions related to vesicles, such as 'trans golgi network transport vesicle' (GO:0030140) or 'coated vesicle'(GO:0030135) **(Figure 11H)**.

In addition, we also studied the top canonical KEGG pathways that were enriched in each module **(Figure 11I)**. We found that both the Yellow and the Brown module shared some pathways such as EIF2 (eukaryotic initiation factor 2) and mTOR (mammalian target of rapamycin) signaling. The Brown module also presented changes in Oxidative Phosphorylation, suggesting that phagocytic microglia might undergo metabolic modifications. As stated above **(section 3.1)**, DAVID analysis also showed changes in metabolism, such as an increase in glycolysis. In addition, the Blue module revealed changes in different signaling pathways and in Fcγ (fragment crystallizable γ) receptor-mediated phagocytosis. The Turquoise module showed

changes in pathways related to NGF (nerve growth factor) signaling and glucocorticoid receptor signaling.

Thus, the three functional analyses performed (DAVID, ClueGO and IPA) revealed several neurogenesis-related functions in phagocytic microglia suggesting that phagocytic microglia might be involved in the maintenance and correct regulation of the neurogenic niche.

Figure 11. Functional analysis of phagocytic microglia by WGCNA and IPA analysis. [A] Yellow module obtained by WGCNA analysis presented an up-regulatory pattern. [B] Brown module obtained by WGCNA analysis presented a down-regulatory pattern. [C] Blue module obtained by WGCNA analysis presented a transient up-regulatory pattern. [D] Turquoise module obtained by WGCNA analysis presented a transient down-regulatory pattern. [E] IPA analysis showing top 20 significantly changed GO terms associated to Yellow module. GO:0022613, ribonucleoprotein complex biogenesis; GO:0006396, RNA processing; GO:0043603, cellular amide metabolic process; GO:0034622, cellular macromolecular complex assembly; GO:0044265, cellular macromolecule catabolic process; GO:0007005, mitochondrion organization; GO:0006974, cellular response to DNA damage stimulus; GO:0031175, neuron projection development; GO:0090150, establishment of protein localization to membrane; GO:0016569, covalent chromatin modification; GO:0048167, regulation of synaptic plasticity; GO:1905114, cell surface receptor signaling pathway involved in cell-cell signaling; GO:0060341, regulation of cellular localization; R.HSA.1852241, organelle biogenesis and maintenance; GO:0007017, microtubule-based process; GO:0043086, negative regulation of catalytic activity; GO:2000573, positive regulation of DNA biosynthetic process; GO:1900006, positive regulation of dendrite development; GO:0051338, regulation of transferase activity; ko05010, Alzheimer's disease. [F] IPA analysis showing top 20 significantly changed GO terms associated to Brown module. GO:0030054, cell junction; GO:0005657, replication fork; GO:0016323, basolateral plasma membrane; GO:0031252, leading edge; GO:0005912, adherens junction; GO:0005924, cell substrate adherens junction; GO:0009989, cell matrix junction; GO:0001726, ruffle; GO:0005925, focal adhesion; GO:0044427, chromosomal part; GO:0006886, intracellular protein transport; GO:0032270, positive regulation of cellular protein metabolic process; GO:0015031, protein transport; GO:0019955, cytokine binding; GO:0005794, golgi apparatus; GO:0007599, hemostasis; GO:0017038, protein import; GO:0051325, interphase; GO:0044431, golgi apparatus part; GO:0006915, apoptotic program. [G] IPA analysis showing top 20 significantly changed GO terms associated to Blue module. GO:0051321, meiotic cell cycle; GO:0051318, G1 phase; GO:0004422, metalloendopeptidase activity; GO:0000794, condensed nuclear chromosome; GO:0004527, exonuclease activity; GO:0004707, MAP kinase activity; GO:0000775, chromosome rericentric region; GO:0000776, kinetochore; GO:0000910, cytokinesis; GO:0051301, cell division; GO:0008237, metallopeptidase activity; GO:0000793, condensed chromosome; GO:0006310, DNA recombination; GO:0007565, female pregnancy; GO:0000279, M phase; GO:0007093, mitotic cell cycle checkpoint; GO:0022402, cell cycle process; GO:0051017, actin filament bundle formation; GO:0051640, organelle localization; GO:0001934, positive regulation of protein amino acid phosphorylation. [H] IPA analysis showing top 20 significantly changed GO terms associated to Turquoise module. GO:0044437, vacuolar part; GO:0005765, lysosomal membrane; GO:0005774, vacuolar membrane; GO:0030136, clathrin coated vesicle; GO:0043235, receptor complex; GO:0030140, trans golgi network transport vesicle; GO:0008360, regulation of cell shape; GO:0022604, regulation of cell morphogenesis; GO:0046519, sphingoid metabolic process; GO:0006959, humoral immune response; GO:0007423, sensory organ development; GO:0016627, oxidoreductase activity acting on the ch-ch group of donors; GO:0005179, hormone

*activity; GO:0016830, carbon carbon lyase activity; GO:0005048, signal sequence binding; GO:0000151, ubiquitin ligase complex; GO:1903622, transcription from RNA polymerase III promoter; GO:0004930, G protein coupled receptor activity; GO:0051087, chaperone binding; GO:0030135, coated vesicle. **[I]** IPA analysis showing significantly changed top 5 canonical pathways associated to each module.*

6.3.4. Functional analysis of the phagocytic microglia transcriptome reveals changes in apoptosis

The functional analysis of the phagocytic microglia transcriptome using DAVID analysis **(sections 6.3.1)** showed significant up-regulated changes in functions related to the regulation of cell death. The genes associated to these functions encoded a mixture of cytoplasmic and nuclear proteins i.e., genes that would act directly on the cell expressing them (**autologous genes**); as well as released proteins or membrane proteins acting on neighbor cells, i.e., genes whose protein would exert their function outside the expressing cell (**heterologous genes**) **(Figure 6)**. We found that the majority of the cell death-related heterologous genes were anti-apoptotic, suggesting that the effect that microglial phagocytosis might exert over the neurogenic cascade could be related to an increase in neuroprotection or survival. On the other hand, the majority of the autologous genes were pro-apoptotic; however, autologous genes expressed by phagocytic microglia can only affect the expressing cell, and therefore, these pro-apoptotic genes would not modulate neurogenesis **(Figure 12)**. Nevertheless, the fact that phagocytic microglia expressed autologous pro-apoptotic genes could explain the increase in microglial apoptosis that we observed upon phagocytosis **(Figure 6, 7)**.

Gene Symbol	FC Ph3h	FC Ph24h	Location	Effect on apoptosis
BAD	1,1	1,1	autologous	pro-apoptotic
FAS	1,5	1,5	autologous	pro-apoptotic
FOXO3	21	38	autologous	pro-apoptotic
GAS1	2,7	3	autologous	pro-apoptotic
NLRP3	12	19	autologous	pro-apoptotic
PPP2CB	1,5	1,6	autologous	pro-apoptotic
PTEN	1,1	1,6	autologous	pro-apoptotic
RHOA	1	1,7	autologous	pro-apoptotic
SCRIB	1,9	2,4	autologous	pro-apoptotic
STK3	1	1,5	autologous	pro-apoptotic
TFPT	2,4	3	autologous	pro-apoptotic
RARG	1,3	3	autologous	pleiotropic
PRDX1	1,1	2	autologous	anti-apoptotic
SIRT1	2	1,6	autologous	anti-apoptotic
SMO	1	3,6	autologous	anti-apoptotic
SOD2	1,2	2	autologous	anti-apoptotic
SPHK1	4,4	1,6	autologous	anti-apoptotic
UBE2B	1,2	1,1	autologous	anti-apoptotic
XRCC5	2,1	3,8	autologous	anti-apoptotic
PRNP	1,8	1,3	autologous and heterologous	anti-apoptotic
TGM2	5,5	4,5	autologous and heterologous	anti-apoptotic
GAL	1,2	1,8	heterologous	pro-apoptotic
IL6	18,7	15,8	heterologous	pleiotropic
CNTF	1,5	2,6	heterologous	anti-apoptotic
FGF2	4,2	3,7	heterologous	anti-apoptotic
FGF8	1,7	1,6	heterologous	anti-apoptotic
VEGFA	4,5	1,8	heterologous	anti-apoptotic

FC < 2	FC < -2
FC 2-4	FC -2-4
FC > 4	FC > -4

Figure 12. Functional analysis of the phagocytic microglia transcriptome reveals changes in apoptosis. Classification of the genes related to death cell obtained from DAVID analysis. The genes were classified according to their FC, to the effects on microglia (autologous) or on the surrounding cells (heterologous) and to the positive or negative effect on apoptosis.

6.3.5. Functional analysis of the phagocytic microglia transcriptome reveals changes in metabolism

The functional analysis of the phagocytic microglia transcriptome using different bioinformatic tools **(sections 6.3.1, 6.3.2 and 6.3.3)** revealed significant changes in different metabolic pathways such as glycolysis and oxidative phosphorylation, which highly suggested that phagocytosis might trigger some metabolic modification in microglia. In order to analyze these changes in depth, we searched in the array for the main genes involved in glycolysis, oxidative phosphorylation, and glucose and lactate transport **(Figure 13A)**. We found that in general terms, genes encoding glycolytic enzymes were up-regulated, whereas mitochondrial Krebb's cycle and oxidative phosphorylation were down-regulated. In addition, lactate shuttles were up-regulated, suggesting that instead of processing pyruvate in the mitochondria, phagocytic microglia might convert pyruvate to lactate through lactic fermentation and release it through lactic shuttles. The possible metabolic switch from oxidative phosphorylation to glycolysis in phagocytic microglia might be a key signature of phagocytosis but is out of the

scope of this thesis and will be studied in the future by analyzing mitochondrial respiration and glycolysis in live naïve and phagocytic microglia cells with an Agilent Seahorse XF Analyzer.

Interestingly, our arrays suggest one key candidate to regulate metabolic adaptations in phagocytic microglia: the Hif-1α signaling pathway **(Figure 13B)**. Hif-1α (Hypoxia-inducible factor-1-α) is a heterodimeric transcription factor considered the master transcriptional regulator in response to hypoxia (Nizet and Johnson, 2009). Under hypoxic conditions, Hif1α induces the transcription of more than 60 genes, including VEGF and erythropoietin, which are involved in biological processes such as angiogenesis and erythropoiesis (Lee et al., 2004), and neurogenesis (Nowacka and Obuchowicz, 2012). In addition, Hif-1α also exerts hypoxia-independent functions by inducing transcription of genes involved in cell proliferation and survival, as well as glucose and iron metabolism (Weidemann and Johnson, 2008). More specifically, Hif-1α promotes anaerobic metabolism (glycolysis) and inhibits Krebb's cycle, functions that highly resembles the gene expression pattern found in our array data.

Therefore, since phagocytic microglia presents a transcriptional profile suggestive of enhanced glycolysis and decreased Krebb's cycle, and it also displays a variety of functions that might regulate neurogenesis, we propose that Hif-1α might be a possible master regulator of the metabolic and neurogenic functions of phagocytic microglia. Along with the metabolic switch, the role of Hif-1α in phagocytic microglia will be studied in depth outside this project.
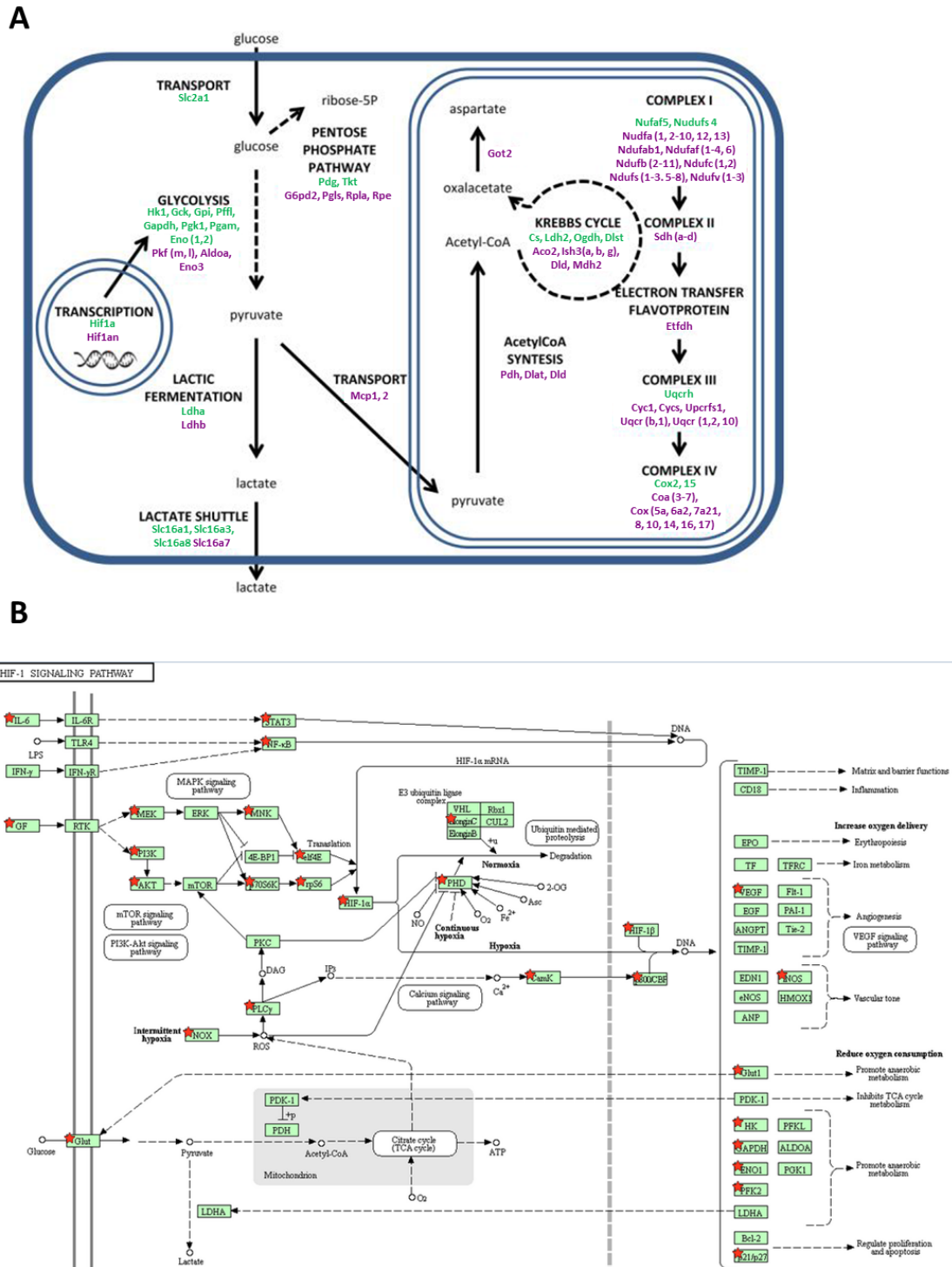
Figure 13. Functional analysis of the phagocytic microglia transcriptome reveals changes in metabolism. [A] *Schematic representation of the metabolic pathways of the phagocytic microglial cell and the main genes changed in each of them. Up-regulated genes are shown in green and down-regulated genes are shown in purple.* **[B]** *Hif1 signaling pathway scheme obtained by DAVID software. The red star shows the significantly changed molecules of the array in the pathway.*

6.4. THE POTENTIAL MODULATORS OF NEUROGENESIS ARE MAINLY PEPTIDES AND HORMONES

6.4.1. The validation of phagocytosis-related neurocandidates confirms the expression of several potential modulators of neurogenesis triggered by microglial phagocytosis

After confirming the existence of an up-regulation in the function of neurogenesis by phagocytic microglia in the three functional analyses with DAVID, ClueGO, and IPA, we focused on the identity of the neurogenesis-related genes. In order to avoid excluding any candidate gene due to our initial highly strict filtering (rsq > 0.9), we stepped back and used a less restrictive (rsq > 0.7) gene list that contained 20,800 probes with no further screening of the fold change.

To identify the potential regulators of neurogenesis, we compared our database with MANGO (The Mammalian Adult Neurogenesis Gene Ontology), a database of genes already described to be involved in the regulation of adult hippocampal neurogenesis (Overall et al., 2012). The MANGO database enlists 259 neurogenesis-related genes that include a large range of molecules such as transcription factors (E2F1, E2F transcription factor 1; Atf1, activating transcription factor 1), enzymes (Plcγ, phospholipase C γ; Rrm1, ribonucleotide reductase catalytic subunit M1), cell cycle molecules (Cyclins), receptors (dopamine, glutamate or ephrin receptors), growth factors (IGF1, VEGF) etc. We compared our list of 20,800 probes with the MANGO database and found that 213 MANGO genes were differentially expressed in naïve vs phagocytic microglia.

In this list of 213 MANGO genes, there were two different types of neurogenesis-related genes. One set of genes encoded receptors as well as cytoplasmic and nuclear proteins, i.e., genes that would act directly on the cell expressing them (<u>autologous genes</u>). An example of an autologous gene is the transcription factor REST (RE1 silencing transcription factor), which is a molecule involved in neurogenesis when expressed by neuroprogenitor cells since it represses neuronal genes {Kohyama, 2010 #12027}. The other set of genes encoded released proteins or membrane proteins (ligands, metalloproteases, etc.) acting on neighbor cells, i.e., genes whose protein would exert their function outside the expressing cell (<u>heterologous genes</u>). An example of a heterologous gene is the trophic factor EGF (epidermal growth factor),

129

which can be expressed by different types of cells within the neurogenic niche in order to affect neurogenesis. We focused on heterologous genes, as only their expression in microglia could influence neurogenesis.

Using the criteria defined above (where we only focused on proteins that were released or bound to the membrane), we found that the MANGO database contained 26 heterologous genes that had previously been involved in regulating neurogenesis. To further extend the list of heterologous genes in our array that could be potential regulators of neurogenesis genes outside of the MANGO database, we developed the following strategy. First, we found the GO terms associated with each MANGO heterologous gene and selected those terms that could be related to different steps of the neurogenic process (proliferation, differentiation, migration, chemotaxis, survival and development). The MANGO heterologous genes encompassed 57 different neurogenesis-related GO terms, such as growth factor activity (GO:0008083), nervous system development (GO:0007399), learning (GO:0007612), memory (GO:0007613), cell proliferation (GO:0008283), cell differentiation (GO:0030154), neuron development (GO:0048666), etc. Next, we found in our arrays the genes with differential expression between naïve and microglia that were associated to each neurogenic GO term, generating 57 gene lists in our arrays. As each gene could be classified under more than one neurogenesis-related GO term, we next double crossed the 57 gene lists to obtain a single list which comprised 1,669 non-repeated neurogenic genes.

At this point, our neurogenic gene list from the array contained both heterologous and autologous genes; therefore, in order to select only the heterologous genes, we again double-crossed the neurogenic gene list with two gene lists obtained by the search of the GO terms extracellular region (GO:0005576) and extracellular space (GO:0005615), and obtained 281 neurogenic heterologous genes in our arrays. Finally, genes that encoded receptors were discarded manually with the help of Protein Analysis Through Evolutionary Relationships (PANTHER, http://pantherdb.org/), a comprehensive software system for inferring the functions of genes based on their evolutionary relationships. In addition PANTHER also provides information about each gene, allowing us to discard 57 genes that encoded receptors [Note: it is coincidental that 57 genes were discarded and that 57 gene lists were generated from the GO terms contained in MANGO]. In order to validate our strategy, we checked the 26 heterologous MANGO genes in our screened neurogenic list and found that all had been selected, confirming our methodology as a valid filtering approach to discover heterologous neuroregulatory genes. Therefore, we finally selected 224 genes with differential expression

between naïve and phagocytic microglia, which were heterologous, non-receptors, and whose function had been previously involved in neurogenesis. The 224 neurocandidate genes were classified according to the main regulatory expression patterns: 94 genes UP, 73 DOWN, 28 Transient-UP and 29 Transient-DOWN **(Figure 14)**.
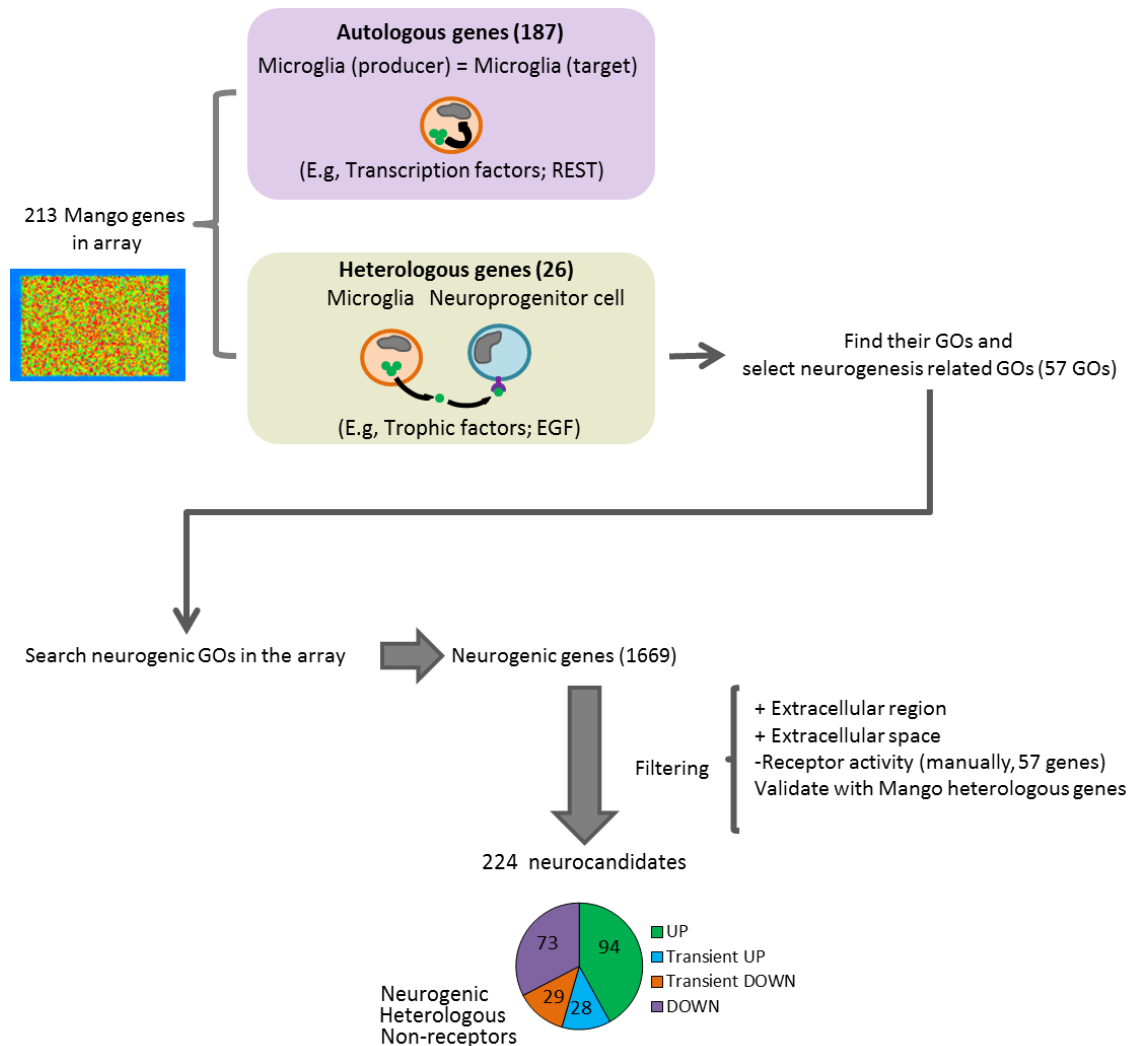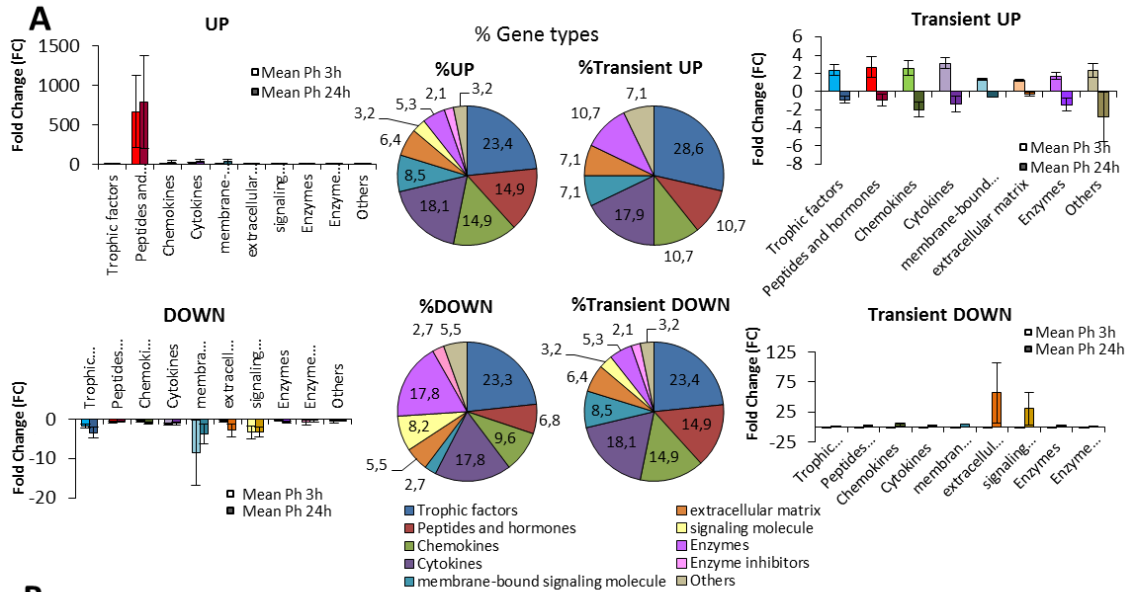

Figure 14. Phagocytic microglia express several potential modulators of neurogenesis. Diagram depicting the strategy followed to search for potential modulators of neurogenesis produced by phagocytic microglia in the arrays. The filtering started by differentiating the heterologous and autologous genes in the MANGO database. Then, GO terms related to neurogenesis were selected for the heterologous MANGO genes. Afterwards, the molecules that presented the neurogenic GO terms were searched in the array. Finally, the genes were filtered only to select those that appeared extracellularly (heterologous genes), and genes with receptor activity were discarded.

6.4.2. The potential modulators of neurogenesis are mainly peptides and hormones

After identifying the 224 neurocandidate genes, we performed a further classification to determine their nature. Using again PANTHER, the neurocandidates were grouped according to Panther Protein Class, which gives information about the type of protein that encoded by a gene (for example, growth factor, chemokine, membrane-bound signaling molecule, etc.). Ten different categories were established: trophic factors, peptides and hormones, chemokines, cytokines, membrane-bound signaling molecules, extracellular matrix, signaling molecules, enzymes, enzymes inhibitors, and others. We found that in the four regulation patterns, the majority of the genes were categorized as trophic factors (between 23-29% in all regulatory patterns). We also found cytokines, chemokines and peptides and hormones as the main gene types of the neurocandidates. Despite the fact that trophic factors were found to be the largest percentage in each regulation pattern, they showed a similar and rather lower mean FC (4.7 ± 1.2% in UP, 3.5 ± 1.4% in DOWN, 2.4 ± 0.6% in Transient-UP and 1.6 ± 0.2% in Transient-DOWN) than the other categories. Only the up-regulated peptides and hormones revealed a large mean of 800 FC at both 3h and 24h of phagocytosis **(Figure 15A, B)**. This data suggests that peptides and hormones are the most likely molecules to perform neurotrophic functions in the neurogenic niche by phagocytic microglia.

Figure 15. Phagocytosis-related neurocandidates include trophic factors and peptides and hormones.

[A] A further classification of the 224 potential modulators of neurogenesis. 'Trophic factor' is the category with the highest percentage of genes in every regulatory pattern, however, the category

*'Peptides and Hormones' includes genes with the highest FC changes in the UP regulation pattern. **[B]** The 224 neurocandidates classified by their identity and FC.*

6.4.3. The validation of phagocytosis-related neurocandidates confirms the mRNA expression of several potential modulators of neurogenesis triggered by microglial phagocytosis

Next, we validated the mRNA expression of the neurocandidates in naive and phagocytic microglia by RT-qPCR. We selected a subset of genes for validation considering both the high FC obtained in the array and/or the well-known neuromodulatory potential described in the literature: VGF (non-acronym), Cartpt (cocaine- and amphetamine-regulated transcript prepropeptide), FGF2 (fibroblast growth factor), VEGFa (vascular endothelial growth factor A), PDGFa (platelet-derived growth factor A), IGF-1 (insulin growth factor 1), MMP3 (matrix metallopeptidase 3), Jag1 (jagged 1), CSF3 (colony stimulating factor 3), IL-1β (interleukin-1β), IL-6 (interleukin-6), TNF-α (Tumor necrosis factor-α), and TGF-β (transforming growth factor-β). We also included some classical trophic factors (Brain Derived Neurotrophic Factor, BDNF; Epidermal Growth Factor, EGF; Glial Cell Derived Neurotrophic Factor, GDNF; Nerve Growth Factor, NGF; and Neurotrophin 4, NT4) that were not detected in the array, probably due to any technical problem that lead to missing values of the probes in the array.

We found that the expression pattern of the selected neurocandidates determined by RTqPCR was largely in agreement with that obtained in the arrays. Among the genes with the largest mRNA expression were the neuropeptide VGF (no abbreviation), the matrix metalloprotease 3 (MMP3), and the cytokine colony stimulating factor 3 (CSF3) **(Figure 16A, B)**. Some of the high FC genes from the array (Secretogranin II, SCG2; Delta-like4, Dll4; and Delta-like3, Dll3) were not significantly changed, while some of the added classical trophic factors (BDNF, NT4, and NGF) were not detected by RT-qPCR either **(data not shown)**. This validation of neurocandidates reinforced the idea that phagocytosis promotes the production of neurotrophic molecules by microglia.
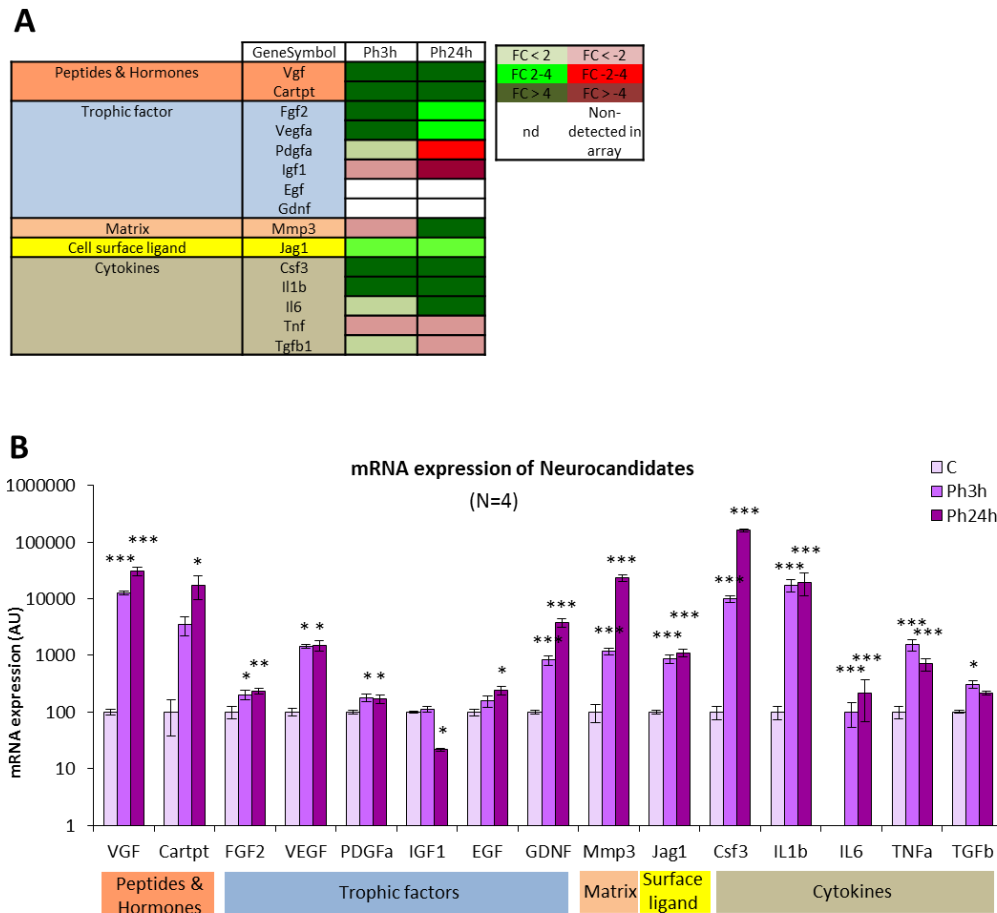
Figure 16. Validation of phagocytosis-related neurocandidates by RT-qPCR. *[A] Summary of the FC in the array of the neurocandidate genes selected for validation. Up-regulated FCs are displayed in green and down-regulated FCs in red. Different color intensities show ranges of FC: <2FC (light), 2-4FC (medium), >4FC (dark). Those genes not detected in the array have no color. [B] mRNA expression levels of the neurocandidates selected for validation by RT-qPCR. N=4. HPRT was selected as a reference gene. Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA was significant at p < 0.05). Only significant effects are shown.*

6.4.4. The increase of potential modulators of neurogenesis by phagocytic microglia is also triggered by physiological levels of apoptotic cells and even before the engulfment of apoptotic cells

Up until now, we had worked with the most classically used in vitro phagocytosis assay in which microglia were fed with a large amount of phagocytic targets (10:1 apoptotic cells to microglia (Witting et al., 2000). Since in physiological conditions in adult mice microglia is not challenged with such high rates of apoptotic cells, we switched our assay to a more physiological paradigm; thus, we varied the proportion of apoptotic cells to 1:1. In this model,

we first tested whether the features of the 10:1 model were conserved. We fed primary microglia with apoptotic SH-SY5Y and allowed them to phagocytose through a time course of 1, 3, 5, 15 and 24h **(Figure 17A, B)**. In the 1:1 model, microglia showed a very similar response compared to the previous paradigm. As early as 1h, 49.2 ± 2.7% of microglia were already engaged in phagocytosis, and as expected, this percentage kept increasing over time reaching 92.2 ± 4.5% of phagocytosis at 24h **(Figure 17C)**. A further analysis of the different engulfed particles (DAPI, CM-DiI or DAPI and CM-DiI) revealed that the proportion of each inclusion type varied throughout the time course. At early time points, more and larger DAPI and CM-DiI particles were observed, whereas at later time points, as apoptotic cells degrade within microglia, small and numerous CM-DiI inclusions were detected **(Figure 17D)**. There was a small decrease in microglia cell numbers along the time course **(Figure 17E)**, which was in accordance with a small increase in microglial apoptosis. Microglial apoptosis never reached more than the 6.9 ± 4.8 % of the total **(Figure 17F)**.

We also validated the expression of the neurocandidates by RT-qPCR in the 1:1 model **(Figure 17G)**. The neuropeptide VGF, metalloprotease MMP3, and cytokine CSF3 were some of the genes with largest increase in mRNA expression. Overall, phagocytosis induced similar changes in neurocandidates regardless of the amount of apoptotic cells. In addition, we delved into the requirements for triggering the neurogenic program, whether a physical contact between microglia and apoptotic cells (tethering and engulfment) was necessary or whether the program started in microglia as soon as they sensed the find-me signals released from apoptotic cells. For this purpose, we also added media obtained from apoptotic cells to naïve microglia and analyzed the mRNA expression of neurocandidates after 24h **(Figure 17G)**. We found that candidates from extracellular space (matrix and cell-surface) and cytokines were highly up-regulated after culturing microglia with apoptotic cell media. The obtained mRNA levels in this group were very similar to those at 24h of phagocytosis (Ph24h). However, the majority of 'peptides and hormones' and 'trophic factors' were not significantly changed. These results suggest that the neurogenic program triggered by microglial phagocytosis might be initiated by the detection of find-me signals, but further steps in the phagocytic process are required to develop a complete neurogenic program.
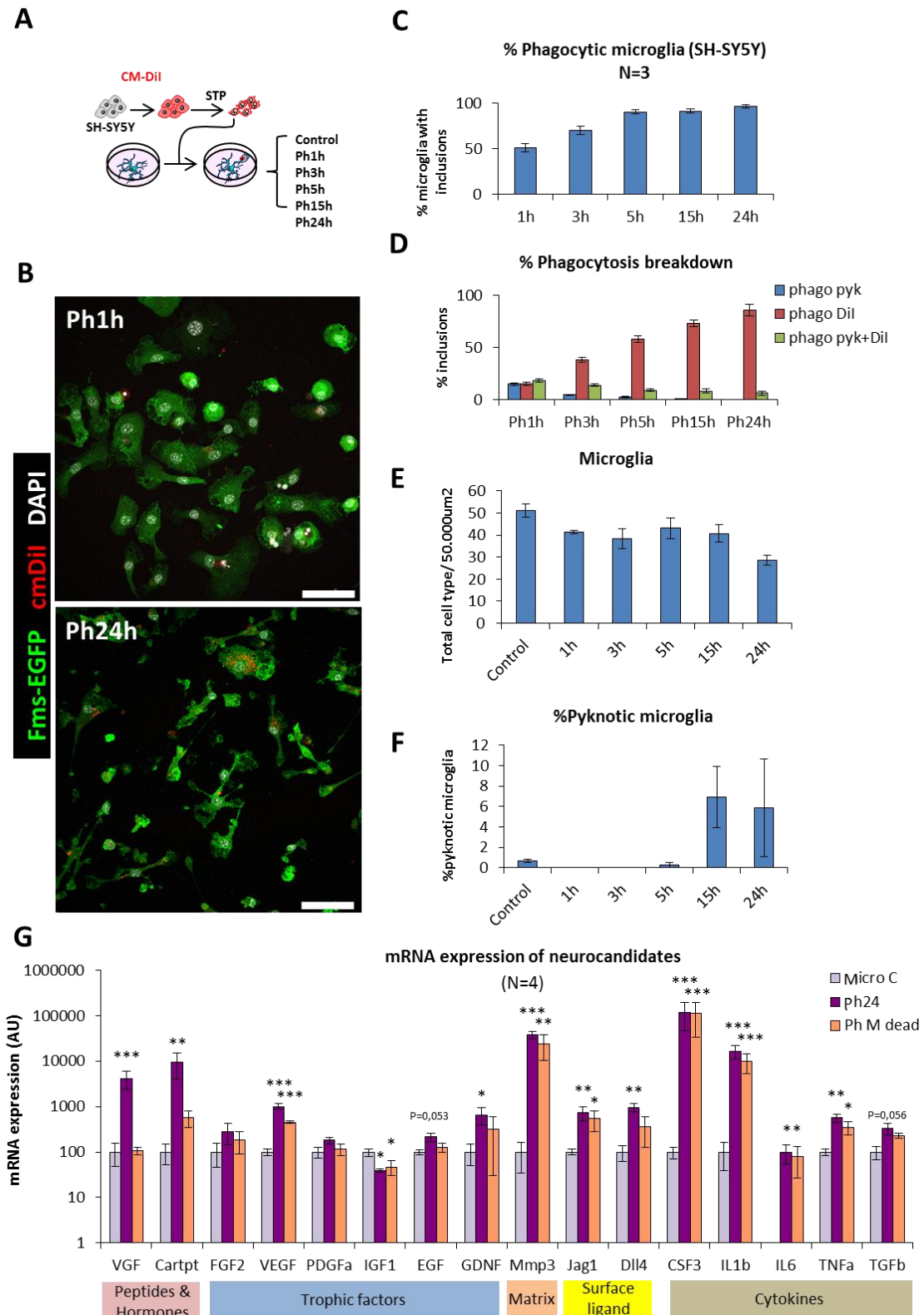
Figure 17. Validation of a physiological model of phagocytosis using a 1:1 proportion of apoptotic cells and microglia. *[A] Experimental design of the phagocytosis assay. [B] Representative confocal microscopy images of primary microglia (GFP, green) fed with SH-SY5Y, which were previously labeled with CM-DiI (red) and treated with STP for the induction of apoptosis (pyknosis/karyorrhexis, DAPI, white). N, microglial nucleus; white arrowheads, phagocytosed apoptotic SH-SY5Y cells; blue*

137

arrowheads, phagocytosed CM-DiI; scale bars, 30μm. z=6.3 μm. **[C]** *Quantification of the percentage of microglia with CM-DiI and/or DAPI inclusions along a time course. Only fully closed pouches with particles within were identified as phagocytosis.* **[D]** *Breakdown of the percentage of each inclusion type (DAPI, CM-DiI or DAPI and CM-DiI).* **[E]** *Quantification of average microglia density in control and different phagocytic time points.* **[F]** *Quantification of the percentage of pyknotic microglia. N=3. Bars represent mean ± SEM.* **[G]** *mRNA expression levels of the neurocandidates selected for validation by RT-qPCR in control microglia (microC), 24h phagocytic microglia (Ph24h) and microglia cultured with apoptotic cell media (Ph M dead). N=4 independent experiments. HPRT was selected as a reference gene. Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA was significant at p < 0.05). Only significant effects are shown.*

6.5. CONDITIONED MEDIA FROM PHAGOCYTIC MICROGLIA AND NAÏVE MICROGLIA DRIVE NEUROPROGENITOR CELLS TOWARDS DIFFERENT PHENOTYPES

Once we had validated that phagocytosis triggers the increase in the expression of several potential modulators of neurogenesis, we tested the effect of microglial phagocytosis-related neurocandidates on neurogenesis in vitro. To model neurogenesis in vitro, we used neurospheres obtained from whole P0-P1 brains. Neuroprogenitor cells from disaggregated neurospheres were allowed to proliferate 48h in DMEM/F12 with trophic factor EGF/FGF2 (Babu et al., 2011). We performed a neurogenesis differentiation assay in which neuroprogenitors were allowed to differentiate during a time course (1-5 days) in the presence of conditioned media from control (naïve microglia; CM microC) and phagocytic (CM microPH) microglia. DMEM was used as an internal control, as microglia were cultured in this media **(Figure 18A, B)**. Cell death (apoptosis) was observed in all conditions, but higher rates of apoptosis were found in neuroprogenitor cells cultured in CM microPH, with a peak of 57.1 ± 10.7% apoptosis at 3d of differentiation **(Figure 18C, D)** that diminished to 34.8 ± 8.3% at 5d **(Figure 18E, F)**. On the contrary, apoptosis was maintained at both 3d and 5d and never reached more than 10.2 ± 3.8% in CM microC and DMEM **(Figure 18D-F)**. However, apoptosis was quantified as pyknotic and karyorrhectic nuclei attached to the coverslips, therefore, total cell numbers do not match between different conditions because cells that were detached prior to fixation were not included, which led to an underestimation in the proportion of apoptotic cells in every condition **(Figure 18C, E)**.
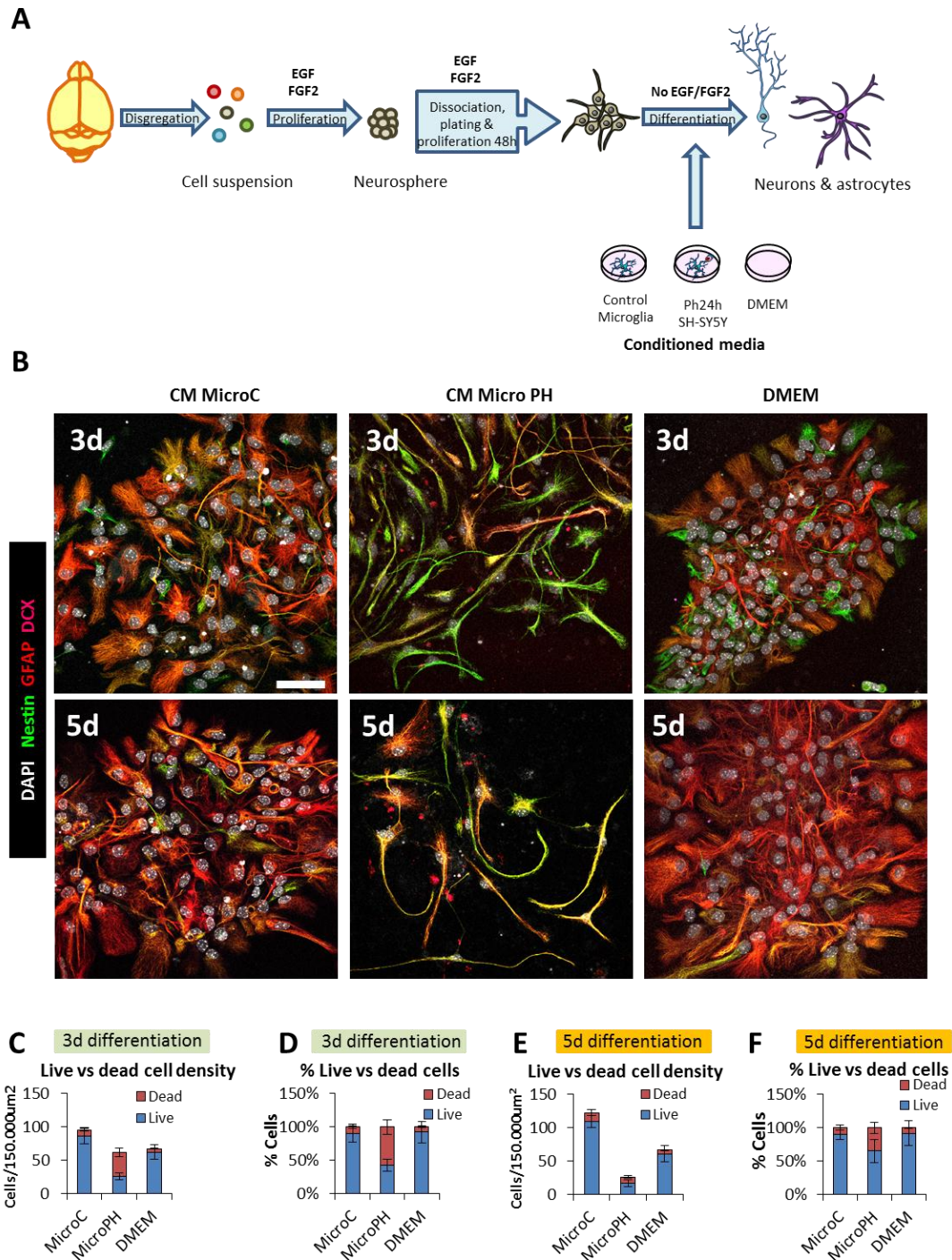
Figure 18. Effect of phagocytic microglia secreted factor on neurogenesis in vitro. **[A]** *Experimental design of the in vitro neurogenesis assay.* **[B]** *Representative confocal microscopy images of neuroprogenitors treated with CM microC or microPH. DMEM was used as control. Scale bars, 20µm. z=9 µm.* **[C]** *Total numbers of live and dead cells (determined by pyknosis/karyorrhexis) after CM treatment in 3d of differentiation.* **[D]** *Proportion of live and dead cells (determined by pyknosis/karyorrhexis) after CM treatment in 3d of differentiation.* **[E]** *Total numbers of live and dead cells (determined by pyknosis/karyorrhexis) after CM treatment in 5d of differentiation.* **[F]** *Proportion of live and dead cells (determined by pyknosis/karyorrhexis) after CM treatment in 5d of differentiation. Bars represent mean*

± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA was significant at p < 0.05).

We next analyzed the cell types produced in the neurogenesis differentiation assay. We observed 4 major types of cells based on morphology and marker expression: bipolar, stellate, early-ramified, and late-ramified **(Figure 19A)**. Unlabeled nuclei and cells with unclear morphology were not taken into account for the quantification (14.6 ± 4.1%).

1. Bipolar cells presented an elongated shape and expressed high levels of nestin, an intermediate filament protein that is involved in proliferation and is commonly used as a stem cell marker (Lopez-Atalaya et al., 2017). They also expressed GFAP (glial fibrillary acid protein), another intermediate filament protein involved in migration and proliferation, which is generally used to distinguish astrocytes from other cell types, although is also expressed in the radial process of NSCs (Encinas and Sierra, 2012; Moeton et al., 2016). However, none of them expressed DCX (doublecortin), a microtubule-associated protein implicated in neuroblast migration (Brown et al., 2003).

2. Stellate cells displayed a starry morphology with or without processes; they expressed high levels of GFAP, but showed variable levels of nestin and never presented DCX staining.

3. Ramified cells had very thin processes and never expressed GFAP. Early ramified cells were less complex, expressed nestin, and could also express low levels of DCX; in contrast, late-ramified cells exhibited more process complexity. Some of the late-ramified cells had already lost nestin expression and all of them had high DCX expression.

After identifying the different cell types, we quantified total numbers and the proportion of each of the cell types in the different CM media **(Figure 19B-E)**. We found that CM microC treatment mainly produced stellate cells both at 3d and 5d (73.60% ± 6.90% and 69.81% ± 2.18% respectively). Control (DMEM) conditions showed very similar results to CM microC, as stellate cells were the main phenotype (54.4 ± 2.9% at 3d). On the contrary, in CM microPH cultures, 72.8% ± 4.0% and 79.1% ± 8.9 cells were bipolar, whereas 14.9% ± 4.5% and 8.7% ± 2.7% were stellated at 3d and 5d of differentiation, respectively. Therefore, we concluded that CM microC mainly drove cells towards a stellate phenotype (GFAP^{high}, nestin$^{+/-}$, DCX$^-$) as well as ramified DCX$^+$ cells, whereas CM microPH drove cells towards a bipolar phenotype (nestin^{high} GFAP$^+$, DCX$^-$).

Taking the morphology and the cell markers into account, stellate cells resemble to astrocytes in culture, whereas ramified DCX$^+$ cells would belong to immature neuronal lineage.

However, the identity of bipolar cells remained elusive and thus, we performed further studies to characterize this phenotype.
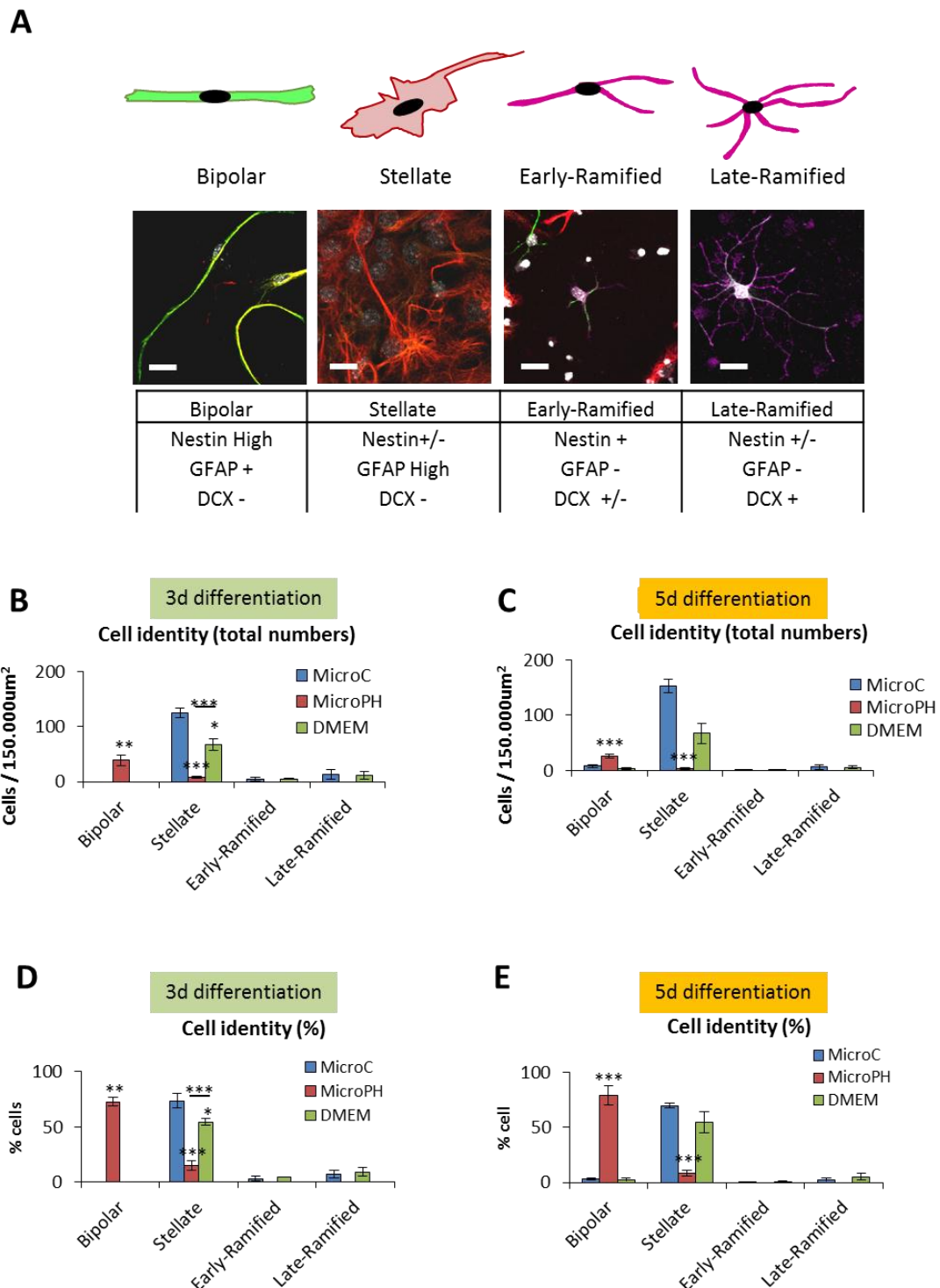
Figure 19. CM microC and microPH give rise to different cell phenotypes in vitro. *[A] Schematic shapes, representative confocal microscopy of the different morphologies observed in the neurogenesis assay images and a summary of the markers expressed by each cell type. [B] Quantification of total numbers of the different cell types found after 3d treatment with CMmicroC or microPH. [C] Quantification of the*

141

proportion of the different cell types found after 3d treatment with CM microC or microPH. [D] Quantification of total numbers of the different cell types found after 5d treatment with CM microC or microPH. [E] Quantification of the proportion of the different cell types found after 5d treatment with CM microC or microPH. Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA was significant at p < 0.05).

6.5.1. Identifying the nature of the bipolar phenotype triggered by conditioned media from phagocytic microglia

In order to understand the identity of the bipolar cells produced by CM microPH, we performed several studies to characterize their phenotype. Since bipolar cells expressed nestin and GFAP but never showed DCX staining, we considered that these cells could be either astrocytes or still be progenitor cells. Therefore, we followed different strategies in order to prove this hypothesis.

6.5.1.1. Bipolar cells triggered by conditioned media from phagocytic microglia are likely to be immature astrocytes

First, we tested whether bipolar cells could be mature astrocytes. Since GFAP staining did not provide sufficient information on the maturity levels of astrocytes, as it labels both stem cells, mature astrocytes, and reactive astrocytes (Sierra et al., 2015), we stained the CM-treated cultures with S100β, a marker of mature astrocytes and oligodendrocytes (Wang and Bordey, 2008) **(Figure 20A)**.

We found that in CM microC very few stellate cells (Nestin$^{+/-}$, GFAP^{High}) were S100β$^+$ (0.7 ± 0.7% and 4.6 ±1.3% at 3 and 5d, respectively). However, the labeling in these cells was notorious but rather dim, suggesting that stellate cells were still immature astrocytes **(Figure 20B, C)**. In addition, we also found that 3.1 ± 1.3% and 1.6 ± 0.3% of cells at 3 and 5d, respectively were stained only by S100β but not by Nestin or GFP. Therefore, these cells were included in the unlabeled nuclei in the first differentiation experiment **(Section 5)**. These cells expressed high levels of S100β and had several processes with smaller branches in each of them. Due to their well-defined morphology and expression marker, we identified these cells as oligodendrocytes (Wang and Bordey, 2008). On the other hand, in CM microPH, no oligodendrocytes were found and 64.0 ± 24.0% at 3d and 45.4 ±7.9% at 5d of bipolar and 91.7 ± 8.3 at 3d and 63.3 ± 3.3% at 5d of stellate cells presented a very dim S100β staining (all the pictures were compared to a negative control without primary antibody in the

immunofluorescence). Results with DMEM were very similar to those obtained with CM microC **(Figure 20B, D)**. Therefore, the faint S100β expression in bipolar cells triggered by conditioned media from phagocytic microglia suggested that they could either be immature astrocytes or neuroprogenitors.
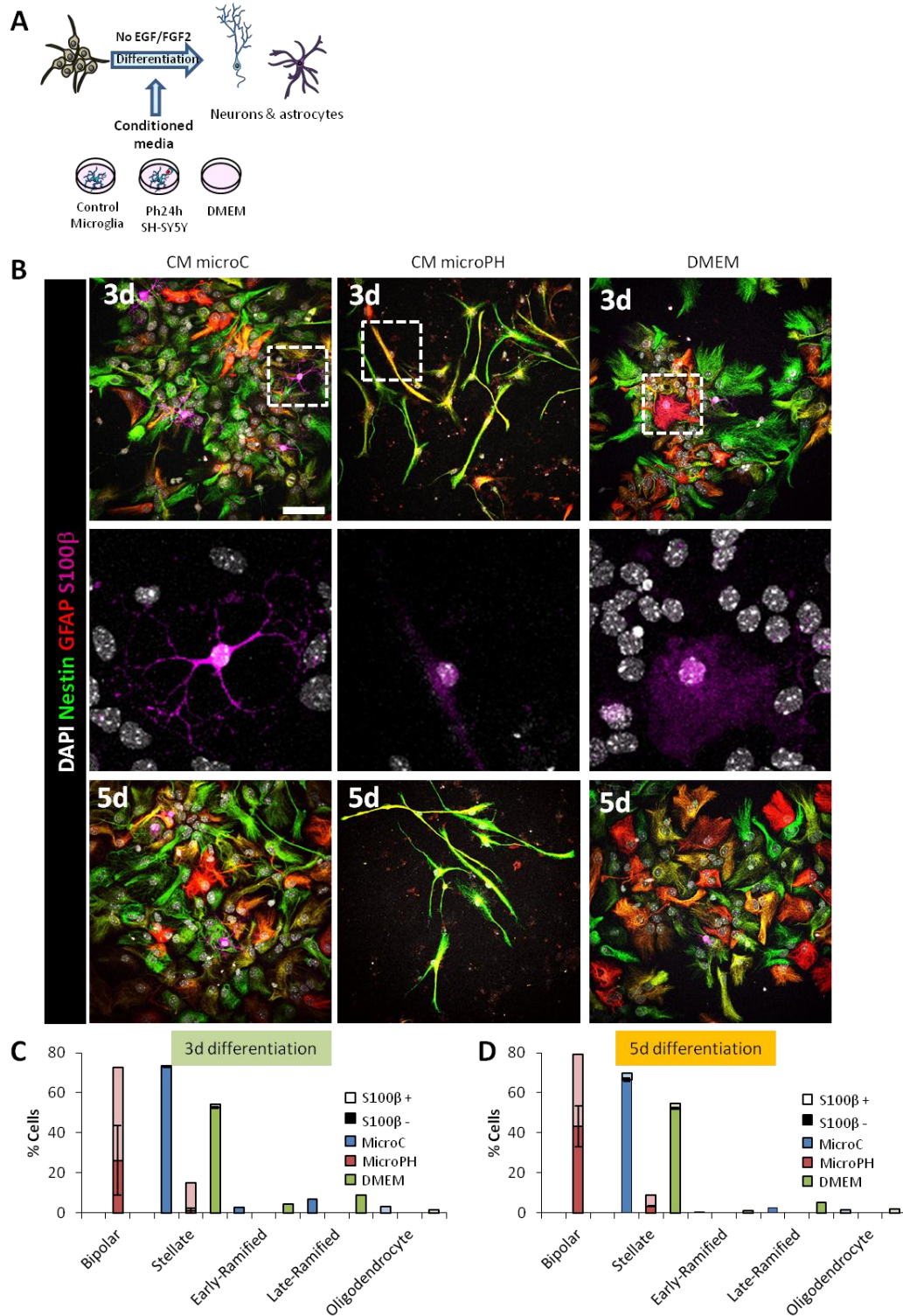
Figure 20. S100β expression in CM cell types. **[A]** *Experimental design of the in vitro neurogenesis assay for S100β staining.* **[B]** *Representative confocal microscopy images of neuroprogenitors treated with CM*

microC or microPH. DMEM was used as control. Scale bars, 20µm. z=9 µm. [C] Quantification of the proportion of S100β in the different cell types found after 3d treatment with CM microC or microPH. [D] Quantification of the proportion of S100β in the different cell types found after 5d treatment with CM microC or microPH. N = 2 independent replica. Bars represent mean ± SEM.

6.5.1.2. Bipolar cells triggered by conditioned media from phagocytic microglia are unlikely to be pluripotent

As mentioned above, bipolar cells expressed high amounts of nestin and GFAP. Hippocampal NSCs also display these markers in vivo (Encinas and Sierra, 2012) and thus, it was possible that bipolar cells represented an undifferentiated phenotype. In order to answer this question, we hypothesized that if bipolar cells were an undifferentiated form of neuroprecursors, they would possess the potential to give rise to neurons and astrocytes. Therefore, we differentiated the neuroprogenitor cells into stellate, ramified, and bipolar cells using CM microC and microPH for 3d. We then studied the pluripotency of these phenotypes by switching the culture media to DMEM/F12 culture media (the regular media to grow neurospheres) without trophic factors. We allowed cells to differentiate for another 5d and 9d. Treatments with CM 3d were always performed in parallel as control **(Figure 21A, B)**.

We found differences in cell density between the cultures that had been treated with CM microPH and CM microC or DMEM **(Figure 21C)**. After the treatment with 3d CM microC or DMEM followed by 5d DMEM/F12 the cultures presented a density of 99.3 ± 9.3 and 104.5 ± 7.0 of live cells respectively, a density that was maintained over time (9d). In contrast, culturing with 3d CM microPH followed by 5d DMEM/F12, live cells were at a density of 11.8 ± 0.3 cells. The differences in cell density among treatments were the result of the culturing with the different CMs, as observed in **Figure 19**, and those differences were maintained after the switch to the DMEM/F12 media. We then analyzed the cell types resulting from differentiation after switching to DMEM/F12 in the pluripotency assay. The cultures that had been treated with CM microC presented a large proportion of stellate cells both at 5d (78.9 ± 1.1%) and 9d (92.8 ± 3.4%) and a small percentage of ramified cells (14.8 ± 0.9% at 5d; 3.8 ± 0.1% at 9d) **(Figure 21D, E)**. Interestingly, after CM microPH treatment followed by DMEM/F12 we found that now the proportion between bipolar and stellate cells was inverted compared to the neurogenesis assay **(Figure 19)**. We found a majority of stellate cells after 5d of DMEM/F12 (57.4 ± 0.9%) and 42.6 ± 0.9% of bipolar cells, percentages that were maintained until 9d of DMEM/F12 **(Figure 21D, E)**. No DCX[+] cell was found after CM microPH followed by DMEM/F12 treatment. This data strongly suggested that bipolar cells are unlikely to still be

neuroprogenitor cells since they only give rise to astrocytes and never to neuron-committed cells.

These results, along with the S100β data, led us to perform a further characterization of the cells using calcium imaging to study the calcium response of these cells to certain stimuli, as we will explain in the next sections.
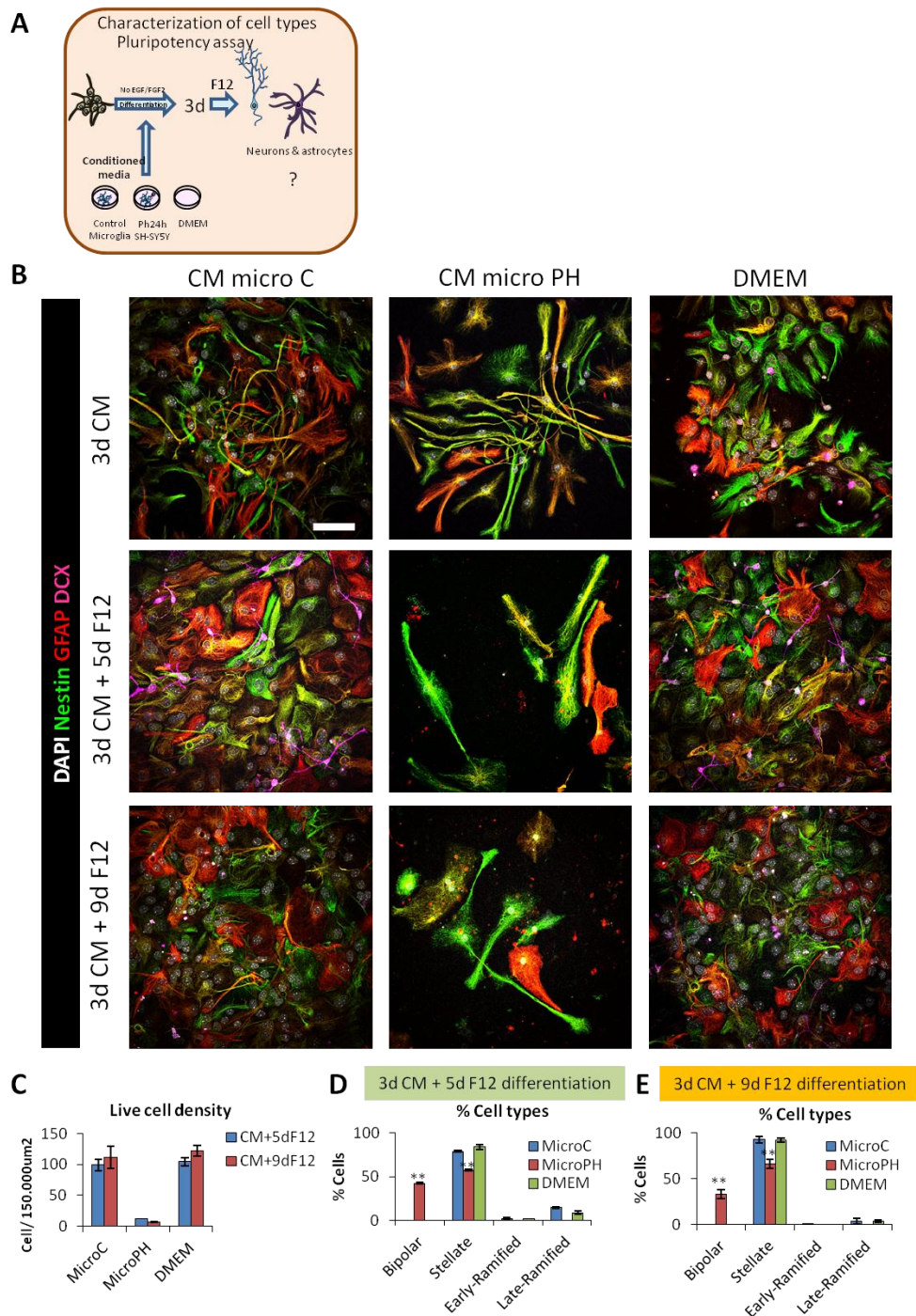
Figure 21. Pluripotency assay in CM cell types. [A] *Experimental design of the in vitro pluripotency assay.* **[B]** *Representative confocal microscopy images of neuroprogenitors treated with CM microC or*

145

microPH followed by 5 or 9d of DMEM/F12. DMEM was used as control. Scale bars, 20µm. z=9 µm. **[C]** *Quantification of the cell density after each treatment* **[D]** *Quantification of the proportion of the different cell phenotypes found after 3d treatment with CM microC or microPH followed by 5d DMEM/F12.* **[E]** *Quantification of the proportion of the different cell phenotypes found after 3d treatment with CM microC or microPH followed by 9d DMEM/F12. N = 2 independent replica. Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA was significant at p < 0.05).*

6.5.1.3. Bipolar cells triggered by conditioned media from phagocytic microglia belong to an astrocytic linage

In order to further unravel the identity of bipolar cells, we characterized their calcium response to different insults: KCl, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), ATP (adenosine triphosphate), histamine, and NMDA (N-methyl-D-aspartate). KCl would trigger an intracellular Ca^{+2} response in excitable cells; AMPA and NMDA would depolarize neurons expressing the corresponding receptors (Bloodgood and Sabatini, 2008); ATP would activate puringeric receptors in astrocytes and neurons (De Melo Reis et al., 2011); and histamine would trigger intracellular Ca^{+2} response in immature cells through histamine receptor, which are highly expressed on immature/stem cells and embryonic stem cells (Eiriz et al., 2011). In order to visualize the Ca^{+2} response of the different cells, CM-treated neuroprogenitors were incubated and loaded with 5µM Fura-2 AM, a radiometric compound that changes its conformation upon Ca^{+2} binding and consequently changes its maximum excitation from 340nm to 380nm. Freshly dissociated neuroprogenitor cells were used to characterize the responses of neuroprogenitor cells to the different insults used and compare with the Ca^{+2} responses of bipolar cells **(Figure 22A)**. In total, 38 stellate cells, 8 ramified cells, 19 bipolar cells and 33 neuroprogenitor cells were analyzed in 2 independent experiments. We found that 69.7% neuroprogenitor cells responded to ATP and histamine and presented a hyperpolarization to KCl. In CM microC treated cultures (5d), 76.3% of stellate cells depolarized in response to KCl, AMPA and ATP, and 62.5% of ramified cells responded to every stimuli but NMDA, most likely because they were still immature astrocytes **(Figure 22B,C)**. On the other hand, in CM microPH treated cultures, 89.5% of bipolar cells highly depolarized when incubated with ATP, and, in addition, they also presented a hyperpolarization in response to KCl **(Fig 22B,C)**. These data showed that bipolar cells presented similarities with both neuroprogenitor cells and astrocytes, suggesting that bipolar cells are immature astrocytes.
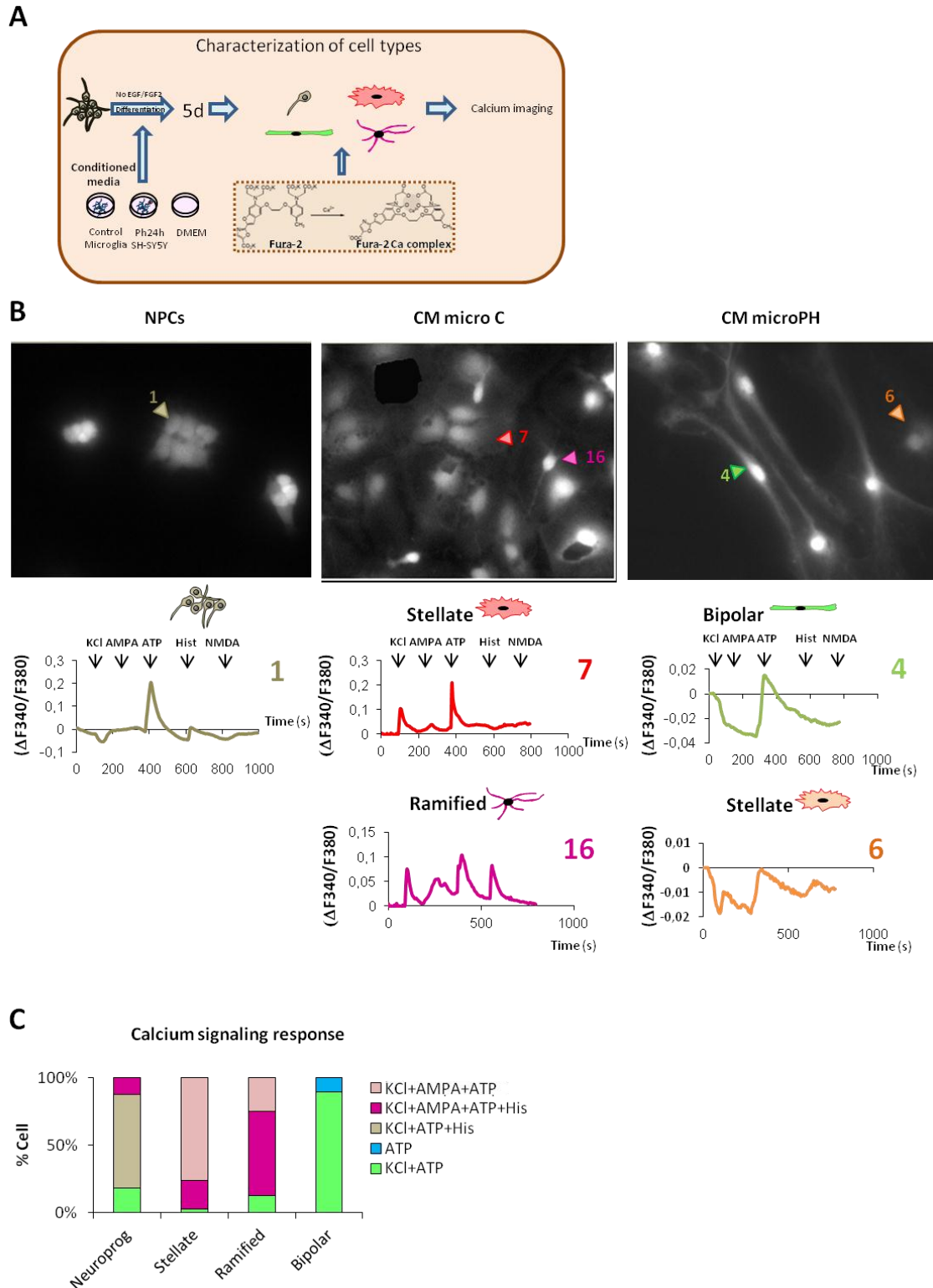
Figure 22. Calcium imaging in CM cell types. *[A] Experimental design of the in vitro calcium imaging assay. Neuroprogenitor cells were treated with CM microC or microPH for 5d and the resulting stellate, ramified and bipolar cells were incubated and loaded with Fura-2 AM and afterwards, cells were challenged with KCl, AMPA, ATP and Histamine in order to measure their Ca^{+2} response. [B] Representative epifluorescence microscopy images of neuroprogenitors treated with CM microC or*

147

*microPH for 5d. Freshly dissociated neuroprogenitor cells were used as control. **[C]** Quantification of the proportion of the different cell phenotypes responding to each stimulus. The baseline was calculated as the mean of the first 60sec of recording for each cell. Only peaks that increase or decrease 3 times the SEM of the baseline were considered as response. N = 2 independent replica.*

6.5.2. Identification of the molecules that drive the bipolar phenotype triggered by phagocytic microglia conditioned media

6.5.2.1 Cytokines are unlikely to drive the bipolar phenotype triggered by phagocytic microglia conditioned media

After characterizing bipolar cells as astrocyte-committed cells, we next focused on the identity of the molecules that could give rise to this phenotype. As we observed in the arrays **(Figure 23)**, phagocytic microglia produce several cytokines, such as Csf3, IL-1β, IL-6, TNF-α, or TGF-β among others. In addition to phagocytosis, these molecules are also released by microglia upon an inflammatory stimulus and have already been reported to affect neurogenesis (Sierra et al., 2014; Valero et al., 2017). Thus, we focused our attention on these cytokines as the possible mechanism that drives the bipolar phenotype.

Since cytokine expression after phagocytosis shares similarities with their expression in inflammation, we compared the phenotypes obtained from CM microPH, with the ones obtained from CM of microglia treated with LPS, a well-known inductor of inflammation and cytokine release (Cavaillon, 2017).

First, we studied the mRNA expression of the cytokine neurocandidates by RT-qPCR in naïve, Ph24h or LPS-treated (150ng/ml;(Fraser et al., 2010)) microglia. Moreover, we added a group of cultures of Ph24h followed by LPS in order to determine whether microglial phagocytosis could alter the cytokine production induced by LPS exposure (Ph+LPS) **(Figure 23A)**. We found that Csf3, IL-1β and IL-6 presented significantly higher mRNA expression in all the treatments (Ph24h, LPS or Ph+LPS) compared to the control group. On the other hand, TNF-α showed a significantly increased expression in every treatment compared to naïve microglia and, additionally, LPS treatment induced higher levels of this cytokine than Ph24h or Ph+LPS. TGF-β mRNA increased after LPS and Ph+LPS treatments compared to the control group.

After determining that both phagocytic and LPS-treated microglia presented similar mRNA expression levels of the neurocandidate cytokines, we treated our neuroprogenitor culture with the LPS treated microglia CM (CM microLPS) to test whether these cytokines could be responsible of the bipolar phenotype found in CM microPH **(Figure 23B)**. LPS was used as control and was maintained for the whole duration of the experiment. The first characteristic that we observed was that neither LPS nor CM microLPS treated neuroprogenitors presented a bipolar phenotype. Moreover, the vast majority of the cells in LPS and CM microLPS treatments, after both 3d (65.5 ± 7.8% in LPS and 63.9 ± 4.7% in CM microLPS) and 5d (75.2 ± 4.4 in LPS and 63.8 ± 9.0% in CM microLPS), presented a stellate phenotype indistinguishable from the stellate phenotype previously found in CM microC and DMEM **(Figure 23C)**. However, DCX$^+$ cells were highly more abundant in LPS and CM microLPS treatments compared to CM microC and DMEM and also showed a shorter and thicker morphology than the ones observed in CM microC. There were no differences between LPS and CM microLPS **(Figure 23D)**. Therefore, CM LPS did not promote the same phenotype as CM microPH, which may suggest that cytokines are not responsible for the effect of CM microPH.

A

mRNA expression of cytokine neurocandidates

B

C

LPS   MicroLPS

D

3d differentiation

5d differentiation
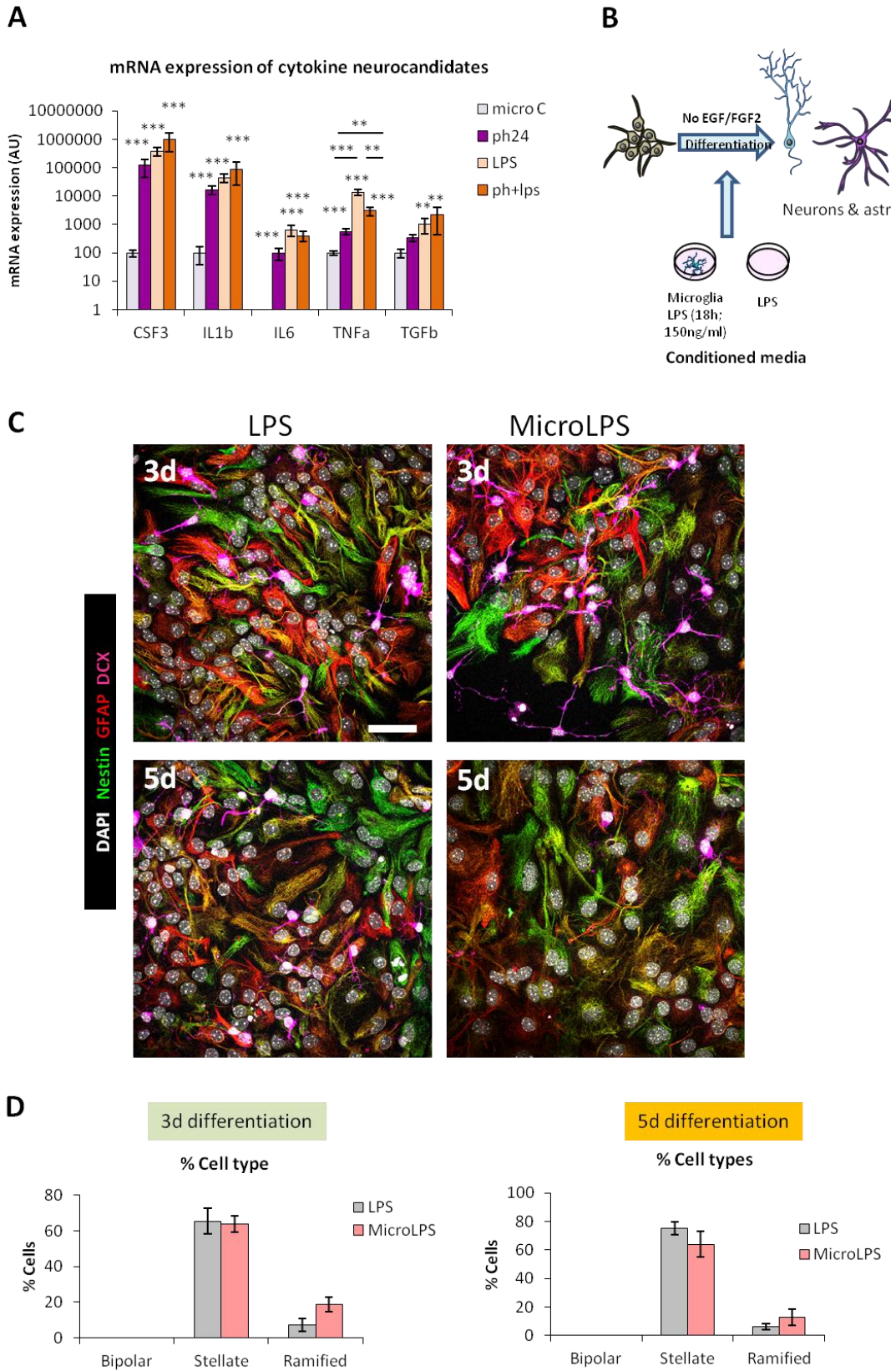
Figure 23. Effect of CM microLPS on neurogenesis in vitro. [A] *mRNA expression levels of the cytokine neurocandidates by RT-qPCR in naïve, phagocytic, LPS treated and phagocytic followed by LPS treated microglia. N=4 independent replica. HPRT was selected as a reference gene. **[B]** Experimental design of*

the in vitro neurogenesis assay. [C] Representative confocal microscopy images of neuroprogenitors treated with CM from LPS treated microglia or LPS (150ng/ml; 18h). N=2 independent replica. Scale bars, 20µm. z=6.3 µm. [D] Quantification of the different cell types found after 3d or 5d treatment with CM from LPS treated microglia or LPS. Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA or Student's t-test was significant at p < 0.05).

Interestingly, the levels of cytokines after phagocytosis were as high as those with LPS and the DCX$^+$ cells were much more abundant after CM microLPS treatment than in CM microC. These were surprising results because pro-inflammatory cytokines are well-documented to exert detrimental consequences for neurogenesis. We first thought that these unexpected results might be due to the low LPS dosage. Therefore, we performed the same experiments with a higher concentration of LPS (1ug/ml; 24h) as described by Monje (Monje et al., 2003), where they found a reduction in DCX$^+$ cells after CM microLPS treatment.

We treated the neuroprogenitor cell culture with CM from microglia incubated with a high concentration of LPS (CM microLPS^{high}) to test whether we could find any LPS dose-dependent differences in CM cell phenotype. Again LPS was used as control and was maintained for the whole duration of the experiment **(Figure 24A)**. Similar to the experiment with the low concentration of LPS, neither LPS^{high} nor CM microLPS^{high} resulted in the formation of bipolar cells **(Figure 24B)**. In addition, the majority of the cell phenotypes after LPS and CM microLPS^{high} treatments were stellate cells (70.7 ± 7.4 in LPS^{high} and 71.9 ± 2.6% in CM microLPS^{high} at 5d), identical to those observed in CM microC and DMEM **(Figure 19)**. Similar to the low dose LPS experiment, ramified cells presented a larger proportion compared to CM microC and DMEM. No differences were found between LPS and CM microLPS^{high} treatments **(Figure 24C)**.

These results strongly suggested again that cytokines were not responsible for the bipolar phenotype induced by CM microPH. However, we found again a higher proportion of DCX$^+$ cells in CM microLPS^{high} than in CM microC (9.9 ± 3.5% and 2.5 ± 1.7% at 5d respectively)), in disagreement with previous findings (Monje et al., 2003). To further analyze this difference, we mimicked Monje's paradigm by switching primary microglia to BV2 (Monje et al., 2003), a microglia cell line, in order to determine whether the differences between our results and the literature might be originated by the use of different cells.
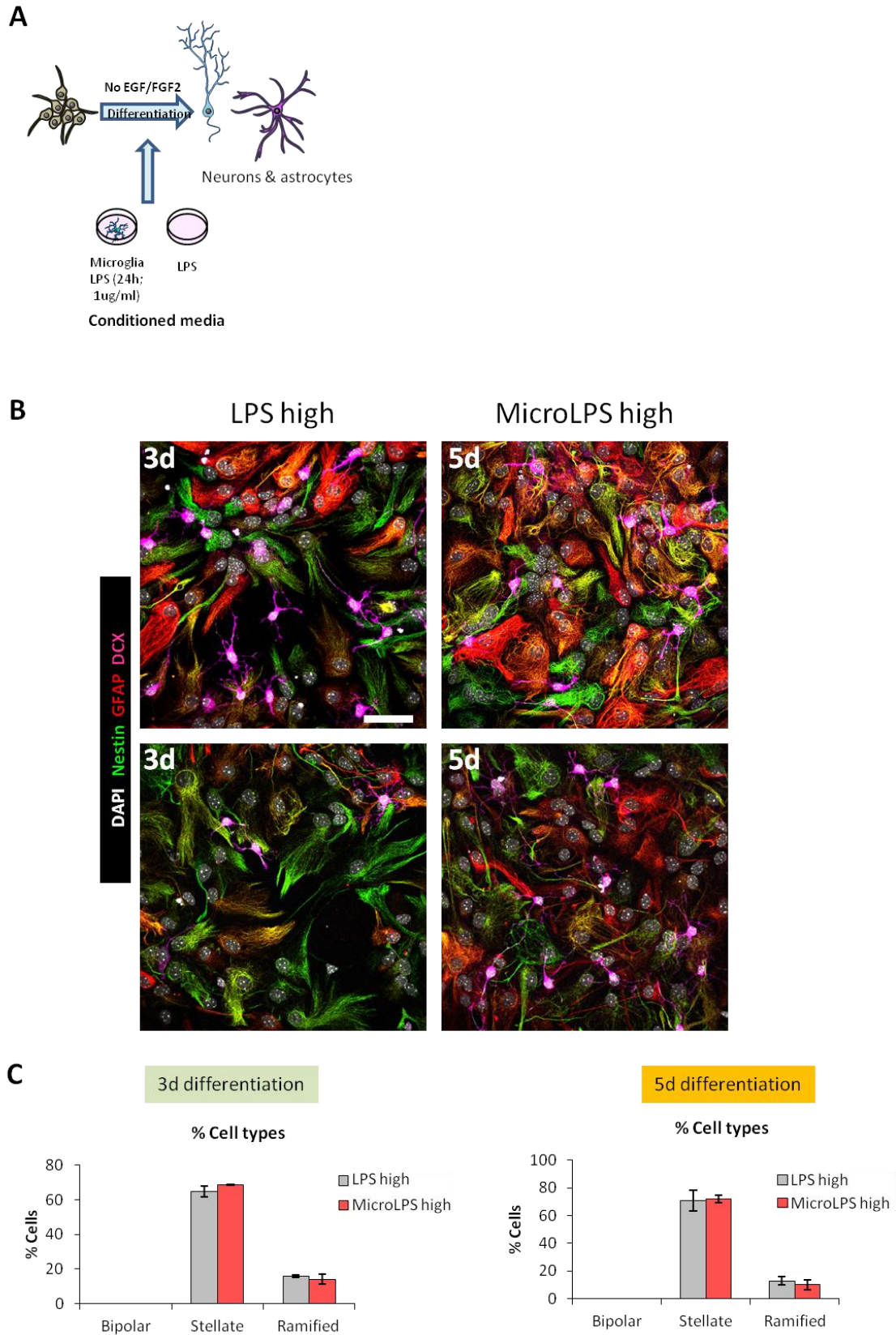
Figure 24. Effect of conditioned media from LPS^high treated primary microglia on neurogenesis in vitro.
[A] Experimental design of the in vitro LPS^high neurogenesis assay. [B] Representative confocal microscopy images of neuroprogenitors treated with CM MicroLPS^high or LPS^high (1µg/ml; 24h). Scale bars,

20µm. z= 7.4µm. **[C]** *Quantification of the different cell types found after 3d or 5d treatment with CM MicroLPS^{high} or LPS^{high}. Bars represent mean ± SEM.*

Thus, we cultured the neuroprogenitors with CM from BV2 cells treated the high concentration of LPS (CM BV2 LPS^{high}) or naïve BV2 CM (CM BV2). LPS^{high} was used as control, and was maintained for the whole duration of the experiment **(Figure 25A)**. Similar to the experiments performed in primary microglia, no bipolar cells were found after the treatment with CM BV2 LPS^{high} or CM BV2 **(Figure 25B, C)**. Moreover, all the treatments induced a large proportion of stellate cells (65.4 ± 2.0% in LPS^{high}; 67.2 ± 0.8% in CM BV2^{high} and 84.2 ± 10.7% in BV2 at 5d) and a smaller proportion of ramified cells. There were no differences among the treatments, but all of them produced a higher proportion of ramified cells than CM microC or DMEM **(Figure 19)**. Therefore, neither by mimicking Monje's (Monje et al., 2003) by increasing the LPS concentration nor by using BV2 cells did we observe the reduction in neuronal-committed cells that they found, suggesting that the effects of inflammation might be more complex than what previously thought, and should be a matter of study in the future.
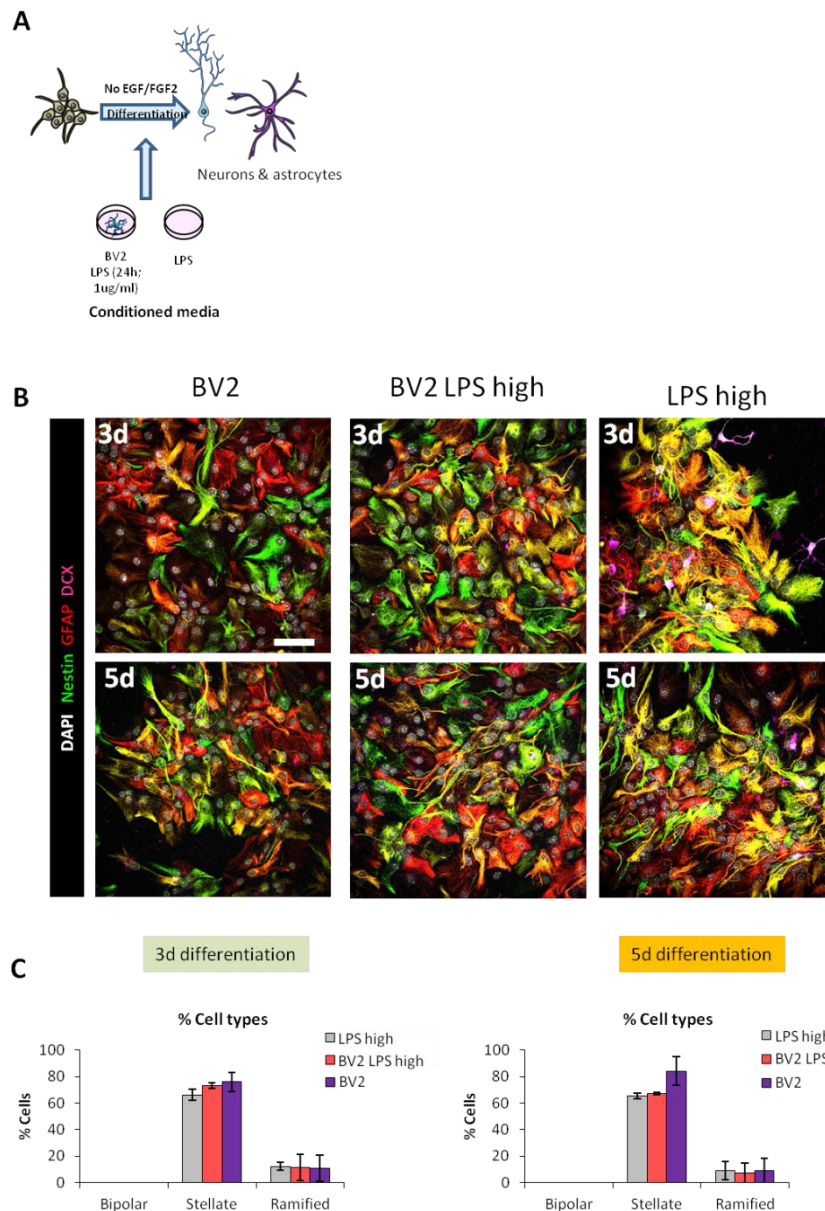
Figure 25. Effect of CM BV2 LPS^{high} on neurogenesis in vitro. *[A] Experimental design of the in vitro CM BV2 LPS high neurogenesis assay. [B] Representative confocal microscopy images of neuroprogenitors treated with CM BV2, CM BV2 LPS high or LPS high (1ug/ml; 24h). Scale bars, 20µm. z= 6.3µm. [C] Quantification of the different cell types found after 3d or 5d treatment with CM BV2, CM BV2 LPS^{high} or LPS^{high}. N = 2 independent replica. Bars represent mean ± SEM.*

In summary, the experiments above suggest that neither LPS per se, nor LPS-induced microglial inflammation differentiated neurospheres into bipolar cells, which highly suggests that cytokines are not responsible for the induction of this phenotype by phagocytic microglia. Importantly, the results obtained with MicroLPS or BV2 CM were very similar to the ones obtained with neuroprogenitor cells directly treated with LPS. This fact led us to hypothesize that LPS might have not been degraded in the CMs and that the results we observed might be

due to the remaining LPS within the CMs rather than the effect of released cytokines. Thus, we developed another paradigm in which we treated primary microglia with LPS[high] (1µg/ml) for 6h in order to trigger the inflammatory response and then change to fresh media for another 18h, which would not contain any LPS. Next, we treated neuroprogenitor cells with either LPS[high] or CM microLPS[high] 6h + 18h. In addition, we also added another experimental group in which we treated neuroprogenitor cells with the CM from apoptotic SH-SY5Y in order to discard the possibility that bipolar cells could be the result of the molecules released by apoptotic cells **(Figure 26A)**. We found that both LPS[high] and CM microLPS[high] 6h + 18h presented a majority of stellate cells (87.4 ± 1.9% and 86.0 ± 3.0% at 5d, respectively) and a small proportion of ramified cells (12.6 ±1.8% and 14.1 ± 2.9% at 5d, respectively), and there were no differences between the two treatments in terms of cell type proportion **(Figure 26B, C)**. However, after 5d of CM microLPS[high] 6h + 18h, all the ramified cells expressed high levels of nestin. In addition, apoptotic SH-SY5Y CM treated neuroprogenitor cells did not show differences compared to CM microC **(Figure 26B, C)**. Neither LPS[high], CM microLPS[high] 6h + 18h nor apoptotic SH-SY5Y CM treatments give rise to bipolar cells, which highly suggest that cytokines or molecules released from apoptotic cells are not responsible for this phenotype and show a unique effect of microglial phagocytosis released molecules on neuroprogenitors.
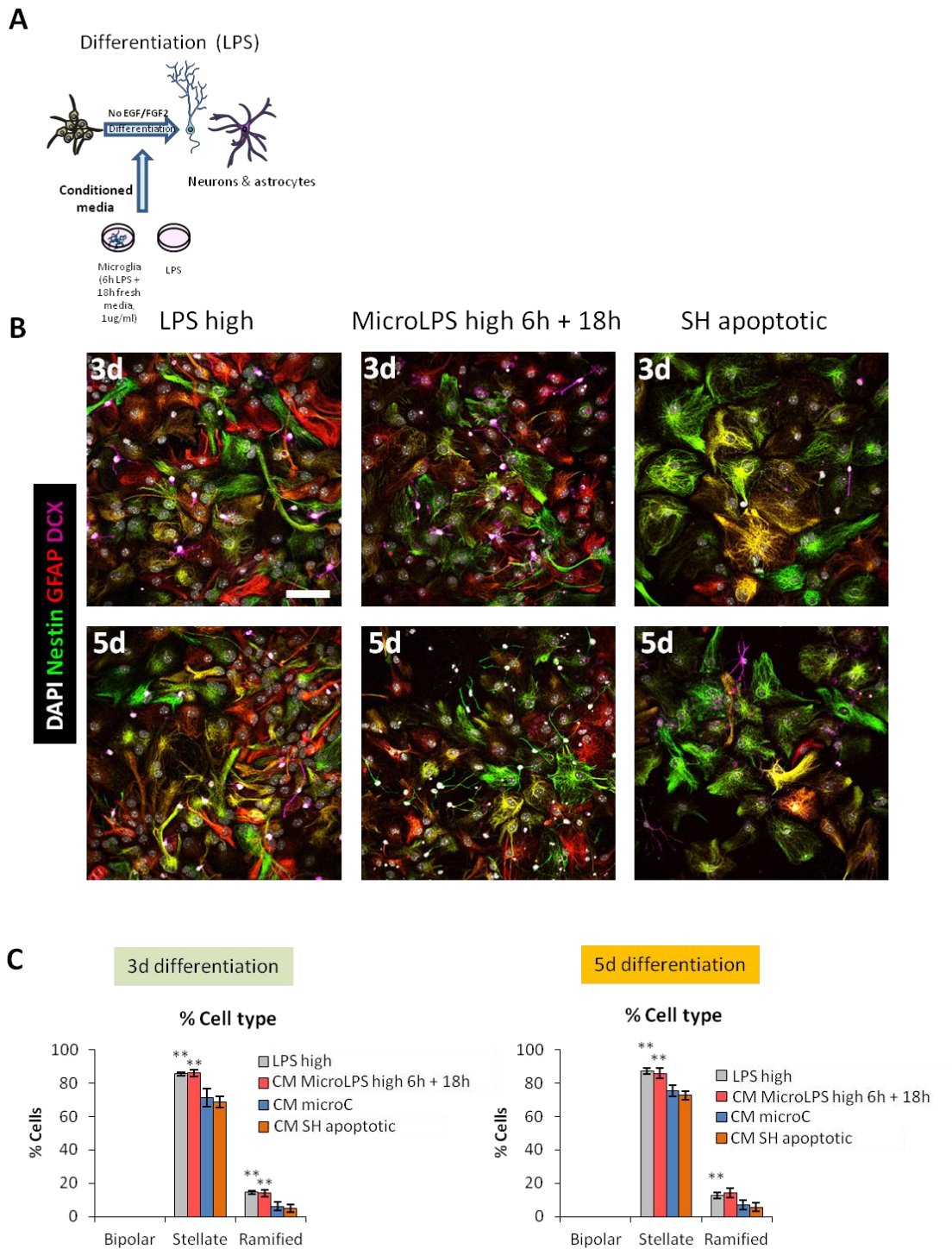
Figure 26. Effect of the CM MicroLPS^high 6h + 18h on neurogenesis in vitro. *[A] Experimental design of the in vitro neurogenesis assay. [B] Representative confocal microscopy images of neuroprogenitors treated with CM MicroLPS^high 6h + 18h or LPS^high (1ug/ml; 24h), CM MicroC and CM SH apoptotic. Scale bars, 20µm. z= 6.3µm. [C] Quantification of the different cell types found after 3d or 5d treatment with CM MicroLPS^high 6h + 18h or LPS^high (1ug/ml; 24h), CM MicroC and CM SH apoptotic. N = 3 independent replica. Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA was significant at p < 0.05).*

6.5.2.2 The neuropeptide VGF is highly increased in phagocytic microglia

After discarding cytokines as the possible mechanism that drives CM microPH effects on in vitro neurogenesis, we studied the role of the neurocandidate that showed the highest FC changes in both the arrays and RT-qPCR, the neuropeptide VGF **(Figure 16)**. VGF is a neuropeptide that has been implicated in metabolism and neurogenesis (Foglesong et al., 2016; Thakker-Varia et al., 2014), and we have identify its increased expression in phagocytic microglia in vitro in the gene arrays **(Figure 15 and 16)**. In order to determine whether VGF could play a role in the effects we observed in the neurogenesis assay in vitro, we first studied the production of VGF by microglia at the protein level. Therefore, we performed an ELISA of the microglia control and phagocytic CMs in order to analyze the release of this neuropeptide. However, the concentration of VGF in the samples was below the detection level of the ELISA kit **(data not shown)**. VGF is post-translationally processed, which give rise to a variety of VGF peptides, and in addition, the variety of VGF that is detected by the VGF ELISA kit is undetermined. Therefore, we set up a collaboration with Gian Luca Ferri and Christina Cocco (University of Cagliari, UNICA, Italy), experts in VFG signaling (Brancia et al., 2018), who have developed antibodies against different terminus of the different VGF varieties **(Figure 27A)**. Using these antibodies, we tested the production of VGF in control and phagocytic (24h) microglia. Five antibodies were used:

1. <u>BQ11</u> (selective for the VGF C-terminal 3-5 amino acids)

2. <u>BQ17</u> (selective for the cleaved N-terminus of TLQP-peptide variants)

3. <u>BQ42</u> (selective for the N-terminus of the TLQP-variants)

4. <u>BQ22</u> (selective for the N-terminus of rat VGF)

5. <u>BQ28</u> (selective for the N-terminus of an internal VGF peptide, repeatedly identified in proteomic studies).

We found that both control and phagocytic microglia were labeled with all the VGF antibodies. The expression in control microglia was rather faint, whereas phagocytic microglia expressed higher levels of VGF, which was especially noticeable with the antibodies BQ17 and BQ42, both selective for TLQP variants **(Figure 27 A, B)**. This data suggested that upon phagocytosis, microglia triggers the production of some of the VGF variants. However, whether phagocytic microglia drives its effects on neurogenesis in vitro and in vivo will remain to be studied in future experiments.
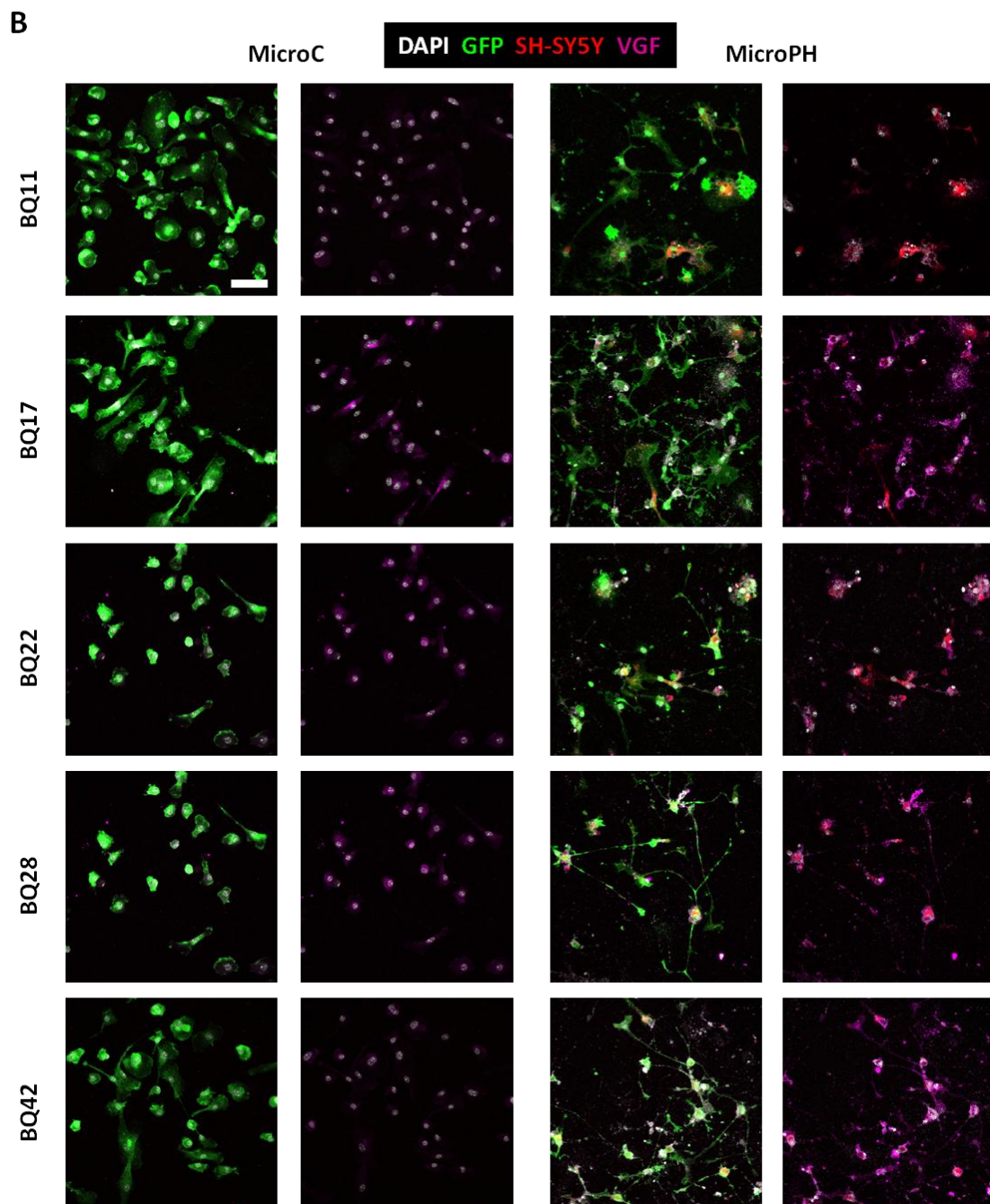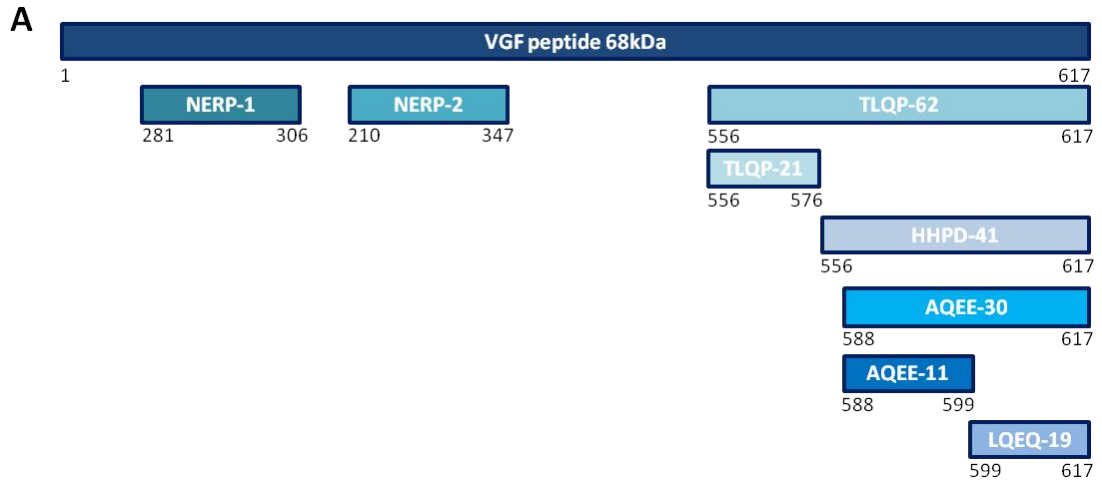
A

B

6.5.2.3 Lactate is not increased in phagocytic microglia

In addition to cytokines and VGF, we also focused our attention on the metabolite lactate, which is a by-product of the glycolytic process. As we mentioned in **Figure 13**, phagocytic microglia showed major transcriptional changes in genes related to metabolism; genes related to mitochondrial pathways such as Krebb's cycle and oxidative phosphorylation were downregulated, whereas glycolysis genes were increased. These alterations in metabolism could lead to the production of lactate by phagocytic microglia. Lactate has been described to exert hormone functions and it has been related to memory formation and neuroprotection (Proia et al., 2016), and it might also support neurogenesis in vivo (Alvarez et al., 2014). Therefore, we studied the production and release of this metabolite by control, and phagocytic microglia **(Figure 28A)**. In addition, the metabolic switch towards glycolysis had been described in macrophages upon LPS stimulation (Palsson-McDermott et al., 2015), and therefore, CM MicroLPS^{high} 6h+18h was also added to the study in order to validate this response in LPS stimulated microglia. We found no significant differences between the lactate concentration produced by control and phagocytic microglia **(Fig 28B)**. In addition, CM MicroLPS^{high} 6h+18h lactate production was significantly enhanced compared to control microglia. Since phagocytic microglia did not produce higher levels of lactate, it is unlikely that lactate mediated the neuromodulatory effects triggered by phagocytosis.
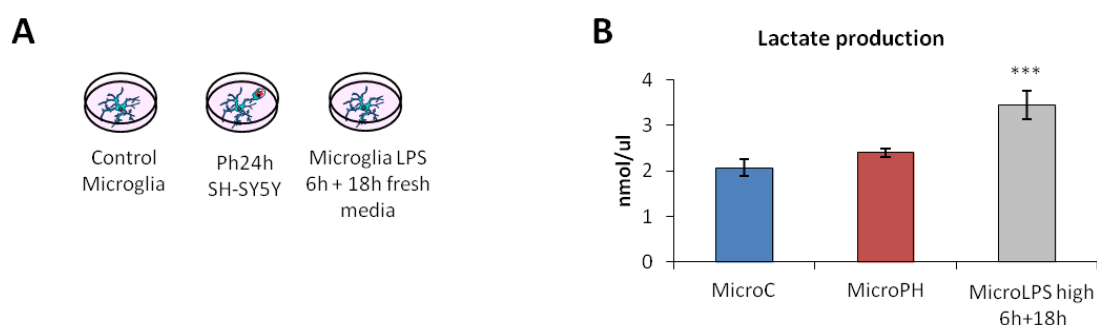
Figure 27. Lactate release in conditioned media from control and phagocytic microglia in vitro. [A] Experimental design of lactate production. [B] Quantification of lactate concentration in CM from control, phagocytic and LPS 6h+18h-treated microglia N=4 independent replica. Bars represent mean ±

SEM. *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA was significant at p < 0.05).

6.5.3. Phagocytosis conditioned media induces astrogenesis

After determining that CM microPH affects neurogenesis in vitro by inducing the differentiation of neuroprogenitors towards astrocyte-commited bipolar cells, we next characterized the effect of the CMs at later stages of neurogenesis. Therefore, we performed a late survival and differentiation assay in which neuroprogenitor cells were allowed to differentiate for 10d into neuroblasts and astrocytes using DMEM/F12 without trophic factors. After this period, differentiated cultures were treated for 3 and 5d with CM microC and microPH as well as DMEM for positive control **(Figure 29A, B)**. Importantly, contrary to what we found in the early differentiation assay, culturing differenciated astrocytes and neuroblast with CM microPH did not result in an increase in apoptosis compared to CM microC **(Figure 29C, D)**. In addition, we found that after 10d in DMEM/F12 the culture showed both stellate and ramified cells in a similar proportion between CM microC and DMEM **(Figure 29E, F)**. After 3 and 5 days of treatment with CM microC and DMEM, the cultures presented 96.8 ± 3.4 % of stellate cells and 1.9 ± 1.9% of ramified cells. Interestingly, the treatment with CM microPH give rise to a different phenotype from the ones observed until this point. These cells shared similarities with the stellate cells in marker expression and morphology; however, they displayed even a more starry morphology than the stellate cells and, thus, we identified them as mature stellate cells. The CM microPH culture showed 21.7 ± 2.7% stellate cells and 78.3 ± 4.14% mature stellate cells at 5d, and DCX^+ cells were never found. These data suggest that CM microPH induces astrogenesis.
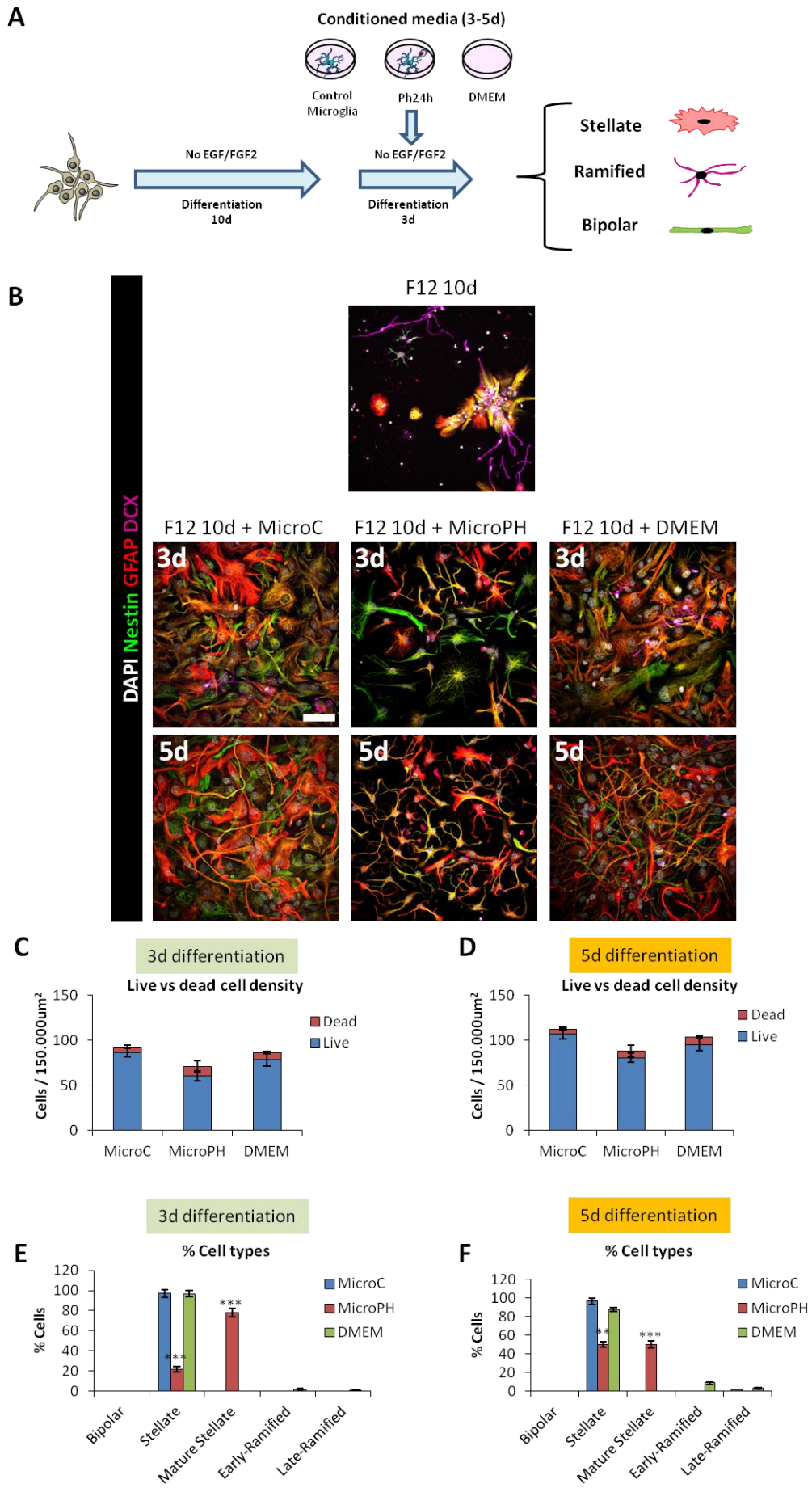
*Figure 29. Effect of CM microC and microPH in a late survival and differentiation assay. [A] Experimental design of the in vitro late survival and differentiation assay. [B] Representative confocal microscopy images of neuroprogenitors treated with 10d of DMEM/F12 followed by 3-5d of CM microC or microPH and DMEM. The upper image represents the DMEM/F12 treatment of 10d, prior to adding any CM. Scale bars, 20µm. z= 6.3µm. [C] Quantification of the different cell types found after 10d of DMEM/F12 followed by 3-5d of CM microC or microPH and DMEM. N = 2 independent experiments. Bars represent mean ± SEM. ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after one-way ANOVA was significant at p < 0.05).*

6.6. VALIDATION OF THE EFFECTS OF MICROGLIAL PHAGOCYTOSIS ON NEUROGENESIS IN VIVO

To sum up, we have confirmed that microglia phagocytosis triggers a neuromodulatory program in vitro by the production of several molecules. Our next step was to validate this program in vivo.

In order to replicate the expression of neuromodulatory factors by phagocytic microglia in vivo, we resorted to utilize as a model the adult hippocampal neurogenic cascade. In young mice, SGZ neural precursors are abundant and proliferate at high rates, generating many apoptotic cells (Sierra et al., 2010). Thus, in the DG of young mice (1mo), microglial phagocytosis can be frequently observed. Therefore, we utilized an ex vivo approach consisting in analyzing the transcriptomic profile in microglia acutely isolated by fluorescent activated cell sorting (FACS). We compared a population enriched in phagocytic microglia isolated from the dentate gyrus (DG), where neurogenesis occurs and apoptosis is abundant; with non-phagocytic microglia isolated from the CA region of the hippocampus, where there is no neurogenesis and therefore no apoptosis **(Figure 30A)**. We dissected the DG and CA from 1-month-old fms-EGFP mice and microglia cells were acutely purified in a FACS sorter **(Figure 30B)**. Next, the expression of neuromodulatory molecules was quantified by RT-qPCR. While we found that DG microglia showed a tendency to express higher levels of trophic factors than CA microglia the results were not significant **(Figure 30C)**. However, it must be noticed that the DG contains a highly heterogeneous population of microglia. In the 1mo hippocampus, only 30% of microglia is engaged in phagocytosis in the SGZ (Sierra et al., 2010). In addition, as phagocytosis is fast (around 1.5h) (Sierra et al., 2010) many microglia that do not appear phagocytic (i.e., do not contain phagocytic pouches) may have just finished phagocytosing. Furthermore, the DG includes many non-phagocytic cells from the hilus and the molecular

layer. This heterogeneity likely interferes with our ability to detect transcriptional changes associated to phagocytosis in vivo. For these reasons, we pursue the phagocytic microglia expression analysis by utilizing a combination of single-cell transcriptomics with bioinformatics in collaboration with Dr. Florent Ginhoux (Agency for Science, Technology, and Research, Singapore). For this task, we followed the same methodology as in **Figure 30A** and we acutely isolated microglia cells using FACS-sorting from the DG and CA of heterozygous CX3CR1-GFP mice, a mouse strain in which the microglia-specific *CX3CR1* gene is replaced by a green fluorescent protein (*GFP*) reporter gene. The single-cell RNA seq was performed in a total of 192 cells from DG and 96 cells from CA. Our strategy was aimed at identifying those microglia cells with a gene-expression profiles ranging from phagocytic and non-phagocytic stages usingthe NBOR algorithm ('neighborhood-based ordering of single cells') developed in the Ginhoux laboratory (Schlitzer et al., 2015). This algorithm can objectively determine the position of a given cell in a developmental continuum and calculate the similarity of each single cell's gene-expression profile to a defined gene set of a particular cell population (landmark) and then order each cell according to such similarity score into a spatial continuum around those landmarks. This algorithm would help us to determine the transcriptional changes occurring along the process of phagocytosis. However, we did not observed differences between the two populations **(Figure 30D, E)**, most likely because the DG is not sufficiently enriched in phagocytic microglia.
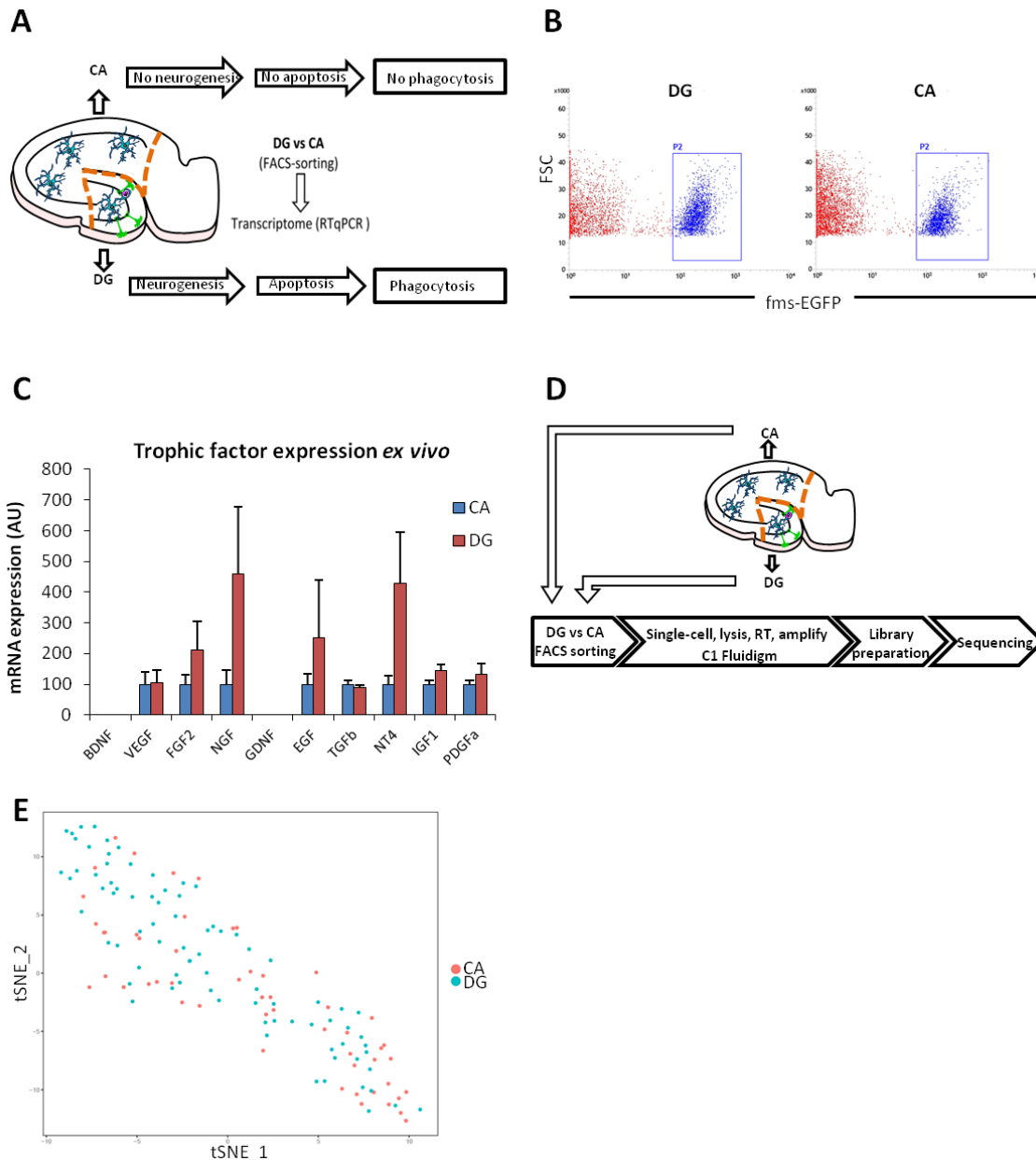
Figure 30. Transcriptional analysis of control and phagocytic microglia in vivo. [A] *Experimental design used for the transcriptional analysis of control and phagocytic microglia in vivo.* **[B]** *Experimental design used to isolate microglia (GFP+) vs non-microglial cells (GFP-) from 1 mo fms-EGFP mice. Flow cytometry analysis of the expression of neuromodulatory molecules from dentate gyrus (DG) microglia and CA microglia. Gates for GFP+ microglia cells (P2, blue) and GFP- non-microglial cells (red) were defined based on the distribution of the fms-EGFP+ cells in EGFP vs FSC for both DG and CA (left and right panel respectively).* **[C]** *Expression of neuromodulatory molecules in DG microglia vs CA microglia by RTqPCR in FACS-sorted cells from fms-EGFP mice hippocampi (n = 4, each from 20 pooled hippocampi). OAZ1 was selected as a reference gene. Bars represent mean ± SEM.* **[D]** *Experimental design used for the single-cell RNA sequencing of control and phagocytic microglia in vivo.* **[E]** *Distribution of the 96 cells from CA and 192 cells from DG used for single cell RNA sequencing in a tSNE (t-distributed Stochastic Neighbor Embedding) analysis.*

Since the in vivo transcriptional analysis presented high variability and the methods used did not allowed us to validate the transcriptional program of phagocytosis, we switched the strategy and we focused on confirming the neuromodulatory role of the molecules secreted by phagocytic microglia in vitro on adult hippocampal neurogenesis. Therefore, we injected CM microC and microPH into 2mo fms-EGFP mice for 6d using osmotic pumps. After this period, BrdU, was administered to track proliferating cells and mice were sacrificed 2h later **(Figure 31A)**. We observed no differences in total BrdU proliferative cells in CM microPH treated mice compared to CM microC treatment **(Figure 31B, C).** Importantly, contrary to what we observed in vitro, CM microPH did not induce apoptosis in vivo **(Figure 31D)**. In addition, there was a tendency (p=0.1849) to find fewer total numbers of NSCs labeled with nestin and GFAP **(Figure 31E, F)** and a decreased the number of proliferating stem cells (p=0.1637) **(Figure 31G)** CM microPH compared to CM microC. Moreover, when analyzing neuroblast cells, we found no differences between CM microC and microPH, neither in the total number of neuroblasts nor in the proportion of the different neuroblast subpopulations (AB, CD and EF), nor in neuroblast proliferation **(Figure 31H-I)**. Further experiments are currently being performed to increase the 'n' and confirm the statistically significance of the downregulation of proliferative cells and proliferating stem cells in CM microPH compared to CM microC.
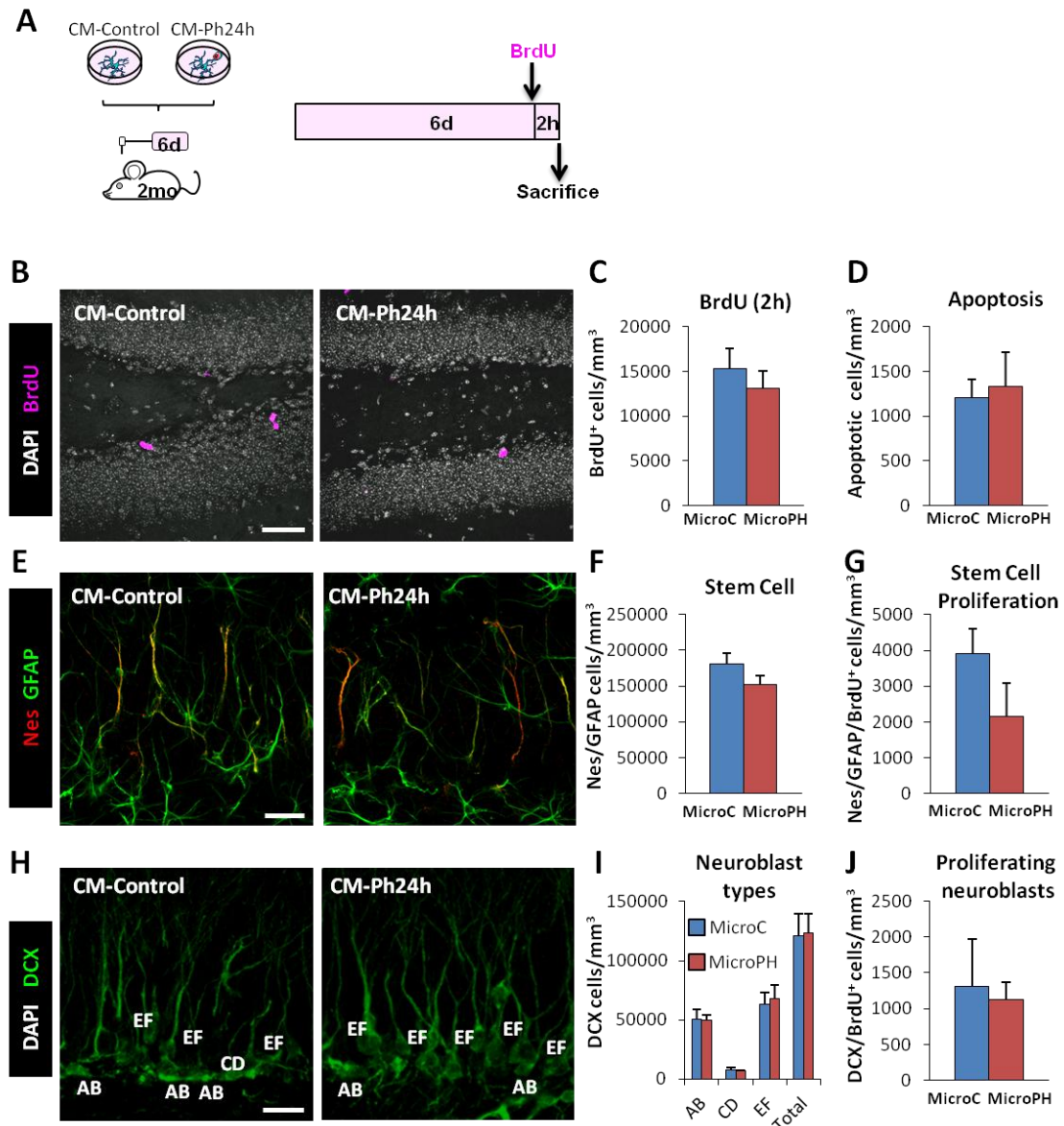
Figure 31. Effects of phagocytic microglia secreted molecules on neurogenesis in vivo. [A] Experimental design used for the administration of CM microC or microPH by osmotic pumps to 2mo fms-EGFP mice. [B] Representative confocal images of cell proliferation after the CM treatments for 6d. Cell nuclei were labeled with DAPI (white) and BrdU was used as a proliferative marker (magenta). Scale bars, 50µm. z= 12µm. [C] Quantification of BrdU+ cells per mm[3] after CM microC or microPH treatment. N = 7 independent experiments. [D] Quantification of apoptotic cells after the CM microC or microPH treatment. N= 3 independent experiments. [E] Representative confocal images of stem cells labeled with nestin (Nes, Red) and GFAP (Green). Scale bars, 20µm. z= 12µm. [F] Quantification of stem cells per mm[3] after the CM microC or microPH treatment. N= 7 independent experiments. [G] Quantification of proliferation of stem cells after the CM microC or microPH treatment. N= 7 independent experiments. [H] Representative confocal images of neuroblast cell populations AB, CD and EF. Neuroblast cells are labeled with DCX (green). Scale bars, 20µm. z= 12µm. [I] Quantification of the proportions of neuroblast cell population AB, CD and EF. N = 4 independent experiments. [J] Quantification of total number of

BrdU$^+$/DCX$^+$ cells per mm^3 after treatment with CM microC or microPH. Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by after Student's t-test was significant at p < 0.5 in all the comparisons with the exception of figure [I] in which Bonferroni posthoc test was applied (after two-way ANOVA was significant at p < 0.05).

Because we observed a tendency to find decreased NSCs after 6ds in the presence of CM microPH, we hypothesized that the induced alterations would accumulate over time. Therefore, we performed a long-term experiment in which mice were treated with CM microC and microPH in osmotic minipumps for 6h, followed by BrdU administration, and sacrificed 28d later to allow the differentiation of the labeled cells **(Figure 32A)**. The total number of BrdU$^+$ cells was significantly reduced in mice treated with CM microPH compared to CM microC **(Figure 32B, C).** Importantly, CM microPH did not induce apoptosis in vivo **(Figure 32D)**. In addition, there was a tendency (p=0.1383) to find fewer total numbers of NSCs labeled with nestin and GFAP **(Figure 32E, F).** In addition, we also found a significant reduction in the most mature neuroblast cell subpopulation (EF) in mice treated with CM microPH compared to CM microC **(Figure 32G-H)**. Moreover, after 28d, newborn neurons (NeuN$^+$/BrdU$^+$) showed a tendency to decline (p= 0.119, respectively) in CM microPH compared to CM microC **(Figure 32I, J)**. No proliferating NSC, neuroblast or astrocytes were found in any of the treatments **(data not shown)**.

All these data, together with the tendency to observe fewer proliferative cells found at 2h after BrdU, suggest that the acute (24h) release of mediators by phagocytic microglia limits neurogenesis via the reduction in the survival of neuronal-committed cells.
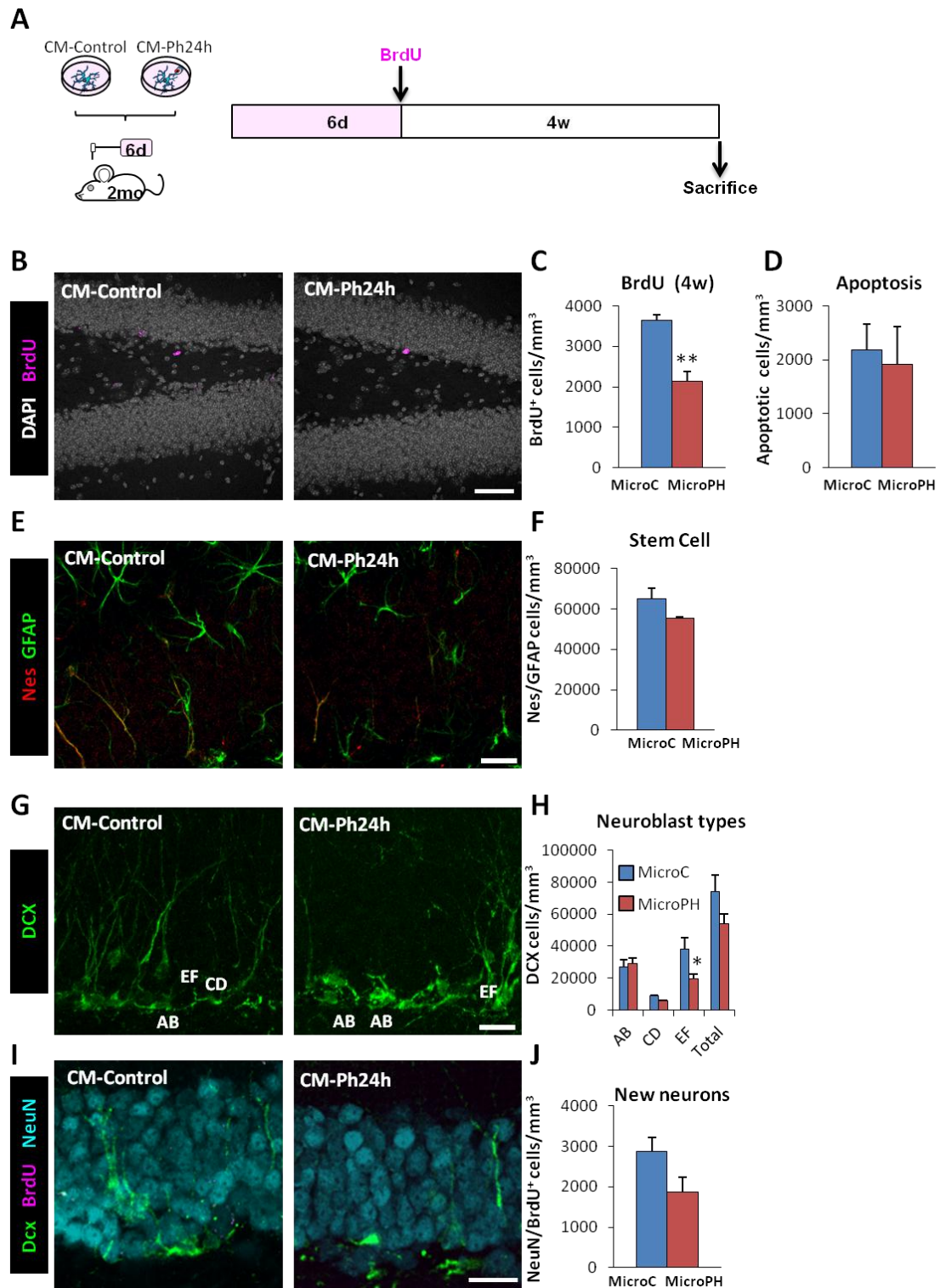
Figure 32. Long term effects of phagocytic microglia secreted molecules on neurogenesis in vivo. *[A] Experimental design used for the administration of CM microC or microPH by osmotic pumps to 2mo fms-EGFP mice. [B] Representative confocal images and quantification of total BrdU⁺ cells. Scale bars, 50µm. z= 12µm. [C] Quantification of BrdU⁺ cells per mm³ after CM microC or microPH treatment. N = 3 independent experiments. [D] Quantification of apoptotic cells after CM microC or microPH treatment. N= 3 independent experiments. [E] Representative confocal images of stem cells labeled with nestin (Nes,*

168

*Red) and GFAP (Green). Scale bars, 20μm. z= 12μm. **[F]** Quantification of stem cells per mm³ after CM microC or microPH treatment. N = 3 independent experiments. **[G]** Representative confocal images of neuroblast cell populations AB, CD and EF. Neuroblast cells are labeled with DCX (green). Scale bars, 20μm. z= 10μm. **[H]** Quantification of the proportions of neuroblast cell population AB, CD and EF. N = 3 independent experiments. **[I]** Representative confocal images and quantifications of new neurons labeled with BrdU (magenta) and NeuN (cyan). Scale bars, 20μm. z= 10μm. **[J]** Quantification of new neurons per mm³ after CM microC or microPH treatment. N = 3 independent experiments. Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by after Student's t-test was significant at p < 0.5 in all the comparisons with the exception of figure [H] in which Bonferroni posthoc test was applied (after two-way ANOVA was significant at p < 0.05).*

6.7. Characterization of microglial receptors

Finally, we developed alternative strategies to demonstrate the role of microglial phagocytosis on neurogenesis in vivo, based on the genetic knock-down or pharmacological manipulation of key phagocytic receptors. In the next section we will focused on the characterization of several of these microglial receptors.

Our group has recently reported an impairment of microglial phagocytosis in a mouse model of epilepsy by intrahippocampal injection of the glutamate agonist kainic acid (Abiega et al., 2016) and thus we hypothesized that hippocampal microglia may express glutamate receptors, which had been shown in vitro (Beppu et al., 2013; Fontainhas et al., 2011; Kaindl et al., 2012). Thus, we analyzed by RT-qPCR the expression of the 23 glutamate receptor subunits (both ionotropic and metabotropic) in FACS-sorted microglia from the hippocampus and cortex of 2mo fms-EGFP mice. We detected a residual expression of all ionotropic and metabotropic subunits in hippocampal and cortical microglia **(Figure 33)**, however, this poor expression was unlikely to lead to the formation of functional receptors. Therefore, it is unlikely that microglia interacts with the neurogenic niche via glutamate signaling.

We next focused on the receptors involved in finding and tethering apoptotic cells, and characterized their expression on acutely purified microglia from the hippocampus of control and kainic mice. We selected purinergic receptors such as P2X4, P2X7, P2Y6 or P2Y12, which are able to sense ATP and UTP, well-described 'find-me' signals released by apoptotic cells (Elliott et al., 2009); as well as recognition receptors such as triggering receptor expressed in myeloid cells 2 (Trem2) (Hsieh et al., 2009), Mer Tyrosine Kinase (MerTK) (Scott et al., 2001),

complement receptor 3 (CR3) (Veldhoen et al., 2007), and the G protein coupled receptor (GPR34)(Preissler et al., 2015).

We found that purinergic receptors (P2X4, P2Y6, and P2Y12) were significantly increased, while the apoptotic cell recognition receptors (Trem2, MerTK, CR3, and GPR34) were significantly decreased in microglia from KA mice **(Figure 33)**. These results suggest that modulating the expression or function of purinergic and recognition receptors may alter microglial phagocytosis and allow us to further test its impact on neurogenesis.
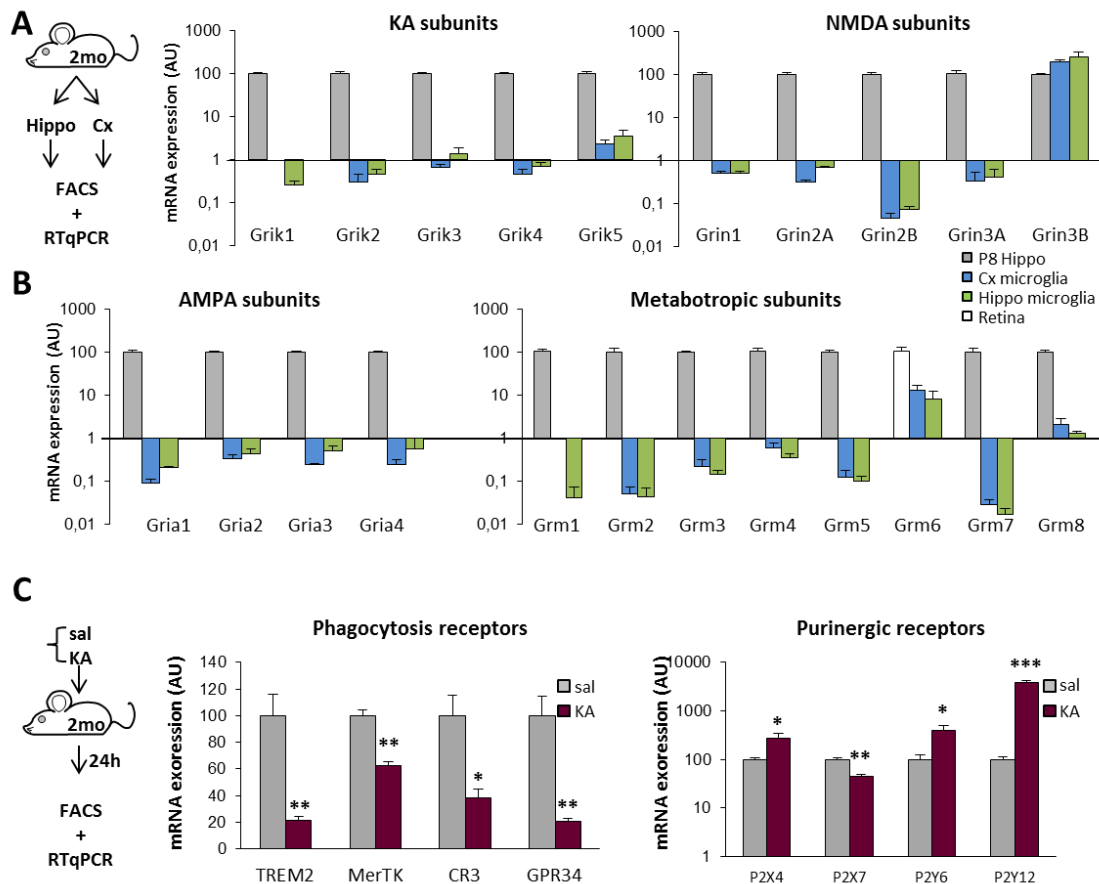
*Figure 33. Characterization of microglial receptors. [A, B]Experimental design for RT-qPCR expression of KA; NMDA, AMPA and metabotropic receptor subunits in acutely purified microglia (FACS-sorted) from the hippocampus and the cortex of 2mo mice. N = 4 from 8 pooled hippocampi and cortices each. The relative expression was compared to a positive control, a P8 hippocampus,except for Grm6, in which the retina from a 2mo mouse was used. L27A was used as a reference gene. [C] Experimental design and expression of phagocytic and purinergic receptors by RT-qPCR in FACS-sorted microglia from control and KA mice at 1 dpi. N = 3 from 8 pooled hippocampi. HPRT was used as a reference gene. Bars represent mean ± SEM. * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 by Holm-Sidak posthoc test (after Student's t-test or one-way ANOVA was significant at p < 0.05).*

7. DISCUSSION

7. DISCUSSION

During adult hippocampal neurogenesis, the majority of the newborn cells undergo apoptosis. To avoid disturbing the surrounding neurons, these apoptotic cells are quickly and efficiently removed by phagocytosis by resident microglia. Here we propose that phagocytosis is not merely a passive process of corpse removal but has an active role in maintaining the homeostasis of the adult hippocampal neurogenic cascade by producing neurogenic regulators. To test this hypothesis we first performed a genome-wide transcriptomic analysis using gene expression arrays to compare cultured naïve vs. phagocytic microglia. Gene ontology analysis revealed that, in addition to major changes in different metabolic and signaling pathways, phagocytosis triggered a neuromodulatory program in microglia. We found a significant upregulation of the neurogenesis function and identified significant changes in several genes involved in functions related to different stages of the neurogenic cascade, including trophic factors, matrix metalloproteases, neuropeptides, and surface ligands. Furthermore, conditioned media from phagocytic microglia altered differentiation of cultured neural progenitors, which was unrelated to cytokines. Finally, these results were confirmed in vivo by the administration of the conditioned media from phagocytic microglia via osmotic pumps, which resulted in a reduction of proliferation and neuroblast cell population.

Altogether, our data supports that upon engulfment of apoptotic newborn cells, microglia initiates a transcriptional program devoted to control the adult hippocampal neurogenic cascade.

In this Thesis Project we have set up an in vitro model of phagocytosis, studied the transcriptional changes induced in microglia upon phagocytosis, and characterized the modulation that phagocytic microglia exerts over neurogenesis, all of which will be discussed in the following sections.

7.1. IN VITRO MODEL OF PHAGOCYTOSIS

In order to study phagocytosis-induced microglial changes and the interaction between phagocytosis and neurogenesis in vitro, we have developed a model of phagocytosis of apoptotic cells based on the characteristics observed in phagocytosis in the adult hippocampal neurogenic niche (Beccari et al., 2018), based on models previously published (De Simone et

al., 2003; Parnaik et al., 2000). To develop this model we selected several features: the type of microglia; the type of cargo; the culture medium; and the quantification method. All these characteristics play an important role in the outcome of the phagocytic process and influence the strengths and limitations of our in vitro model of phagocytosis.

7.1.1. Selection of microglia: primary cultures

A strong advantage of our model is the use of primary microglia over microglial cell lines such as BV2. Microglia cell lines are commonly used due to their highly proliferative nature and ease of use. However, these immortalized cells usually greatly differ from the in vivo target cell. For these reason, we have used primary microglia grown using a well-established protocol. Different procedures have been used to grow primary microglia. Classically, mixed cultures of astrocytes and microglia are grown in the presence of macrophage colony stimulating factor (M-CSF), a mitogen that allows obtaining high yields of microglial cells (Kloss et al., 1997). We resorted to culture microglia with granule macrophage colony stimulating factor (GM-CSF) (Sierra et al., 2008), to obtain a higher yield of cells. Nonetheless, it has been recommended to culture microglia with a mixture of M-CSF and TGF-β in order to develop the molecular signature of adult microglia (Butovsky et al., 2014). Butovsky et al., compared FACS-sorted adult microglia to adult primary microglia grown with M-CSF, GM-CSF or M-CSF with TGF-β, and newborn primary microglia without any mitogen. They found that the transcriptome of adult primary microglia cultured with M-CSF and TGF-β was the most similar to FACS-sorted adult microglia. However the comparison between GM-CSF treated newborn primary microglia and adult microglia was not assessed. Therefore, in the future it would be interesting to compare our results obtained in primary postnatal microglia grown in GM-CSF with other established protocols to grow adult microglia, and to validate key results in vivo. In addition, in the recent years a new approach has also started to emerge in which microglia is cultured in serum-free conditions because it promotes a ramified microglial morphology (Collins and Bohlen, 2018). However, although it is a morphological resemblance to in vivo microglia in physiological conditions, there is no correlation between morphology and function, as we will also discuss later. Indeed, serum-free cultured microglia have been shown to fail in the phagocytosis of beads or myelin debris regardless of a previous opsonization process of the phagocytic targets with serum (Bohlen et al., 2017). Thus, prior to establishing a methodology to culture microglia, it is necessary to assess whether it disturbs the proper functioning of the cell.

7.1.2. Selection of cargo: early apoptotic cells

Another advantage of our model is that it uses a physiologically relevant target, i.e., apoptotic cells. Traditionally, many in vitro phagocytosis assays have been performed using artificial targets, such as latex beads. These targets can be engulfed by microglia, however, they do not release or express 'find-me' or 'eat-me' molecules and they cannot be degraded within microglia (Diaz-Aparicio et al., 2016). In addition, the engulfment of latex beads triggers a different downstream signaling than apoptotic cells in macrophages (Park et al., 2011). Only the uptake of apoptotic cells, and not of latex beads, leads to the up-regulation of Ucp2 (uncoupling protein 2), which in turn, lowers the mitochondrial membrane potential, necessary for a correct engulfment (Park et al., 2011). Therefore, the results obtained in these assays must be carefully considered, especially if the resulting immunomodulatory effects of phagocytosis are aimed to be addressed because each type of cargo is recognized by different receptors and triggers different signaling pathways. For example, microglia identify invading pathogens through scavenger and Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) (Sierra et al., 2013; Takeda et al., 2003). The receptor-ligand interaction results in the production of pro-inflammatory cytokines, chemokines, and reactive oxygen species, and enhances phagocytic activity and antigen presentation in microglia (Hanisch, 2002; Ribes et al., 2010). On the other hand, microglia identify apoptotic cells through different phagocytic receptors that recognize 'find-me' and 'eat-me' signals produced by apoptotic cells (Diaz-Aparicio et al., 2016; Sierra et al., 2013), and over the past 20 years this process has strongly been proposed to have an anti-inflammatory nature (De Simone et al., 2003; Fadok et al., 1998; Fraser et al., 2010). In this Thesis Project, we specifically focused on the phagocytosis of apoptotic cells (murine NE-4C and human SH-SY5Y) because microglia engulf the excess of newborn cells that undergo apoptosis in the neurogenic niches in physiological conditions, and therefore it is a genuine model to study the relationship between microglial phagocytosis and neurogenesis.

A key feature of microglial phagocytosis in the adult hippocampus is that in physiological conditions is a very fast and efficient mechanism, as we have estimated that microglia remove apoptotic cells in approximately 1.5h (Sierra et al., 2010) and live imaging experiments in vitro have shown phagocytosis to last 25-90min (Parnaik et al., 2000). In addition, around 90% of the hippocampal apoptotic cells are contained within phagocytic pouches (Sierra et al., 2010), ensuring that apoptotic cells do not evolve into secondary necrotic cells and spill over intracellular compounds. In contrast to apoptotic cells, necrotic cell

engulfment is pro-inflammatory, at least in macrophages (Erdman et al., 2009). Therefore, it is important to ensure an early apoptotic cell induction in order to mimic physiological conditions accurately. Different strategies to induce apoptosis have been utilized in the literature. Chemical inductors, such as staurosporine (Abiega et al., 2016) or etoposide (Fraser et al., 2010) are used as cancer treatments due to their ability to inhibit proliferation, which results in cell death. On the other hand, there are also non-chemical inductors of apoptosis, such as irradiation (Zhao et al., 2016), which triggers the accumulation of DNA mutations in proliferative cells, eventually causing cell death. For this reason, we avoided the use of irradiation, since apoptosis initiation would depend on the number of mutations in each cell and therefore, it would be more difficult to synchronize apoptotic induction. Thus, we used staurosporine and we selected a short incubation time (4h) in order to ensure that microglia was fed with early apoptotic cells rather than primary or secondary apoptotic cells.

Importantly, we observed that in our model up to 30% of microglia was already engaged in phagocytosis within the first hour, a percentage that increased over time. Although we did not directly assess the time that our microglia need to completely engulf and degrade an apoptotic cell, both Parnaik (Parnaik et al., 2000) and our assay coincide in around 60% of phagocytic microglia at 5-6h, which suggests that the engulfment and degradation process of our assay might follow a similar timing to Parnaik's results. Therefore, the in vitro microglial phagocytosis assay that we set up also achieves the rapid and early phagocytosis observed in vivo.

7.1.3. Selection of culture media: presence of opsonins

Opsonins are molecules that tag apoptotic cells in order to facilitate their recognition and engulfment. Importantly, the opsonin C1q has recently been shown to modulate the outcome of the phagocytic process, since its presence has been related to an anti-inflammatory response of microglia, whereas its absence is associated to a pro-inflammatory response (Fraser et al., 2010). Taking into account that C1q might exert such a drastic immunomodulatory outcome, we studied the role of C1q and its relationship with phagocytosis in the hippocampus in physiological conditions. We found that most of the phagocytic pouches contained C1q and that the majority of microglial cells presented C1q within the microglial cytoplasm conforming the phagocytic pouch. In addition, we also showed that microglia is the main source of C1q in the hippocampus. The production of C1q by microglia and the macrophage linage had also been reported before, but the majority of the

studies performed in this area are related to the overproduction of C1q upon microglial activation (Lynch et al., 2004) or under pathological conditions such as ischemic stroke (Schafer et al., 2000) or viral infection (Depboylu et al., 2005). Since we found a relationship between C1q and microglial phagocytosis in physiological conditions, C1q was included in the in vitro phagocytosis model by using non-inactivated serum. However, traditionally microglia cultures have been performed in the presence of heat-inactivated serum in order to inhibit complement proteins (Lee and Tansey, 2013). Thus, it is not surprising that the inmunomodulatory outcome of our phagocytic assay differs from the previously described anti-inflammatory response upon phagocytosis of apoptotic cells, as we will further discuss in **section 7.4**.

7.1.4. Selection of quantification method: confocal microscopy

We have strengthened our method by the direct observation and quantification of phagocytic pouches. In contrast, phagocytosis is commonly quantified using indirect methodologies, such as the expression of "activation markers" or changes in microglial morphology of (Diaz-Aparicio et al., 2016). Classical microglial "activation markers", such as CD68 (macrosialin) have traditionally been used as indicators of microglial phagocytosis (Perego et al., 2011; Schafer et al., 2012). However, the expression of CD68 and phagocytosis do not correlate as observed during epilepsy, where CD68 is overexpressed while microglial phagocytosis is impaired (Abiega et al., 2016); or after LPS treatment, where many non-phagocytic microglia overexpress CD68 (Sierra et al., 2010). In addition, microglial morphology has also been used as an indirect method to assess microglial phagocytosis. Traditionally, ameboid-shaped microglia was believed to be phagocytic, whereas ramified microglia was supposed to be quiescent (Bohatschek et al., 2001). Nevertheless, microglial morphology is not an indicator of phagocytosis since in physiological conditions microglial phagocytosis is executed by ramified microglia (Sierra et al., 2010). In contrast, hypertrophic microglia after LPS treatment are highly phagocytic but after epilepsy they are not (Abiega et al., 2016; Sierra et al., 2010). Using indirect techniques as an approximation for phagocytosis can result in misleading conclusions and thus, direct quantifications of this process should always be performed.

One commonly used method is based on using flow cytometry to assess microglial engulfment of fluorescent cargo (either latex beads, transgenic cells that express a fluorescent protein or labeled using membrane dyes such as cm-DiI) (Pul et al., 2013). However, detaching

cultured microglia cells is an extremely complicated task due to their strongly adhesion capacity, reducing their survival. Therefore, to directly assess phagocytosis we used confocal z-stacks and orthogonal projections to confirm the presence of the cargo within the microglial pouch.

A crucial issue when analyzing microglial phagocytosis by confocal imaging is the ability to visualize the phagocytic pouches surrounding the apoptotic cell in order to perform a correct identification of the phagocytosis process. In vivo, phagocytosis is usually executed by microglial terminal branches ("ball-and-chain") (Sierra et al., 2010); these processes are long and very thin, and therefore, failing to correctly label microglial branches would result in an underestimation of phagocytosis. The use of transgenic mice in which the microglial cytoplasm is labeled is a powerful tool to allow the discrimination of the fine branches that generate the phagocytic pouches and, thus, ensure a correct classification of the phagocytosed apoptotic cells (Beccari et al., 2018). The most frequently used transgenic mice are CX3CR1$^{+/GFP}$, in which the fluorochrome GFP is inserted in the locus of one fractalkine receptor allele (Jung et al., 2000). There is an increasing variety of reporter mouse lines available, but we here we have used fms-EGFP mice, in which EGFP is expressed under the control of the macrophage colony-stimulating factor receptor promoter (Geissmann et al., 2010; Sasmono et al., 2003; Sierra et al., 2010) to study microglial phagocytosis.

In conclusion, in vitro models represent a reductionist but valuable approach to unravel different signaling pathways involved in phagocytosis. Thus, although our in vitro model of phagocytosis presents some limitations, it comprises many characteristics of microglial phagocytosis in vivo, which will be advantageous in order to utilize it as a pivotal strategy to unravel the microglial changes triggered by phagocytosis.

7.2. PHAGOCYTOSIS ALTERS GENES INVOLVED IN MICROGLIAL PHYSIOLOGY, INCLUDING METABOLISM AND APOPTOSIS

We next analyzed the phagocytic microglial transcriptome using the in vitro microglial phagocytosis assay described above. We compared genome-wide transcriptome of naïve vs phagocytic microglia using gene expression arrays. We analyzed two time points after engulfment, an early (3h) and a late time point (24h), in order to study transcriptional changes over time.

There are numerous studies in the literature in which the microglial transcriptome has been analyzed in order to find unique microglial signatures compared to macrophages (Butovsky et al., 2014; Wes et al., 2016) or to analyze microglia in different neurodegenerative diseases (Hirbec et al., 2018), or against other brain cell types (Holtman et al., 2015; Zhang et al., 2014), but no systematic transcriptome analysis of phagocytic microglia (in vitro or in vivo) has been performed before. The only study we found on this topic compares ameboid vs ramified microglia using gene arrays by the long-standing assumption that ameboid microglia are phagocytic, whereas ramified microglia present a quiescent non-phagocytic phenotype (Parakalan et al., 2012). In this study, ameboid and ramified microglia from the corpus callosum of adult rats were isolated by laser-capture microdissection in order to perform gene arrays. They found that ameboid (presumably phagocytic) microglia was enriched in functions such as cell cycle, cytoskeleton, ribosome activity or migration, whereas ramified (presumably non-phagocytic) microglia expressed functions related to cellular homeostasis, cell projection, glial cell development, axon ensheathment, and regulation of synaptic transmission and plasticity. On the contrary using our well defined in vitro model of phagocytosis we found that phagocytic (24h) microglia comprised many of those functions that Parakalan (Parakalan et al., 2012) ascribed to both ameboid and ramified cells. The difference between our results might arise from their assumption that microglial morphology (ameboid and ramified) is directly related to a specific function (phagocytosis vs non-phagocytosis respectively). However, as we have mentioned in the previous section, this widely-held belief is changing, as there are growing evidences that confirm that microglia does not present discrete morphological phenotypes, but rather show different responses to different stimuli (Ransohoff, 2016). In agreement, our group has observed microglial phagocytosis regardless of their morphology. For instance, ramified microglia in physiological conditions are efficient phagocytes, whereas hypertrophic microglia during epilepsy are not (Abiega et al., 2016). Therefore, Parakalan's differences in gene expression between ameboid and ramified microglia cannot be ascribed to phagocytosis.

One main function that emerged from our gene arrays comparing naïve and phagocytic microglia is the cell physiology, including genes related to both apoptosis and metabolism. It is important to note that human SH-SY5Y cells do not have active transcription sites labeled with 5-Fluorouridine and their mRNA shows a degraded profile in a Bioanalyzer, suggesting that the transcripts found were specific to microglia, and not to engulfed apoptotic cells.

7.2.1. Phagocytic microglial show expression changes in genes related to apoptosis

Transcriptional analysis of phagocytosis showed genes involved in the regulation of cell death. These apoptosis-related genes could also be classified according to the cell in which they could exert their function: autologous genes would regulate apoptosis inside microglia, whereas heterologous genes would affect cells outside microglia. We found that the majority of the apoptosis-related autologous genes were pro-apoptotic, such as BAD (BCL2 associated agonist of cell death), FAS (Fas cell surface death receptor), FOXO3 (forkhead box O3), GAS1 (growth arrest specific 1), or PTEN (phosphatase and tensin homolog) and were upregulated in phagocytic microglia. The overexpression of these pro-apoptotic autologous genes could explain the microglial cell death that we observed in the in vitro phagocytic assay, as we found increasing levels of microglial apoptosis along the time course of phagocytosis, reaching 10% of apoptosis by 24h. We disregarded that this microglial death was the result of staurosporine present in the SH-SY5Y culture media since similar results were observed after several washes of the apoptotic cells prior to adding them to microglia. Importantly, microglial death was also unrelated to an excessive amount of apoptotic cells, or to the type of apoptotic cell (SH-SY5Y or NE-4C). Nonetheless, it is important to notice that we have never observed microglial apoptosis in vivo in physiological conditions (Abiega et al., 2016; Sierra et al., 2010), suggesting that the microglial death found in the in vitro phagocytic assay might be intrinsic to the culture.

On the other hand, the majority of the apoptosis-related heterologous genes were anti-apoptotic, such as CNTF (ciliary neurotrophic factor), FGF2 (fibroblast growth factor 2), FGF8 (fibroblast growth factor 8), or VEGFa (vascular endothelial growth factor) and were upregulated upon phagocytosis. These heterologous anti-apoptotic genes could support survival of other cells and they might be related to the neuromodulatory functions that phagocytic microglia exerts, and that will be further discussed in **section 7.5**.

7.2.2. Phagocytic microglia show expression changes in genes related to metabolism

Another notorious modification we found in the transcriptional analysis was changes in genes related to metabolism. We found that phagocytic microglia showed genes that suggested an enhanced glycolysis, decreased Krebb's cycle, and shutdown of oxidative phosphorylation. This gene expression pattern is reminiscent of the metabolic switch known as

the Warburg effect, which was originally described in cancer cells (Warburg, 1956). Warburg proposed that, independent of oxygen levels, tumor cells increase glucose uptake and produce ATP through glycolysis and fermentation, which leads to local acidification due to the increase in lactate production. Originally, Warburg proposed that cancer cells performed this metabolic switch due to mitochondrial impairment; however, subsequent studies disregarded mitochondrial malfunction in cancer cells (Moreno-Sanchez et al., 2007). Later on, the Warburg effect was also observed in other cell types, including macrophages upon LPS stimulation (Palsson-McDermott et al., 2015), which was characterized by an increase in glucose uptake, an enhancement of glycolysis, and a reduction in Krebb's cycle (Tannahill et al., 2013). The decrease Krebb's cycle results in an increment of succinate levels, which leads to the stabilization of the transcription factor hypoxia inducible factor-1α (Hif-1α), in turn inducing the production of the pro-inflammatory cytokine IL-1β (Koivunen et al., 2007; Palsson-McDermott et al., 2015; Tannahill et al., 2013). In microglia, the Warbug effect is also triggered by inflammation in vitro (O'Neill and Hardie, 2013; Orihuela et al., 2016). The stabilization of Hif-1α does not only induce the production of IL-1β, but it also triggers the enhancement of different trophic factors related to angiogenesis, cell survival and proliferation, such as VEGF, IGF2, or TGF-β3 (Lee et al., 2004b), which might suggest a role of this metabolic switch in neuroprotection. However, the possible metabolic switch from oxidative phosphorylation to glycolysis in phagocytic microglia might be a key signature of phagocytosis and will be studied in the future by analyzing mitochondrial respiration and glycolysis in live naïve and phagocytic microglia cells.

In addition, the metabolic shift towards glycolysis has been recently shown at the core of trained immunity in monocytes and macrophages (Netea et al., 2015). Trained immunity refers to the memory that immune cells develop upon different stimuli and it has been associated to both beneficial and detrimental consequences, such as the improved response of the immune cells after vaccination (Blok et al., 2015) or the exacerbated pro-inflammatory response in inflammatory diseases (Bekkering et al., 2015). Moreover, trained immunity has been associated to be mediated by mTOR, Hif-1α, and interleukin-1 family cytokines in monocytes and macrophages (Cheng et al., 2014; Moorlag et al., 2018). Moreover, repeated systemic inflammation also triggers trained immunity in microglia, resulting in a subsequent exacerbated inflammatory response, which is also known as priming (Haley et al., 2017; Wendeln et al., 2018). Our gene array data suggest that phagocytosis of apoptotic cells may trigger a similar metabolic switch in microglia, in which glycolysis and lactic fermentation are enhanced over Krebb's cycle and oxidative phosphorylation. This switch might be necessary for

the development of microglial immune memory (i.e., trained immunity), which could lead to a more rapid and efficient response by microglia upon reiterated exposure to apoptotic cells, a hypothesis that will be tested in the future.

7.2.3. Phagocytic microglial show expression changes in genes related to chromatin remodeling

Interestingly, we also found several functions in phagocytic microglia that are related to chromatin modification and transcription factors. The gene expression results suggest that phagocytosis might also trigger long-term transcriptional changes in microglia through epigenetic modifications. In order to test this hypothesis, we performed a DNA methylation analysis of naïve and phagocytic microglia at 24h and 3d post phagocytosis to detect long-term changes. However, this experiment is currently under analysis and thus, it will not be part of the results of this Thesis Project. Nevertheless, these changes in chromatin modification and transcription factors might be related to the metabolism changes that we observed in phagocytic microglia, as trained immunity in macrophages requires the metabolic switch and is based on chromatin remodeling and epigenetic reprogramming (Pennisi, 2014). In more detail, trained immunity in macrophages is supported by epigenetic modifications in positive histone regulatory marks such as H3K4me1, H3K4me and H3K27ac (van der Meer et al., 2015). Similarly, trained immunity has been recently observed in microglia as a result of reiterative exposure to peripheral inflammatory stimuli, and also requires epigenetic modifications such as H3K4me2 and H3K27ac (Haley et al., 2017).

Taking all this data into account, it is not surprising that in the transcriptional analysis of phagocytic microglia we observe changes that point towards chromatin modification. These data suggest that upon phagocytosis microglia may alter their metabolism, becoming more glycolytic, which might be related to epigenetic modifications in order to provide microglia with the capacity to maintain long-term changes. In addition, this memory triggered by the phagocytosis of apoptotic cells could contribute to the establishment of microglial heterogenic populations in different regions of the brain, a hypothesis that would be tested in the future.

7.3. PHAGOCYTOSIS TRIGGERS AN IMMUNOMODULATORY PROGRAM IN MICROGLIA

In addition to changes in apoptosis and metabolism, transcriptomic analysis of microglial phagocytosis also showed changes in inflammation-related genes. Phagocytosis of apoptotic

cells was initially considered as immunologically silent process; however, over the past 20 years phagocytosis has been proposed to trigger an active anti-inflammatory response (De Simone et al., 2003; Fadok et al., 1998; Fraser et al., 2010; Lucas et al., 2006). Fadok and colleagues showed that phagocytosis of apoptotic neutrophils by human monocyte-derived macrophages actively decreased the production of pro-inflammatory mediators such as IL-1β, IL-8, IL-10, TNF-α, or leukotriene C4, whereas in turn, it increased the production of anti-inflammatory TGF-β, PGE2 (prostaglandin E2), or PAF (platelet-activating factor) (Fadok et al., 1998). The addition of any of these three anti-inflammatory mediators to LPS-stimulated macrophages decreased the production of cytokines and therefore, they related the molecules produced upon phagocytosis with an-anti-inflammatory effect. Similarly, Lucas (Lucas et al., 2006) showed that feeding mouse macrophages with human neurophils decreased the production of the pro-inflammatory TNF-α and increased the anti-inflammatory TGF-β. In addition, they reported that this TGF-β release was partially the responsible of the decrease in TNF-α..

7.3.1. Phagocytic microglia present an enhanced expression of several both pro- and anti-inflammatory cytokine genes

Surprisingly we found that the expression of several both pro- and anti-inflammatory cytokines were significantly changed in phagocytic microglia compared to naïve microglia. Contrary to what was reported in the literature we observed an overexpression of pro-inflammatory CSF3, IL-1β, IL-6, TNF-α, whereas in agreement with previous reports, we found an increase in the anti-inflammatory TGF-β. Nevertheless, our gene arrays also showed changes in over 40 different cytokines, such as IL-10 (up-regulated at 3h and down-regulated at 24h) or IL-4 (down-regulated at 3 and 24h).

The majority of the above mentioned studies (De Simone et al., 2003; Fadok et al., 1998; Fraser et al., 2010; Lucas et al., 2006) did not perform genome-wide expression analysis, but focused only on a short list of 4-6 cytokines. In addition, the majority of the studies regarding phagocytosis and cytokine production have been performed in macrophages, with the exception of De Simone and Fraser's works (De Simone et al., 2003; Fraser et al., 2010), which were performed in rat primary microglia. Thus, our data suggest a more complex immunomodulatory effect of phagocytosis on microglia than previously thought.

7.3.2. Phagocytic microglia show alternative responses to inflammatory stimuli such as LPS

The cytokine profile triggered in microglia after phagocytosis that we found in our in vitro assay shares similarities with the cytokine expression pattern associated to inflammation. Inflammatory stimuli such as LPS, peptidoglycan, or poly(I:C) also leads to the increased secretion of IL-1β, IL-6, TNF-α in culture. In addition, inflammatory microglia also release IFN-α, IFN-β, IL-10, IL12, IL-18, nitric oxide (NO), and chemokines such as macrophage-inflammaroty protein (MIP)-1a, monocyte chemotactic protein (MCP)-1, and chemokine ligand 5 (RANTES) (Lehnardt, 2010). Therefore, our phagocytic microglia shows a mixed expression profile of mRNA cytokines which holds some parallelism to the pro-inflammatory profile triggered upon inflammation and to the anti-inflammatory profile that was previously described after phagocytosis of apoptotic cells **(section 7.1)**, rendering microglial phagocytosis an immunomodulatory process.

To directly test this hypothesis, we challenged phagocytic microglia with LPS, and found that phagocytic microglia were overall very similar to naïve microglia, but one key difference was the mRNA expression of the pro-inflammatory TNFα, which was reduced in phagocytic microglia compared to LPS-treated microglia. Our results are to some extent in disagreement with previous reports, possibly because of differences in the culture technique. De Simone and colleagues (De Simone et al., 2003) used a model of cultured rat microglia fed with apoptotic PC12 neurons in heat-inactivated serum and reported a reduced production of the pro-inflammatory molecules NO (nitric oxyde) and TNF-α upon LPS stimulation. Moreover, Fraser and colleges showed that LPS-treated rat microglia increased pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α after the phagocytosis of neuronal apoptotic cells. However, when the coverslips were coated with C1q prior to plating microglia, these pro-inflammatory cytokines were decreased (Fraser et al., 2010). Therefore, all these experimental differences are likely to contribute to the differences in the immunological outcome of phagocytosis that have been previously reported and thus, it could explain the fact that we observed several pro-inflammatory cytokines (CSF3, IL-1β, IL-6, TNF-α) up-regulated after phagocytosis.

In conclusion, we speculate that the immunological outcome of phagocytosis might not be ascribed to the reductionist dichotomy of pro- or anti-inflammatory, but that it is a rather more complex mechanism that merges molecules of different immunological nature. However,

the role of the majority of the immune molecules that we found to be released upon phagocytosis, have been studied in the concept of inflammation.

7.4. MICROGLIAL PHAGOCYTOSIS TRIGGERS A NEUROMODULATORY PROGRAM

7.4.1. Phagocytic microglia regulate the expression of 224 neurocandidates

Our transcriptional analysis also showed that phagocytosis initiated a coordinated transcriptional program in microglia. Using a strategy based on unbiased functional analyses (DAVID, ClueGO, and WGCNA/IPA) as well as a focused search using the neurogenesis-specific MANGO database, we identified up to 224 genes whose expression changed after phagocytosis, and potentially affect neurogenesis. Most of these molecules were peptides (such as VGF), trophic factors (IGF-1), matrix metalloproteases (MMP3), and cytokines (CSF3). Importantly, this neuromodulatory program was also triggered when a physiological model of phagocytosis was used (1:1 proportion of apoptotic cells to microglia). It is also interesting to note that many of the neurocandidate genes did not require full engulfment, as their expression in microglia was induced by the conditioned media from apoptotic cells. In the future, it would be interesting to determine which molecules (either released or membrane-bound) are used by apoptotic cells to initiate and coordinate the neuromodulatory program in microglia.

One interesting candidate is Hif-1α (Hypoxia-inducible factor-1-α), which is the major transcriptional regulator in response to hypoxia (Nizet and Johnson, 2009). Under hypoxic conditions, Hif-1α induces the transcription of several genes, including VEGF and erythropoietin, which are involved in biological processes such as angiogenesis, erythropoiesis (Lee et al., 2004a), and neurogenesis (Nowacka and Obuchowicz, 2012). In addition, Hif-1α also exerts hypoxia-independent functions by inducing transcription of genes involved in cell proliferation and survival, as well as glucose and iron metabolism (Weidemann and Johnson, 2008). Hif-1α promotes glycolysis and inhibits Krebb's cycle, similar to the metabolic changes suggested in our array data. Therefore, since phagocytic microglia presents an enhanced glycolysis and decreased Krebb's cycle, and it also exerts a variety of functions that might regulate neurogenesis, we propose that Hif-1α might be a possible master regulator of the metabolic and neurogenic functions of phagocytic microglia. Nevertheless, the metabolic

switch and the role of Hif-1α in phagocytic microglia require further study and validation that will be performed in the future.

7.4.2. The neuromodulatory program needs to be validated in vivo

To validate in vivo this neuromodulatory program we have used different strategies. We first compared by RT-qPCR a population enriched in phagocytic microglia isolated from the dentate gyrus (DG), where neurogenesis occurs and apoptosis is abundant; with non-phagocytic microglia isolated from the CA region of the hippocampus, where there is no neurogenesis and therefore no apoptosis. After four replicas of 10 mice each, we were only able to observe a tendency to find higher expression of neuromodulatory molecules. However, it must be noticed that the DG contains a highly heterogeneous population of microglia, including many non-phagocytic cells from the hilus and the molecular layer. For instance, in the SGZ proper, only 30% of microglia are engaged in phagocytosis (Sierra et al., 2010).

Therefore, in order to overcome this limitation, we performed a single cell RNA-sequencing of the same regions (CA and DG). Our purpose was to discriminate the expression profile of microglia population at different phagocytic stages using a new bioinformatics approach (the NBOR algorithm: 'neighborhood-based ordering of single cells'; (Schlitzer et al., 2015)). This approach can objectively determine the position of a given cell in a developmental continuum and calculate the similarity of each single cell's gene-expression profile to a defined gene set of a particular cell population (landmark) and then order each cell according to such similarity score into a spatial continuum around those landmarks. The single-cell RNASeq was performed in a total of 192 cells from DG and 96 cells from CA in collaboration with Dr. Florent Ginhoux (A*Star, Singapore). However, we did not observed differences between the expression of CA and DG population, most likely because the DG is not sufficiently enriched in phagocytic microglia.

Since none of these two approaches proved valuable for our validation, we also utilized alternative strategies in order to validate in vivo the phagocytic microglial neurogenic program. We hypothesized that if the apoptosis of newborn cells could be enhanced, consequently microglial phagocytosis would increase and therefore, we could obtain a DG population highly enriched in phagocytic microglia. We administered IGF-1 via osmotic pumps into the hippocampus of 1mo mice. IGF-1 is a neurogenic factor that increases neurogenesis and might subsequently increase apoptosis (Sierra et al., 2015). However, we could not find the exact

time point in which apoptosis was increased, probably due to an efficient phagocytosis **(data not shown)**. Following a similar strategy, we also tried to induce an increase in apoptosis by the administration of temozolomide, an apoptotic inductor in proliferative cells (Garthe et al., 2009). Nevertheless, as with IGF-1, we were not able to find the time point of increased apoptosis and phagocytosis **(data not shown)**. An alternative model that we are planning to use to enrich phagocytosis is cranial irradiation. In a recent collaboration with Dr. Klas Blomgren (Karolinska Institute, Sweeden), our group had the opportunity to analyze microglial phagocytosis of irradiated mice and we have discovered that there is a large increase in apoptosis of DG newborn cells that is matched by increased microglial phagocytosis **(Figure 34; data from Sol Beccari's PhD Thesis)**. However, as large doses of irradiation have been reported to trigger microglial immune response (Monje et al., 2003), we will first determine the minimal irradiation dosage necessary to induce DG apoptosis and microglial phagocytosis to use this model to eventually confirm that phagocytosis of apoptotic cells triggers a neuromodulatory program in microglia in vivo. Another strategy that we are planning to pursue in order to validate the phagocytic microglial transcriptome in vivo is the analysis of the microglial ribosome associated mRNA by RiboTag procedure, which would allow the analysis of the active mRNA that is being translated (Haimon et al., 2018). RiboTag mice possess a modified RPL22 protein (Ribosomal protein L22), which participates in the formation of the ribosome. The final exon of this gene is flanked by LoxP recombination sites followed by an identical exon that also encodes hemagglutinin (HA) epitope before the stop codon of the gene. When RiboTag mice are crossed with a Cre mouse of the cell of interest, the LoxP flanked RPL22 exon is removed, resulting in the expression of RPL22-HA protein. Thus, the mRNA that is being translated within the RPL22-HA ribosome can be purified and analyzed, which will allow us to selectively study the active mRNA of microglia.
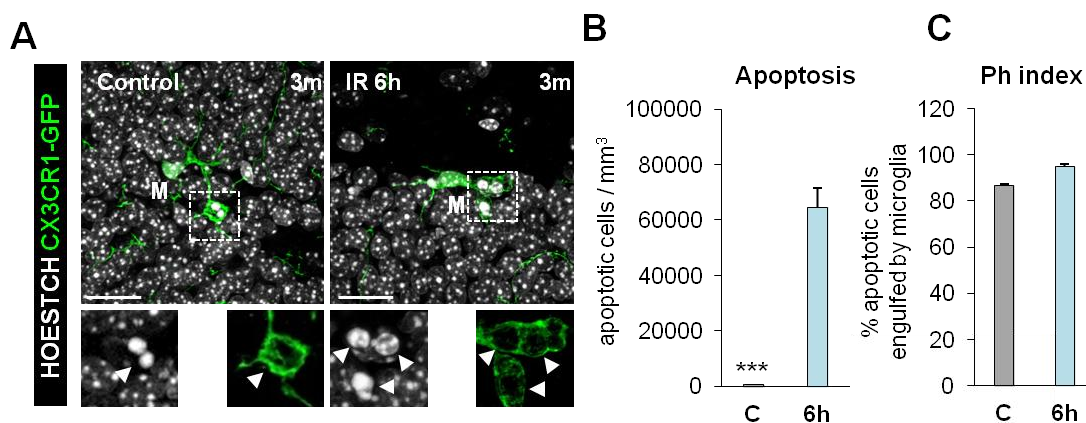
Figure 34. Microglial phagocytosis efficiency after irradiation. [A] Representative confocal z-stack of 6h irradiated mice immunofluorescence in the mouse hippocampal DG. Microglia were labeled with fms CX3CR1-GFP (green) and apoptotic nuclei were detected by pyknosis/karyorrhexis (white, DAPI). [B] Quantification of apoptosis in control and after 6h of irradiation. [C] Percentage of apoptotic cells engulfed by microglia (Ph-index) in control and after 6h of irradiation. Data from Sol Beccari´s PhD Thesis.

7.5. MICROGLIAL PHAGOCYTOSIS MODULATES NEUROGENESIS AND GLIOGENESIS

In order to test the effect of our microglial phagocytosis-related neurocandidates on neurogenesis, we analyzed the effects of control and phagocytic CM both in vitro and in vivo. In vitro we cultured neuroprogenitor cells with CM obtained from either control or phagocytic microglia, whereas in vivo, we injected the CM from control and phagocytic microglia into 2mo fms-EGFP mice for 6d using osmotic pumps and assessed neurogenesis 2h and 28d after BrdU administration. We found that the regulation of the neurogenic niche by the secretome of phagocytic microglia might be exerted by the modulation of neurogenesis or gliogenesis, as we will discuss in the next sections.

7.5.2. Phagocytosis triggers gliogenesis in vitro but not in vivo

In vitro, naive microglia CM gave rise to a large proportion of astrocytes characterized by a stellate morphology and high expression of GFAP; and a small proportion of neuron-commited cells (neuroblasts), ramified cells with high level of DCX. In contrast, neuroblasts were never observed in cultures treated with phagocytic microglia CM, which gave rise to astrocyte-committed cells. These cells presented a bipolar phenotype reminiscent of radial glia (Encinas and Enikolopov, 2008); expressed astrocyte markers, such as GFAP, and S100β (Encinas and Enikolopov, 2008; Raponi et al., 2007); and showed a strong increase in intracellular calcium in response to ATP, typical of astrocytes (De Melo Reis et al., 2011). However, bipolar cells also showed high levels of nestin, an intermediate filament that is typically expressed in neuroprogenitors and other mitotic cells (Lopez-Atalaya et al., 2017). To determine whether bipolar cells were neuroprogenitors, we performed a pluripotency assay by allowing them to differentiate in conventional media. We found that bipolar cells resulting from treatment with phagocytic CM gave rise to stellate cells and none neuroblasts suggesting that they are indeed committed to the astrocyte lineage. In order to further characterize the bipolar phenotype induced by phagocytic CM, we are currently performing Western Blot

analysis of different fate-committing transcription factor, REST (RE1 silencing transcription factor 1), Ascl (ASC1 like protein) and p-Smad (phospho-Smad) in the CM-treated cultures. REST is a repressor of neuronal genes and is highly expressed in astrocytes (Kohyama et al., 2010); Ascl induces neuronal differentiation and therefore is elevated in neurons (Liu et al., 2015); and p-Smad is highly expressed in astrocytes (Kohyama et al., 2010). The levels of these transcription factors in the CM-treated samples will be compared to those in neurospheres, astrocytes, and neurons in order to further elucidate the nature of the bipolar cells induced by phagocytic microglia. Therefore, these in vitro data suggest that microglial phagocytosis might modulate the neurogenic cascade by promoting the differentiation of neuroprogenitor cells into astrocytes.

The potential gliogenic effect of phagocytic microglia was also suggested by our transcriptional assays, since we found the functions 'gliogenesis' and 'glial cell differentiation' significantly up-regulated in the ClueGo analysis. Nevertheless, the majority of the genes found under those categories were autologous, and therefore, their overexpression would only modulate the microglia cell expressing them. We were only able to find four genes within the 'gliogenesis' and 'glial cell differentiation' categories that could exert heterologous functions: NLGN3 (neuroligin 3), NRG1 (neuregulin 1), S100a8 (S100 calcium binding protein A8), and S100a9 (S100 calcium binding protein A9). Both NLGN3 and NRG1 are post-synaptic adhesion molecules, mainly expressed in neurons, although glial expression has also been reported, and they promote maturation of functional synapses (Medina et al., 2018; Ting et al., 2011). NLGN3 is cleaved in an activity dependent manner and the soluble form mediates glioblastoma growth (Jeong et al., 2017). In addition, NRG1 can drive microglial proliferation after nerve injury (Calvo et al., 2011) and NRG1 signaling downregulation can trigger the stem cell transformation into astrocytes (Schmid et al., 2003). The mechanism by which these two molecules cleave in non-neuronal cells has not been studied, and therefore, whether their overexpression in phagocytic microglia could also consequently lead to an enhancement of their soluble forms is yet to be determined. If the soluble forms of NLGN3 or NRG1 were to be detected in the conditioned media from phagocytic microglia, NLGN3 might act as a mitogen and contribute to the expansion of astrocytes in culture. However, the overexpression of NRG1 should not drive neuroprogenitor cells towards astrocytes, and therefore, none of these two molecules could explain the development of neuroprogenitor cells into the bipolar astrocytes that we observed after phagocytic CM treatment. On the other hand, S100a8 and S100a9 are calcium binding proteins usually localized within the cell cytoplasm, but they can also be released. These two molecules can form a heterodimer (S100a8/a9) and its release has been

related to the priming of microglia after a neuroinflammatory stimulus (Denstaedt et al., 2018). Therefore, none of these four candidate molecules are likely to promote the neuroprogenitor differentiation towards astrocytes that we found in the in vitro differentiation assay.

In addition, in vivo, astrocytes are produced directly from the NSCs, after they have exhausted their mitotic potential (Encinas et al., 2011). However, CM from phagocytic microglia administration did not increase newborn astrocytes in the 28d experiment, highly suggesting that phagocytic microglia does not promote the direct conversion of NSC into astrocytes. Therefore, the gliogenesis observed in the in vitro studies might occur as an indirect consequence of the modulation of neurogenesis by phagocytic microglia, as we will discuss in the next section.

7.5.2. Phagocytosis limits neurogenesis in vivo by the regulation of the survival of the neuronal-committed cells

The idea that microglia could exert beneficial functions on neurogenesis in physiological conditions is not new. There are some evidences in the literature suggesting that macrophages are able to produce different trophic factors upon phagocytosis. For instance, phagocytic microglia in culture produce TGF-β and NGF (De Simone et al., 2003) and, similarly, phagocytic hepatic macrophages produce VEGF (Golpon et al., 2004). These three factors are regulators of hippocampal neurogenesis in vivo (Buckwalter et al., 2006; Cao et al., 2004). In addition, upon IL-4 or IFNγ administration, cultured microglia decrease levels of TNFα and produce IGF-1, which help to maintain critical functions such as neurogenesis or oligodendrogenesis (Butovsky et al., 2006). Thus, trophic factors released from phagocytic microglia may contribute to the regulation of neurogenesis (Sierra et al., 2014). Therefore, since our data suggest that it is unlikely that phagocytic microglia regulates the neurogenic niche by an induction of gliogenesis, we also focused on the possibility that this modulation is mediated by the regulation of neurogenesis by affecting different steps of the cascade such as proliferation or survival of the newborn cells.

Although we have not directly assessed neuroprogenitor proliferation, the cell density between 3 and 5d increases in both naïve microglia CM and DMEM, suggesting that there is an intrinsic continuous proliferation of the culture. On the contrary, in phagocytic microglia CM the cell density between 3 and 5d decreases. However, we cannot discard that the bipolar cell

culture does not divide because any virtual proliferation would be masked by the excess of apoptosis unless the proliferation matched the apoptosis observed in this culture. In addition, in vivo experiments showed no differences in proliferation at 2h after BrdU (although there is a tendency to find fewer BrdU cells, which will be further validated by increasing the 'n' in order to obtain a higher statistical power) and a decrease of BrdU cells after 28d, suggesting that microglial phagocytosis might not be modulating proliferation, but rather the survival of the the neuronal-committed cells.

Indeed, one important effect of the phagocytic microglia CM in vitro is that it resulted in the death of up to 50% of neuroprogenitors in early differentiation assays. In contrast, naïve microglia have been shown to promote survival of cultured neuroprogenitors. Microglia CM from primary rat cultures promoted neuronal survival after 7d in culture compared to non-treated neurons (Morgan et al., 2004). Similarly, neuroblast production in a SVZ-derived culture was increased when culturing with microglia CM (Walton et al., 2006). However, several lines of evidence argue against a toxic effect of phagocytic microglia CM. First, the majority of the pro-apoptotic genes that were up-regulated in phagocytic microglia were autologous, with the exception of pro-inflammatory cytokines. However, as we discussed above, inflammatory microglia CM not only did not induce cell death, but it also increased neuroblast population in vitro, suggesting that pro-inflammatory cytokines do not promote apoptosis in our in vitro model. Second, in vivo administration of phagocytic microglia CM does not increase the total number of apoptotic cells. However, it must be noted that in vivo in physiological conditions, microglial phagocytosis is a fast process that lasts around 1.5h (Sierra et al., 2010), and therefore increases in apoptosis might be masked by the high efficiency of removal. And third, in the in vitro late survival neurogenesis assay the phagocytic microglia CM did not induced cell death. Therefore, the phagocytic microglia CM is unlikely to be toxic per se but rather seems to affect the survival/differentiation of early neuroprogenitor cells.

Thus, we studied those molecules of our neurocandidates that could potentially alter the survival/differentiation of the neurogenic cascade. We focused on the up-regulated neurocandidates because this was the only gene group in which the term neurogenesis showed up. Among the up-regulated neurocandidates, we found that IL-1β, IL-6 and TNFα have been reported to decrease survival of neuroprogenitor cells in vitro (IL-1β, TNFα) and in vivo (IL-6) (Breton and Mao-Draayer, 2011), which results in an inhibition of adult neurogenesis. However, in our hands inflammatory microglia CM did not trigger a reduction in the survival of neuroprogenitor cells, and thus the effects of these candidates on the

191

neuroprogenitor cell culture should be extensively studied in the future. Next, we hypothesized that the lack of an essential factor in the media obtained from phagocytic microglia could also be responsible for the reduction in survival. IGF-1, for example, enhances proliferation and survival of stem cells (Huat et al., 2014) and is down-regulated upon phagocytosis, therefore it is likely that the absence or reduction of key molecules could also mediate the phagocytic microglial effects on the modulation of neurogenesis.

Since both in vitro and in vivo experiments support the hypothesis that the regulation of neurogenesis by phagocytic microglia might be mediated by the modulation of the survival/differentiation of newborn cells, we next focused on analyzing whether the identity of the cells in the neurogenic cascade were being affected (NSCs, ANPs, or neuroblasts). In vivo experiments showed no changes in NSCs between CM from naïve and phagocytic microglia suggesting that they are not the target cells of the neuromodulatory program. Nevertheless, both at 6d and 4w, NSCs presented a tendency to decline, which needs to be further studied in order to validate any differences in these cells. Moreover, ANPs are unlikely to have been affected by the CM microPH treatment because their immediate product, the AB neuroblasts, are not altered at 6d. Finally, in vivo experiments showed a decreased in the most mature neuroblasts (EF) at 28d, suggesting that neuroblasts are the cell population that is mostly affected by the phagocytic microglia CM. Overall, these results suggest that phagocytic microglia modulates neurogenesis via the reduction in the survival of neural-committed cells.

It is important to note that in order to understand the relevance of this neuromodulatory program, as well as the discrepancies between in vitro and in vivo data, first, we must take into account that our in vitro analysis is a reductionist approach of what can be observed in vivo. During adult neurogenesis, microglia phagocytose the excess of newborn cells that are generated (Sierra et al., 2010). The peak of neurogenesis, and consequently apoptosis and phagocytosis is reached at 1mo, in which at most, around 30% of phagocytic microglia (containing a phagocytic pouch) can be observed in the subgranular layer of the DG. This means that there are a great number of non-phagocytic microglia merged in the neurogenic cascade, which are in the subgranular layer, molecular layer and the hilus. Therefore, in vivo neuromodulatory molecules released by phagocytic microglia would have a localized effect on neurogenesis since only cells in close proximity to a phagocytic site would be affected. On the contrary, in vitro we have generated an exaggerated model in which almost every single microglia is phagocytic, therefore all of them produce the different factors that might modulate neurogenesis; and every single neuroprogenitor cell in the culture is

affected by the conditioned media. Thus, what in physiological conditions might be a more localized action, in the in vitro study gets completely exacerbated in the culture, leading to the idea of a detrimental effect of the phagocytic conditioned media if it is not put into context.

7.6. POTENTIAL MECHANISMS UNDERLYING THE EFFECT OF PHAGOCYTOSIS ON NEUROGENESIS

In order to find the possible mechanism that exerts neuromodulatory function of phagocytic microglia, we searched among the 224 neurocandidates identified in our transcriptional assay. We will focus on three main mechanisms: inflammatory cytokines; the metabolite lactate; and the neuropeptide VGF.

7.6.1. Inflammatory cytokines are unlikely to mediate the effect of phagocytic microglia CM on neurogenesis in vitro

Inflammation is a crucial mechanism triggered in response to stimuli that are recognized as noxious, such as pathogens, cell death or injury. In the presence of an inflammatory insults, microglia release different inflammatory mediators such as cytokines, proteins of the complement cascade or chemokines (Nayak et al., 2014). The majority of the molecules released upon inflammation are almost undetectable in physiological CNS; nevertheless, these molecules are rapidly produced in response to an inflammatory stimulus. An excessive and chronic release of these cytokines results in detrimental consequences for the tissue, which have been demonstrated to contribute to tissue damage in several neurodegenerative diseases such as AD, Parkinson's, stroke, MS, epilepsy (Amor et al., 2010; Vezzani et al., 2011).

Inflammatory microglia have previously been reported to modulate neurogenesis in vivo. As stated above, microglial activation upon inflammatory stimuli has considered to trigger deleterious consequences for adult neurogenesis. Cranial irradiation therapy results in cognitive decline and chronic inflammation and it has been associated to adult neurogenesis impairment in vivo, which was reestablished after the blockade of neuroinflammation by indomethacin, which inhibits the synthesis of proinflammatory prostaglandin (Monje et al., 2003). Moreover, upon inhibition of microglial activation by the administration of the antibiotic minocycline neurogenesis was restored in epileptic rodents (Ekdahl et al., 2003). This data linked microglia to a detrimental effect on neurogenesis, however, our data suggests that the relationship between inflammation and neurogenesis is more complex.

193

DISCUSSION

To test whether the immunodulatory effects of phagocytosis were responsible for the in vitro effects we observed on neurogenesis after treatment with phagocytic microglia CM, we performed a neurogenesis assay in which we compared the neuroprogenitor cell differentiation when cultured with phagocytic and LPS-treated microglial conditioned media, using LPS as control. To our surprise, not only did we not observe a reduction in neuroblast cells upon LPS-treated microglia CM or LPS culturing, but we found an increase in neuroblasts, in disagreement with influential papers such as (Monje et al., 2003), where they showed that CM from LPS-treated microglia (BV2 cells) reduced neuroblast survival, an effect that was mediated by IL-6. In order to confirm these results we mimicked Monje´s protocol (BV2 cells, high LPS dose), but we still found an increase in the neuroblast population. Thus, our results suggest that inflammation may not be as detrimental for neurogenesis as previously thought.

Interestingly, LPS per se increased the number of neuroblasts in the culture. An increase in neurogenesis 28d after LPS administration in vivo was reported by (Bland et al., 2010), which might be the result of a compensatory boost in neurogenesis after an initial decrease of the neural progenitor cell population (Valero et al., 2014). Moreover, neural progenitor cells also express TLR receptors (Toll like receptor), which recognize the conserved structural motifs (so called PAMPs, pathogen-associated molecular patterns) in pathogens and trigger the inflammatory response in microglia and macrophages (Rolls et al., 2007). TLRs are activated by LPS, and therefore, the augmented neuroblast cell population in our LPS-treated control group could be due to the direct effect of LPS on the neuroprogenitor cells rather than being mediated by cytokines. In addition, the neuroblasts that we observed in both LPS-treated microglia CM and LPS neuroprogenitor cultures, showed a shorter and thicker morphology than those grown in the naïve microglia CM, which suggests that, although abundant, these neuroblasts might be somehow aberrant and might not develop a correct function and connectivity.

In order to discard the possibility that the neuroblast increase that we observed in LPS-treated microglia CM and LPS neuroprogenitor cultures was due to a direct effect of any remaining LPS on neuroprogenitor cells, we developed another paradigm in which we treated primary microglia with LPS for 6h in order to trigger the inflammatory response and then change to fresh media for another 18h, which would not contain any LPS. Next, we treated neuroprogenitor cells with either LPS or CM microLPS 6h + 18h. Similar to the previous LPS paradigm, we found an increase in neuroblast cell population, although this time, LPS-treated microglia CM triggered neuroblasts presented a more immature phenotype that the LPS

treated cultured, as shown by an increase levels of nestin cell marker in LPS-treated microglia CM culture. Overall, these data suggested that our primary microglia enhanced the production of neuroblast cell population. The discrepancies of these results with the already published literature might reside on our strategies for culturing microglia detailed in **section 7.1**. In addition, the observed enhancement of neuroblast population does not ensure the correct function and connectivity of the cells, and therefore, it would not necessarily translate into an increase in neurogenesis. Thus, further research is necessary in order to unravel the role of microglial produced cytokines in the modulation of neurogenesis.

7.6.2. The metabolite lactate does not mediate the effect of phagocytic microglia CM on neurogenesis in vitro

Another possible mechanism driving the modulation of neurogenesis by phagocytic microglia is the metabolite lactate, which is a product of glycolysis. As we have discussed in the **section 7.2**, phagocytic microglia showed major transcriptional changes in metabolism; mitochondrial processes such as Krebb's cycle and oxidative phosphorylation were downregulated, whereas glycolysis was increased. These alterations in metabolism could lead to the production of lactate by phagocytic microglia. Lactate has been described to exert hormone functions and it has been related to memory formation and neuroprotection (Proia et al., 2016) and it might also support neurogenesis in vivo (Alvarez et al., 2014). Therefore, we studied the production and release of this metabolite in the CM form naïve, phagocytic, and LPS-treated microglia. There were no differences between the lactate concentration produced by naïve and phagocytic microglia, suggesting that lactate was unlikely responsible to mediate the neuromodulatory effects triggered by phagocytosis. Interestingly, lactate production was enhanced in LPS-treated microglia. As mentioned in **section 7.2**, the Warburg effect, which is characterized by an enhancement in glycolysis and a reduction in Krebb's cycle (Tannahill et al., 2013), has been observed in LPS-stimulated macrophages (Palsson-McDermott et al., 2015). Therefore, an increased in lactate production suggests that our LPS-treated microglia might have undergone the metabolic switch towards glycolysis and it could be related to a microglial priming induced by LPS in order to trigger an enhanced response upon reiterative inflammatory stimuli.

7.6.3. The peptide VGF is overexpressed in phagocytic microglia in vitro

Finally, we studied the role of the neurocandidate that showed the highest FC changes in both the arrays and RT-qPCR, the neuropeptide VGF. VGF is a neuropeptide that has been

implicated in metabolism and neurogenesis (Foglesong et al., 2016; Thakker-Varia et al., 2014), and to our knowledge, we are the first group who shows that microglia express VGF and that this expression is enhanced upon phagocytosis. In addition, we are currently validating VGF protein expression in phagocytic microglia both in vivo and in vitro. Nonetheless, we have not been able to identify yet the exact mechanism underlying microglial phagocytosis effects on neurogenesis.

In addition, it is possible that the driving mechanism of phagocytic microglia is not merely a single molecule alone, but the result of the combination of several neuromodulatory molecules. We would like to narrow down the search by characterizing the exact proteins that have been released by phagocytic microglia in the CM media. Since we have identified 224 virtual candidates, the study of all of them by single ELISA is not plausible. In addition, the available commercial ELISA multiplex kits offer the possibility to study fixed groups of proteins, such as angiogenic or immune proteins, and there are no offers available for the vast majority of our candidates. Moreover, personalized assays or full murine proteome ELISA are extremely expensive techniques. Furthermore, we had to discard the option of performing Liquid Chromatography followed by Mass Spectrometry due to the presence of serum proteins in our media, since it could mask the results of lower expressed proteins. In future experiments, we will add or block our candidates of interest in the CM in order to test whether they are responsible of the phenotypes we observed in phagocytic CM. In this task we will either try to modulate single molecules by addition or deprivation or we will use Seppro protein depletion resins (Sigma Aldrich) in order to remove several neuromodulatory molecules at the same time from the CMs.

7.6.4. Alternative approaches to validate the role of phagocytosis on neurogenesis in vivo

We are currently developing alternative strategies to demonstrate the role of microglial phagocytosis on neurogenesis in vivo, based on the genetic knock-down or pharmacological manipulation of key phagocytic receptors. To identify suitable targets, we have focused on a model of microglial phagocytosis impairment, a mouse model of epilepsy by intrahippocampal injection of the glutamate agonist kainic acid (Abiega et al., 2016). We first focused on glutamate receptors, whose expression in microglia had been shown in vitro (Beppu et al., 2013; Domercq et al., 2013). Thus, we studied the expression of both ionotropic (AMPA, KA, and NMDA) and metabotropic (Grm1-8) in acutely purified microglia from the hippocampus

and cortex of 2mo fms-EGFP mice. We detected a residual expression of mRNA of all subunits in hippocampal and cortical microglia by RTqPCR, however, this poor expression was unlikely to lead to the formation of functional receptors. Therefore, it is unlikely that microglia interacts with the neurogenic niche via glutamate signaling.

In addition, we focused on phagocytic and purinergic receptors involved in finding and tethering apoptotic cells, and characterized their expression on acutely purified microglia from the hippocampus of control and kainic mice. Purinergic receptors (P2X4, P2Y6, and P2Y12) were significantly increased, while the apoptotic cell recognition receptors (Trem2, MerTK, CR3, and GPR34) were significantly decreased in microglia from KA mice. Furthermore, some microglial purinergic receptors have been described to modulate neurogenesis. For example, the reduction of microglial P2Y13 receptor has been recently shown to enhance neurogenesis in P2Y13 KO mice (Stefani et al., 2018). Interestingly, our arrays showed a downregulation of P2Y13 receptor upon phagocytosis, suggesting that phagocytosis might regulate purinergic receptors in turn impacting on neurogenesis. These results suggest that modulating the expression or function of purinergic and recognition receptors may alter microglial phagocytosis and allow us to further test its impact on neurogenesis.

We have analyzed three different constitutive KO mice, for P2Y12, GPR34 and a double transgenic KO for MerTK and Axl **(Figure 35; data from Victor Sanchez-Zafra and Iñaki Paris PhD Theses)**. All these receptors have been described to actively participate in the phagocytic process (Preissler et al., 2015; Scott et al., 2001). We first demonstrated by RT-qPCR that except for Axl, these receptors are selectively expressed in microglia. Second, we confirmed that the absence of these receptors resulted in an impairment in microglia phagocytosis. Lastly, we studied neurogenesis in these phagocytosis impaired KO models. We found that at 1mo, P2Y12 KO mice presented a decrease in both neuroblast and neuroblast proliferation, MerTK/Axl KO showed a reduction in neuroblast numbers and GPR34 did not show differences until 3mo, in which DCX$^+$ cells were decreased. In addition, in P2Y12 KO mice, BrdU$^+$ cells and newborn neurons (NeuN/BrdU) were diminished. Constitutive KO mice present several limitations, since the reduction in neurogenesis is the result of a continuous decrement in phagocytosis and many compensatory mechanisms may have played a role before the analysis at 1mo. However, this data highlights the importance of microglial phagocytosis for the correct long-term maintenance of the neurogenic cascade.

DISCUSSION
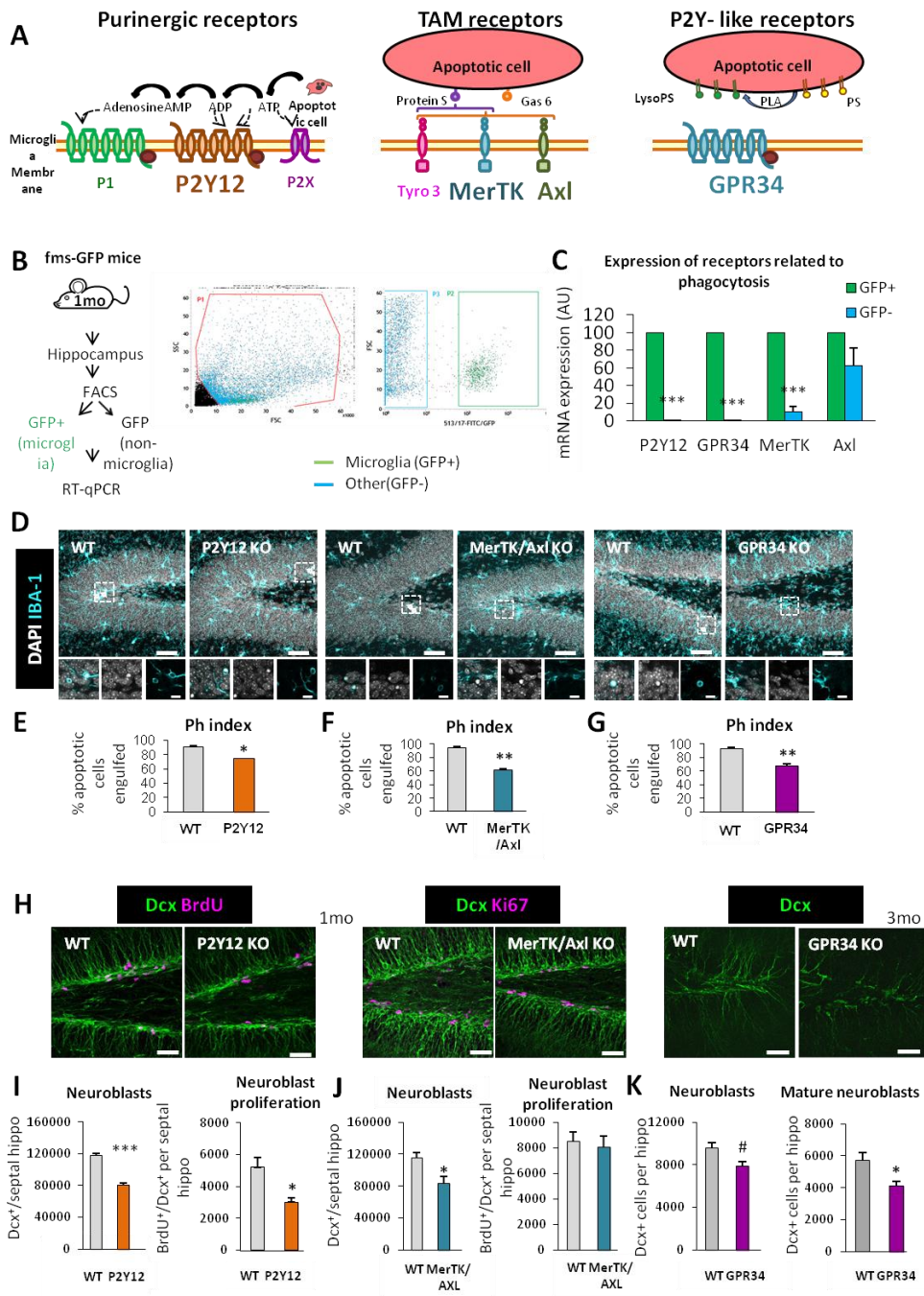
Figure 35. Microglial phagocytosis impairment reduces adult hippocampal neurogenesis. [A] *Schematic representation of the interactions of P2Y12, GPR34 and MerTK/AXL receptors with apoptotic cells.* **[B]** *Experimental design used to isolate microglia (GFP+) vs non-microglial cells (GFP-) from 1 mo fms-EGFP mice. Flow cytometry analysis of the expression of C1q subunits in hippocampal cells. First, debris was excluded using the P1 gate in FSC versus SSC (left panel). Next, gates for GFP+ microglia cells (P3) and GFP- non-microglial cells (P2) were defined based on the distribution of the fms-EGFP+ cells in EGFP vs FSC (right panel).* **[C]** *Expression of P2Y12, MerTK, AXL and GPR34 in microglia (GFP+) vs non-microglial cells (GFP-) by RTqPCR in FACS-sorted cells from fms-EGFP mice hippocampi (n = 3, each from 8 pooled hippocampi). OAZ1 was selected as a reference gene.* **[D]** *Representative confocal z-stack of P2Y12, MerTK/AXL and GPR34 KO mice immunofluorescence in the mouse hippocampal DG in physiological*

198

conditions at 1 month. Microglia were labeled with fms EGFP+ (cyan), C1q (red) and apoptotic nuclei were detected by pyknosis/karyorrhexis (white, DAPI). [E] Percentage of apoptotic cells engulfed (Ph-index) in P2Y12 KO mice. [F] Percentage of apoptotic cells engulfed (Ph-index) in MerTK/AXL KO mice. [G] Percentage of apoptotic cells engulfed (Ph-index) in GPR34 KO mice. [H] Representative confocal z-stack of P2Y12, MerTK/AXL and GPR34 KO mice immunofluorescence in the mouse hippocampal DG in physiological conditions at 1 month. Neuroblasts were labeled with DCX (green) and proliferation was labeled with either BrdU or Ki67 (magenta). [I] Quantifications of neuroblast and neuroblast proliferation in 1mo P2Y12 KO mice. [J] Quantifications of neuroblast and neuroblast proliferation in 1mo MerTK/AXL KO mice. [K] Quantification of neuroblast and mature neuroblast cell population in 3mo GPR34 KO mice. Data from Victor Sanchez-Zafra and Iñaki Paris PhD Theses.

In parallel, we are developing an alternative strategy based on inhibiting microglial phagocytosis in adult mice by injection of a lentiviral vector encoding a truncated dominant form of MGF-E8, an opsonizing molecule that binds to PS of apoptotic cells in order to facilitate phagocytosis (Akakura et al., 2004; Hanayama et al., 2002). The truncated dominant form would be overexpressed by neurons and might prevent microglia from recognizing apoptotic cells. We have some preliminary data in organotypic cultures in which we have observed a diminished microglial phagocytosis. In the future, we will use this strategy to inhibit microglial phagocytosis in vivo and study the effects of microglial phagocytosis impairment in neurogenesis.

7.7. MICROGLIAL PHAGOCYTOSIS ACTS AS THE BRAKE OF NEUROGENESIS

Overall, our data suggest that in physiological conditions, microglial phagocytosis acutely acts as the break of neurogenesis by releasing several neuromodulatory factors. These neuromodulatory factors are likely to aim at reducing the survival of neuronal-committed cells, and therefore, prevent an excessive production of new neurons. Moreover, the effects exerted by phagocytic microglia would be local since only cells from the neurogenic niche in close proximity to an apoptotic cell and, consequently to a phagocytic microglia, might be affected. However, upon microglial phagocytosis impairment, the break exerted on neurogenesis would disappear, allowing the uncontrolled survival of many neural-committed cells, which in turn, would proliferate and differentiate resulting in an increase in neuroblast cell population which could lead to aberrant connections with the hippocampal circuitry and thus, to the disruption of the balance of the neurogenic cascade. Therefore, in phagocytosis impairment conditions, such as epilepsy (Abiega et al., 2016), the phagocytosis impairment could contribute to the disease not only by the lack of clearance of apoptotic cells, but also by the absence of a

neuromodulatory brake. In addition, because the KO models in which microglial phagocytosis is impaired have shown that a decrease in phagocytosis results in a reduced neurogenesis, our data confirm that this regulation is essential for the correct long-term maintenance and functioning of the neurogenic cascade **(Figure 36)**.
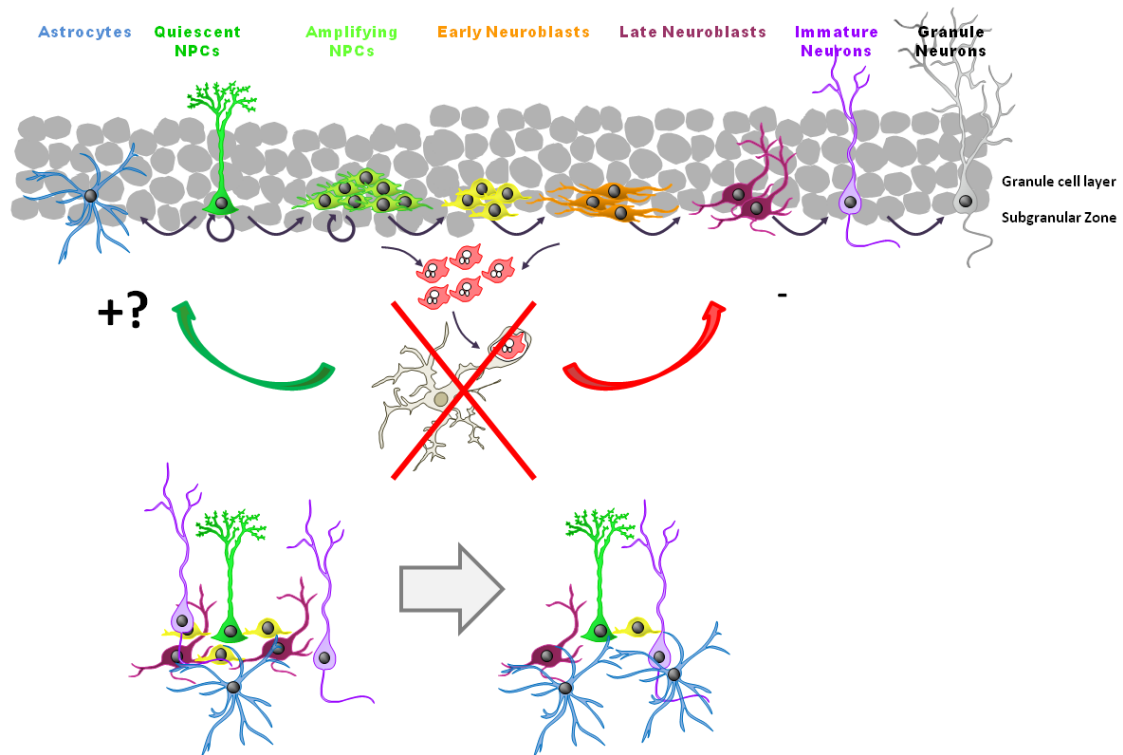
Figure 36. Microglial phagocytosis imposes a brake on neurogenesis. In physiological conditions, microglial phagocytosis of apoptotic cells triggers the production of neuromodulatory molecules that contribute to the maintenance of the neurogenic cascade. However, upon microglial phagocytosis impairment, the balance of the neurogenic cascade is disrupted.

8. CONCLUSIONS

8. CONCLUSIONS

1. REFINEMENT OF AN IN VITRO MODEL OF PHAGOCYTOSIS OF APOPTOTIC CELLS

- We have developed an in vitro model of phagocytosis using primary microglia cultures from fms-EGFP mice fed with apoptotic murine NE-4C neuroprogenitors or human SH-SY5Y neurons that mimics many features of in vivo phagocytosis.

- The opsonizing molecule C1q must be present in the in vitro phagocytosis assay because in the adult hippocampus C1q mRNA is highly enriched in microglia and its protein is present in all phagocytic pouches, suggesting its involvement in phagocytosis.

- We performed a direct quantification of phagocytic pouches using confocal z-stacks and orthogonal projections, a critical procedure to confirm the presence of the cargo within the microglial pouch.

- Similar to phagocytosis in the adult hippocampus, microglial phagocytosis of mouse and human apoptotic cells (NE-4C and SH-SY5Y) is very fast and efficient in vitro.

2. PHAGOCYTOSIS ALTERS GENES INVOLVED IN MICROGLIAL PHYSIOLOGY, INCLUDING METABOLISM AND APOPTOSIS

- Whole genome transcriptome of phagocytic microglia reveals changes in microglial physiology, including cell metabolism and apoptosis.

- Human SH-SY5Y apoptotic cells do not have active transcription sites, their mRNA is degraded, and the gene arrays probes and qPCR primers used are mouse-specific, suggesting that phagocytosis-related transcripts are specific to microglia.

- Phagocytic microglia show expression changes in apoptosis-related genes which might exert pro-apoptotic actions within microglia (autologous genes) and anti-apoptotic actions in the surrounding cells (heterologous genes).

- A small percentage of microglia dies after phagocytosis in vitro, which is unrelated to the amount or type of phagocytosed apoptotic cells.

- Phagocytic microglial show expression changes in genes related to metabolism, which might be related to an enhanced glycolysis, a decreased Krebb's cycle, and a shutdown oxidative phosphorylation.

CONCLUSIONS

- Phagocytic microglia show expression changes in genes related to chromatin modification and transcription factors, which would provide microglia with the capacity to maintain long-term expression and functional changes.

3. PHAGOCYTOSIS TRIGGERS AN IMMUNOMODULATORY PROGRAM IN MICROGLIA

- Phagocytic microglia present an enhanced expression of several both pro- and anti-inflammatory cytokine genes, suggesting that it is a complex mechanism that combines molecules of different immunological nature.
- Phagocytic microglia show alternative responses to inflammatory stimuli such as LPS.

4. PHAGOCYTOSIS TRIGGERS A NEUROMODULATORY PROGRAM IN MICROGLIA

- Phagocytic microglia express a coordinated neuromodulatory program that encompass up to 224 heterologous genes previously shown to modulate neurogenesis (neurocandidates), including peptides, trophic factors, matrix metalloproteases, and cytokines.
- The neuromodulatory program is independent on the amount of cells engulfed by microglia.
- The expression of some neurocandidates requires actual engulfment, whereas others depend on the apoptotic cell conditioned media.
- Comparative RTqPCR or single cell RNASeq of microglia acutely purified from CA and the DG was not sufficient to validate the neuromodulatory program in vivo.

5. PHAGOCYTOSIS TRIGGERS GLIOGENESIS IN VITRO BUT NOT IN VIVO

- Conditioned media from phagocytic microglia and naïve microglia differentiate neuroprogenitor cells into different phenotypes in vitro.
- Naïve microglia drive neuroprogenitor cells towards astrocytes and neuron-committed cells.
- Phagocytic microglia drive neuroprogenitor cells towards a bipolar phenotype of astrocytic lineage characterized by high expression of astrocytic markers such as nestin, GFAP and S100β; their calcium responses to different stimuli; and their ability to differentiate into astrocytes .
- Heterologous gliogenic genes expressed in phagocytic microglia are few and likely not involved in promoting astrocyte differentiation.
- In vivo, phagocytic microglia CM does not induce astrogenesis.

6. PHAGOCYTOSIS LIMITS NEUROGENESIS IN VIVO BY THE REGULATION OF THE SURVIVAL OF THE NEURONAL-COMMITTED CELLS

- Phagocytic microglia conditioned media induces apoptosis of neuroprogenitors in vitro but not in vivo.
- Phagocytic microglia CM reduces differentiation into neuron-committed cells compared with control CM in vitro.
- There is a tendency to find a decrease in stem cells and proliferation of stem cells at 2h in mice injected with phagocytic CM.
- Phagocytic microglia CM does not induce changes in proliferation at 2h, but proliferative cells are diminished after 28d of treatment, suggesting that phagocytosis regulates the survival of neuronal-committed cells..
- Phagocytic microglia CM reduces the most mature neuroblast subpopulation (EF) at 28d.

7. POTENTIAL MECHANISMS UNDERLYING THE MODULATION OF NEUROGENESIS BY MICROGLIAL PHAGOCYTOSIS

- Cytokines are unlikely to give rise to the bipolar cell phenotype observed in phagocytic conditioned media because they are not observed in LPS-treated microglial conditioned media.
- Both LPS and CM from LPS-treated microglia induce an increase of neuroblasts compared to CM from naïve microglia.
- Lactate is not increased in phagocytic microglia, but is enhanced in LPS, which renders lactate unlikely to mediate the modulation of neurogenesis by phagocytic microglia.
- The neuropeptide VGF is highly increased in phagocytic microglia in vitro.
- Glutamatergic receptors are residually expressed in microglia and therefore they are unlikely to form functional receptors and drive the communication between microglia and the neurogenic niche.
- Phagocytic receptors are down-regulated and purinergic receptors are up-regulated in microglia upon microglial phagocytosis impairment after the intrahippocampal injection of Kainic acid, suggesting novel targets to manipulate microglial phagocytosis in vivo.

9. BIBLIOGRAPHY

9. BIBLIOGRAPHY

A

Aarum, J., Sandberg, K., Haeberlein, S.L., and Persson, M.A. (2003). Migration and differentiation of neural precursor cells can be directed by microglia. Proceedings of the National Academy of Sciences of the United States of America *100*, 15983-15988.
Abiega, O., Beccari, S., Diaz-Aparicio, I., Nadjar, A., Laye, S., Leyrolle, Q., Gomez-Nicola, D., Domercq, M., Perez-Samartin, A., Sanchez-Zafra, V.*, et al.* (2016). Neuronal Hyperactivity Disturbs ATP Microgradients, Impairs Microglial Motility, and Reduces Phagocytic Receptor Expression Triggering Apoptosis/Microglial Phagocytosis Uncoupling. PLoS biology *14*, e1002466.

Abraham, H., Orsi, G., and Seress, L. (2007). Ontogeny of cocaine- and amphetamine-regulated transcript (CART) peptide and calbindin immunoreactivity in granule cells of the dentate gyrus in the rat. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience *25*, 265-274.

Aguzzi, A., Barres, B.A., and Bennett, M.L. (2013). Microglia: scapegoat, saboteur, or something else? Science *339*, 156-161.

Aimone, J.B., Wiles, J., and Gage, F.H. (2006). Potential role for adult neurogenesis in the encoding of time in new memories. Nature neuroscience *9*, 723-727.

Aimone, J.B., Wiles, J., and Gage, F.H. (2009). Computational influence of adult neurogenesis on memory encoding. Neuron *61*, 187-202.

Ainscow, E.K., Mirshamsi, S., Tang, T., Ashford, M.L., and Rutter, G.A. (2002). Dynamic imaging of free cytosolic ATP concentration during fuel sensing by rat hypothalamic neurones: evidence for ATP-independent control of ATP-sensitive K(+) channels. The Journal of physiology *544*, 429-445.

Airaksinen, M.S., Holm, L., and Hatinen, T. (2006). Evolution of the GDNF family ligands and receptors. Brain, behavior and evolution *68*, 181-190.

Akakura, S., Singh, S., Spataro, M., Akakura, R., Kim, J.I., Albert, M.L., and Birge, R.B. (2004). The opsonin MFG-E8 is a ligand for the alphavbeta5 integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells. Experimental cell research *292*, 403-416.

Akers, K.G., Martinez-Canabal, A., Restivo, L., Yiu, A.P., De Cristofaro, A., Hsiang, H.L., Wheeler, A.L., Guskjolen, A., Niibori, Y., Shoji, H.*, et al.* (2014). Hippocampal neurogenesis regulates forgetting during adulthood and infancy. Science *344*, 598-602.

Alberdi, E., Wyssenbach, A., Alberdi, M., Sanchez-Gomez, M.V., Cavaliere, F., Rodriguez, J.J., Verkhratsky, A., and Matute, C. (2013). Ca(2+) -dependent endoplasmic reticulum stress correlates with astrogliosis in oligomeric amyloid beta-treated astrocytes and in a model of Alzheimer's disease. Aging cell *12*, 292-302.

Aloisi, F., Care, A., Borsellino, G., Gallo, P., Rosa, S., Bassani, A., Cabibbo, A., Testa, U., Levi, G., and Peschle, C. (1992). Production of hemolymphopoietic cytokines (IL-6, IL-8, colony-stimulating factors) by normal human astrocytes in response to IL-1 beta and tumor necrosis factor-alpha. Journal of immunology *149*, 2358-2366.

Alvarez, Z., Castano, O., Castells, A.A., Mateos-Timoneda, M.A., Planell, J.A., Engel, E., and Alcantara, S. (2014). Neurogenesis and vascularization of the damaged brain using a lactate-releasing biomimetic scaffold. Biomaterials *35*, 4769-4781.

Amor, S., Puentes, F., Baker, D., and van der Valk, P. (2010). Inflammation in neurodegenerative diseases. Immunology *129*, 154-169.

Anderson, H.A., Maylock, C.A., Williams, J.A., Paweletz, C.P., Shu, H., and Shacter, E. (2003). Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nature immunology *4*, 87-91.

Andrae, J., Gallini, R., and Betsholtz, C. (2008). Role of platelet-derived growth factors in physiology and medicine. Genes & development *22*, 1276-1312.

Arandjelovic, S., and Ravichandran, K.S. (2015). Phagocytosis of apoptotic cells in homeostasis. Nature immunology *16*, 907-917.

Arguello, A.A., Fischer, S.J., Schonborn, J.R., Markus, R.W., Brekken, R.A., and Eisch, A.J. (2009). Effect of chronic morphine on the dentate gyrus neurogenic microenvironment. Neuroscience *159*, 1003-1010.

Arsenijevic, Y., Weiss, S., Schneider, B., and Aebischer, P. (2001). Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. The Journal of neuroscience : the official journal of the Society for Neuroscience *21*, 7194-7202.

Asai, T., Wanaka, A., Kato, H., Masana, Y., Seo, M., and Tohyama, M. (1993). Differential expression of two members of FGF receptor gene family, FGFR-1 and FGFR-2 mRNA, in the adult rat central nervous system. Brain research Molecular brain research *17*, 174-178.

Atagi, Y., Liu, C.C., Painter, M.M., Chen, X.F., Verbeeck, C., Zheng, H., Li, X., Rademakers, R., Kang, S.S., Xu, H.*, et al.* (2015). Apolipoprotein E Is a Ligand for Triggering Receptor Expressed on Myeloid Cells 2 (TREM2). The Journal of biological chemistry *290*, 26043-26050.

Azam, F., Mehta, S., and Harris, A.L. (2010). Mechanisms of resistance to antiangiogenesis therapy. European journal of cancer *46*, 1323-1332.

B

Babu, H., Claasen, J.H., Kannan, S., Runker, A.E., Palmer, T., and Kempermann, G. (2011). A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. Frontiers in neuroscience *5*, 89.

Barkho, B.Z., Munoz, A.E., Li, X., Li, L., Cunningham, L.A., and Zhao, X. (2008). Endogenous matrix metalloproteinase (MMP)-3 and MMP-9 promote the differentiation and migration of adult neural progenitor cells in response to chemokines. Stem cells *26*, 3139-3149.

Barreto-Vianna, A.R., Aguila, M.B., and Mandarim-de-Lacerda, C.A. (2016). Effects of liraglutide in hypothalamic arcuate nucleus of obese mice. Obesity *24*, 626-633.

Bean, A.J., Elde, R., Cao, Y.H., Oellig, C., Tamminga, C., Goldstein, M., Pettersson, R.F., and Hokfelt, T. (1991). Expression of acidic and basic fibroblast growth factors in the substantia nigra of rat, monkey, and human. Proceedings of the National Academy of Sciences of the United States of America *88*, 10237-10241.

Beccari, S., Diaz-Aparicio, I., and Sierra, A. (2018). Quantifying microglial phagocytosis of apoptotic cells in the brain in health and disease. Current protocols in immunology *e49*.

Beccari, S., Valero, J., Maletic-Savatic, M., and Sierra, A. (2017). A simulation model of neuroprogenitor proliferation dynamics predicts age-related loss of hippocampal neurogenesis but not astrogenesis. Scientific reports *7*, 16528.

Becker, S., and Wojtowicz, J.M. (2007). A model of hippocampal neurogenesis in memory and mood disorders. Trends in cognitive sciences *11*, 70-76.

Bedard, A., Tremblay, P., Chernomoretz, A., and Vallieres, L. (2007). Identification of genes preferentially expressed by microglia and upregulated during cuprizone-induced inflammation. Glia *55*, 777-789.

Bekkering, S., Joosten, L.A., van der Meer, J.W., Netea, M.G., and Riksen, N.P. (2015). The epigenetic memory of monocytes and macrophages as a novel drug target in atherosclerosis. Clinical therapeutics *37*, 914-923.

Belarbi, K., Arellano, C., Ferguson, R., Jopson, T., and Rosi, S. (2012). Chronic neuroinflammation impacts the recruitment of adult-born neurons into behaviorally relevant hippocampal networks. Brain, behavior, and immunity *26*, 18-23.

Bennett, M.L., and Barres, B.A. (2017). A genetically distinct microglial subset promotes myelination. The EMBO journal *36*, 3269-3271.

Beppu, K., Kosai, Y., Kido, M.A., Akimoto, N., Mori, Y., Kojima, Y., Fujita, K., Okuno, Y., Yamakawa, Y., Ifuku, M.*, et al.* (2013). Expression, subunit composition, and function of AMPA-type glutamate receptors are changed in activated microglia; possible contribution of GluA2 (GluR-B)-deficiency under pathological conditions. Glia *61*, 881-891.

Bergami, M., Masserdotti, G., Temprana, S.G., Motori, E., Eriksson, T.M., Gobel, J., Yang, S.M., Conzelmann, K.K., Schinder, A.F., Gotz, M.*, et al.* (2015). A critical period for experience-dependent remodeling of adult-born neuron connectivity. Neuron *85*, 710-717.

Bernay, B., Gaillard, M.C., Guryca, V., Emadali, A., Kuhn, L., Bertrand, A., Detraz, I., Carcenac, C., Savasta, M., Brouillet, E.*, et al.* (2009). Discovering new bioactive neuropeptides in the striatum secretome using in vivo microdialysis and versatile proteomics. Molecular & cellular proteomics : MCP *8*, 946-958.

Bertrand, J.Y., Jalil, A., Klaine, M., Jung, S., Cumano, A., and Godin, I. (2005). Three pathways to mature macrophages in the early mouse yolk sac. Blood *106*, 3004-3011.

Bialas, A.R., and Stevens, B. (2013). TGF-beta signaling regulates neuronal C1q expression and developmental synaptic refinement. Nature neuroscience *16*, 1773-1782.

BIBLIOGRAPHY

Bindea, G., Galon, J., and Mlecnik, B. (2013). CluePedia Cytoscape plugin: pathway insights using integrated experimental and in silico data. Bioinformatics *29*, 661-663.

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., Fridman, W.H., Pages, F., Trajanoski, Z., and Galon, J. (2009). ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics *25*, 1091-1093.

Bland, S.T., Beckley, J.T., Young, S., Tsang, V., Watkins, L.R., Maier, S.F., and Bilbo, S.D. (2010). Enduring consequences of early-life infection on glial and neural cell genesis within cognitive regions of the brain. Brain, behavior, and immunity *24*, 329-338.

Blobe, G.C., Schiemann, W.P., Pepin, M.C., Beauchemin, M., Moustakas, A., Lodish, H.F., and O'Connor-McCourt, M.D. (2001). Functional roles for the cytoplasmic domain of the type III transforming growth factor beta receptor in regulating transforming growth factor beta signaling. The Journal of biological chemistry *276*, 24627-24637.

Blok, B.A., Arts, R.J., van Crevel, R., Benn, C.S., and Netea, M.G. (2015). Trained innate immunity as underlying mechanism for the long-term, nonspecific effects of vaccines. Journal of leukocyte biology *98*, 347-356.

Bloodgood, B.L., and Sabatini, B.L. (2008). Regulation of synaptic signalling by postsynaptic, non-glutamate receptor ion channels. The Journal of physiology *586*, 1475-1480.

Bodey, B., Kaiser, H.E., and Siegel, S.E. (2005). Epidermal growth factor receptor (EGFR) expression in childhood brain tumors. In vivo *19*, 931-941.

Bohatschek, M., Kloss, C.U., Kalla, R., and Raivich, G. (2001). In vitro model of microglial deramification: ramified microglia transform into amoeboid phagocytes following addition of brain cell membranes to microglia-astrocyte cocultures. Journal of neuroscience research *64*, 508-522.

Bohlen, C.J., Bennett, F.C., Tucker, A.F., Collins, H.Y., Mulinyawe, S.B., and Barres, B.A. (2017). Diverse Requirements for Microglial Survival, Specification, and Function Revealed by Defined-Medium Cultures. Neuron *94*, 759-773 e758.

Boku, S., Nakagawa, S., Takamura, N., Kato, A., Takebayashi, M., Hisaoka-Nakashima, K., Omiya, Y., Inoue, T., and Kusumi, I. (2013). GDNF facilitates differentiation of the adult dentate gyrus-derived neural precursor cells into astrocytes via STAT3. Biochemical and biophysical research communications *434*, 779-784.

Boldrini, M., Fulmore, C.A., Tartt, A.N., Simeon, L.R., Pavlova, I., Poposka, V., Rosoklija, G.B., Stankov, A., Arango, V., Dwork, A.J.*, et al.* (2018). Human Hippocampal Neurogenesis Persists throughout Aging. Cell stem cell *22*, 589-599 e585.

Bonaguidi, M.A., Wheeler, M.A., Shapiro, J.S., Stadel, R.P., Sun, G.J., Ming, G.L., and Song, H. (2011). In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. Cell *145*, 1142-1155.

Bondy, C., and Lee, W.H. (1993). Correlation between insulin-like growth factor (IGF)-binding protein 5 and IGF-I gene expression during brain development. The Journal of neuroscience : the official journal of the Society for Neuroscience *13*, 5092-5104.

Boneberg, E.M., Hareng, L., Gantner, F., Wendel, A., and Hartung, T. (2000). Human monocytes express functional receptors for granulocyte colony-stimulating factor that mediate suppression of monokines and interferon-gamma. Blood *95*, 270-276.

Bonvento, G., Herard, A.S., and Voutsinos-Porche, B. (2005). The astrocyte--neuron lactate shuttle: a debated but still valuable hypothesis for brain imaging. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism *25*, 1394-1399.

Brancia, C., Noli, B., Boido, M., Pilleri, R., Boi, A., Puddu, R., Marrosu, F., Vercelli, A., Bongioanni, P., Ferri, G.L.*, et al.* (2018). TLQP Peptides in Amyotrophic Lateral Sclerosis: Possible Blood Biomarkers with a Neuroprotective Role. Neuroscience *380*, 152-163.

Brandt, M.D., Jessberger, S., Steiner, B., Kronenberg, G., Reuter, K., Bick-Sander, A., von der Behrens, W., and Kempermann, G. (2003). Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. Molecular and cellular neurosciences *24*, 603-613.

Braun, S.M., and Jessberger, S. (2014). Adult neurogenesis: mechanisms and functional significance. Development *141*, 1983-1986.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C.A., Causton, H.C.*, et al.* (2001). Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nature genetics *29*, 365-371.

Breton, J., and Mao-Draayer, Y. (2011). Impact of Cytokines on Neural Stem/Progenitor Cell Fate. Journal of Neurology & Neurophysiology.

Brkic, M., Balusu, S., Libert, C., and Vandenbroucke, R.E. (2015). Friends or Foes: Matrix Metalloproteinases and Their Multifaceted Roles in Neurodegenerative Diseases. Mediators of inflammation *2015*, 620581.

Brocker, C., Thompson, D., Matsumoto, A., Nebert, D.W., and Vasiliou, V. (2010). Evolutionary divergence and functions of the human interleukin (IL) gene family. Human genomics *5*, 30-55. Brockington, A., Lewis, C., Wharton, S., and Shaw, P.J. (2004). Vascular endothelial growth factor and the nervous system. Neuropathology and applied neurobiology *30*, 427-446.

Brown, J.P., Couillard-Despres, S., Cooper-Kuhn, C.M., Winkler, J., Aigner, L., and Kuhn, H.G. (2003). Transient expression of doublecortin during adult neurogenesis. The Journal of comparative neurology *467*, 1-10.

Buckwalter, M.S., Yamane, M., Coleman, B.S., Ormerod, B.K., Chin, J.T., Palmer, T., and Wyss-Coray, T. (2006). Chronically increased transforming growth factor-beta1 strongly inhibits hippocampal neurogenesis in aged mice. The American journal of pathology *169*, 154-164.

Burgess, W.H., and Maciag, T. (1989). The heparin-binding (fibroblast) growth factor family of proteins. Annual review of biochemistry *58*, 575-606.

Burns, K.A., and Kuan, C.Y. (2005). Low doses of bromo- and iododeoxyuridine produce near-saturation labeling of adult proliferative populations in the dentate gyrus. The European journal of neuroscience *21*, 803-807.

BIBLIOGRAPHY

Buss, A., Pech, K., Kakulas, B.A., Martin, D., Schoenen, J., Noth, J., and Brook, G.A. (2008). TGF-beta1 and TGF-beta2 expression after traumatic human spinal cord injury. Spinal cord *46*, 364-371.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L.*, et al.* (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry *55*, 611-622.

Butovsky, O., Jedrychowski, M.P., Moore, C.S., Cialic, R., Lanser, A.J., Gabriely, G., Koeglsperger, T., Dake, B., Wu, P.M., Doykan, C.E.*, et al.* (2014). Identification of a unique TGF-beta-dependent molecular and functional signature in microglia. Nature neuroscience *17*, 131-143.

Butovsky, O., Ziv, Y., Schwartz, A., Landa, G., Talpalar, A.E., Pluchino, S., Martino, G., and Schwartz, M. (2006). Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. Molecular and cellular neurosciences *31*, 149-160.

C

Caberoy, N.B., Alvarado, G., Bigcas, J.L., and Li, W. (2012). Galectin-3 is a new MerTK-specific eat-me signal. Journal of cellular physiology *227*, 401-407.

Cacci, E., Claasen, J.H., and Kokaia, Z. (2005). Microglia-derived tumor necrosis factor-alpha exaggerates death of newborn hippocampal progenitor cells in vitro. Journal of neuroscience research *80*, 789-797.

Caldwell, M.A., He, X., Wilkie, N., Pollack, S., Marshall, G., Wafford, K.A., and Svendsen, C.N. (2001). Growth factors regulate the survival and fate of cells derived from human neurospheres. Nature biotechnology *19*, 475-479.

Calvo, M., Zhu, N., Grist, J., Ma, Z., Loeb, J.A., and Bennett, D.L. (2011). Following nerve injury neuregulin-1 drives microglial proliferation and neuropathic pain via the MEK/ERK pathway. Glia *59*, 554-568.

Camara-Lemarroy, C.R., Guzman-de la Garza, F.J., and Fernandez-Garza, N.E. (2010). Molecular inflammatory mediators in peripheral nerve degeneration and regeneration. Neuroimmunomodulation *17*, 314-324.

Campos, L.S. (2004). Neurospheres: insights into neural stem cell biology. Journal of neuroscience research *78*, 761-769.

Cao, L., Jiao, X., Zuzga, D.S., Liu, Y., Fong, D.M., Young, D., and During, M.J. (2004). VEGF links hippocampal activity with neurogenesis, learning and memory. Nat Genet *36*, 827-835.

Carmeliet, P., and Storkebaum, E. (2002). Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. Seminars in cell & developmental biology *13*, 39-53.

Carnicella, S., and Ron, D. (2009). GDNF--a potential target to treat addiction. Pharmacology & therapeutics *122*, 9-18.

Casano, A.M., and Peri, F. (2015). Microglia: multitasking specialists of the brain. Developmental cell *32*, 469-477.

Cater, H.L., Benham, C.D., and Sundstrom, L.E. (2001). Neuroprotective role of monocarboxylate transport during glucose deprivation in slice cultures of rat hippocampus. The Journal of physiology *531*, 459-466.

Cavaillon, J.M. (2017). Exotoxins and endotoxins: Inducers of inflammatory cytokines. Toxicon : official journal of the International Society on Toxinology.

Chadi, G., Tinner, B., Agnati, L.F., and Fuxe, K. (1993). Basic fibroblast growth factor (bFGF, FGF-2) immunoreactivity exists in the noradrenaline, adrenaline and 5-HT nerve cells of the rat brain. Neuroscience letters *160*, 171-176.

Chambers, R.A., Potenza, M.N., Hoffman, R.E., and Miranker, W. (2004). Simulated apoptosis/neurogenesis regulates learning and memory capabilities of adaptive neural networks. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology *29*, 747-758.

Chekeni, F.B., Elliott, M.R., Sandilos, J.K., Walk, S.F., Kinchen, J.M., Lazarowski, E.R., Armstrong, A.J., Penuela, S., Laird, D.W., Salvesen, G.S.*, et al.* (2010). Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature *467*, 863-867.

Chen, Y.C., Pristera, A., Ayub, M., Swanwick, R.S., Karu, K., Hamada, Y., Rice, A.S., and Okuse, K. (2013). Identification of a receptor for neuropeptide VGF and its role in neuropathic pain. The Journal of biological chemistry *288*, 34638-34646.

Cheng, S.C., Quintin, J., Cramer, R.A., Shepardson, K.M., Saeed, S., Kumar, V., Giamarellos-Bourboulis, E.J., Martens, J.H., Rao, N.A., Aghajanirefah, A.*, et al.* (2014). mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. Science *345*, 1250684.

Choi, J.Y., Lee, W.H., Yoo, T.K., Park, I., and Kim, D.W. (2012). A new severity predicting index for hemorrhagic shock using lactate concentration and peripheral perfusion in a rat model. Shock *38*, 635-641.

Chojnacki, A., Kelly, J.J., Hader, W., and Weiss, S. (2008). Distinctions between fetal and adult human platelet-derived growth factor-responsive neural precursors. Annals of neurology *64*, 127-142.

Chojnacki, A., and Weiss, S. (2004). Isolation of a novel platelet-derived growth factor-responsive precursor from the embryonic ventral forebrain. The Journal of neuroscience : the official journal of the Society for Neuroscience *24*, 10888-10899.

Cocco, C., D'Amato, F., Noli, B., Ledda, A., Brancia, C., Bongioanni, P., and Ferri, G.L. (2010). Distribution of VGF peptides in the human cortex and their selective changes in Parkinson's and Alzheimer's diseases. Journal of anatomy *217*, 683-693.

Collins, H.Y., and Bohlen, C.J. (2018). Isolation and Culture of Rodent Microglia to Promote a Dynamic Ramified Morphology in Serum-free Medium. Journal of visualized experiments : JoVE.

Conesa, A., Nueda, M.J., Ferrer, A., and Talon, M. (2006). maSigPro: a method to identify significantly differential expression profiles in time-course microarray experiments. Bioinformatics *22*, 1096-1102.

Consiglio, A., Gritti, A., Dolcetta, D., Follenzi, A., Bordignon, C., Gage, F.H., Vescovi, A.L., and Naldini, L. (2004). Robust in vivo gene transfer into adult mammalian neural stem cells by lentiviral vectors. Proceedings of the National Academy of Sciences of the United States of America *101*, 14835-14840.

Conti, L., and Cattaneo, E. (2005). Controlling neural stem cell division within the adult subventricular zone: an APPealing job. Trends in neurosciences *28*, 57-59.

Cortes, D., Carballo-Molina, O.A., Castellanos-Montiel, M.J., and Velasco, I. (2017). The Non-Survival Effects of Glial Cell Line-Derived Neurotrophic Factor on Neural Cells. Frontiers in molecular neuroscience *10*, 258.

Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U., Winkler, J., Kuhn, H.G., and Aigner, L. (2005). Doublecortin expression levels in adult brain reflect neurogenesis. The European journal of neuroscience *21*, 1-14.

Cunningham, C.L., Martinez-Cerdeno, V., and Noctor, S.C. (2013). Microglia regulate the number of neural precursor cells in the developing cerebral cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience *33*, 4216-4233.

Cursiefen, C., Cao, J., Chen, L., Liu, Y., Maruyama, K., Jackson, D., Kruse, F.E., Wiegand, S.J., Dana, M.R., and Streilein, J.W. (2004). Inhibition of hemangiogenesis and lymphangiogenesis after normal-risk corneal transplantation by neutralizing VEGF promotes graft survival. Investigative ophthalmology & visual science *45*, 2666-2673.

D

Danial, N.N., and Korsmeyer, S.J. (2004). Cell death: critical control points. Cell *116*, 205-219.
Dantzer, R., and Kelley, K.W. (2007). Twenty years of research on cytokine-induced sickness behavior. Brain, behavior, and immunity *21*, 153-160.

Daria, A., Colombo, A., Llovera, G., Hampel, H., Willem, M., Liesz, A., Haass, C., and Tahirovic, S. (2017). Young microglia restore amyloid plaque clearance of aged microglia. The EMBO journal *36*, 583-603.

De Melo Reis, R.A., Schitine, C.S., Kofalvi, A., Grade, S., Cortes, L., Gardino, P.F., Malva, J.O., and de Mello, F.G. (2011). Functional identification of cell phenotypes differentiating from mice retinal neurospheres using single cell calcium imaging. Cellular and molecular neurobiology *31*, 835-846.

De Simone, R., Ajmone-Cat, M.A., Tirassa, P., and Minghetti, L. (2003). Apoptotic PC12 cells exposing phosphatidylserine promote the production of anti-inflammatory and neuroprotective molecules by microglial cells. Journal of neuropathology and experimental neurology *62*, 208-216.

Demetri, G.D., and Griffin, J.D. (1991). Granulocyte colony-stimulating factor and its receptor. Blood *78*, 2791-2808.

Deng, W., Aimone, J.B., and Gage, F.H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nature reviews Neuroscience *11*, 339-350.

Denstaedt, S.J., Spencer-Segal, J.L., Newstead, M.W., Laborc, K., Zhao, A.P., Hjelmaas, A., Zeng, X., Akil, H., Standiford, T.J., and Singer, B.H. (2018). S100A8/A9 Drives Neuroinflammatory Priming and Protects against Anxiety-like Behavior after Sepsis. Journal of immunology *200*, 3188-3200.

Depboylu, C., Schafer, M.K., Schwaeble, W.J., Reinhart, T.A., Maeda, H., Mitsuya, H., Damadzic, R., Rausch, D.M., Eiden, L.E., and Weihe, E. (2005). Increase of C1q biosynthesis in brain microglia and macrophages during lentivirus infection in the rhesus macaque is sensitive to antiretroviral treatment with 6-chloro-2',3'-dideoxyguanosine. Neurobiology of disease *20*, 12-26.

Desjardins, M., Huber, L.A., Parton, R.G., and Griffiths, G. (1994). Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. The Journal of cell biology *124*, 677-688.

Dhaliwal, J., and Lagace, D.C. (2011). Visualization and genetic manipulation of adult neurogenesis using transgenic mice. The European journal of neuroscience *33*, 1025-1036.

Diaz-Aparicio, I., Beccari, S., Abiega, O., and Sierra, A. (2016). Clearing the corpses: regulatory mechanisms, novel tools, and therapeutic potential of harnessing microglial phagocytosis in the diseased brain. Neural regeneration research *11*, 1533-1539.

Dinarello, C.A. (2011). Blocking interleukin-1beta in acute and chronic autoinflammatory diseases. Journal of internal medicine *269*, 16-28.

Domercq, M., Vazquez-Villoldo, N., and Matute, C. (2013). Neurotransmitter signaling in the pathophysiology of microglia. Frontiers in cellular neuroscience *7*, 49.

Domingues, H.S., Portugal, C.C., Socodato, R., and Relvas, J.B. (2016). Oligodendrocyte, Astrocyte, and Microglia Crosstalk in Myelin Development, Damage, and Repair. Frontiers in cell and developmental biology *4*, 71.

Dono, R., Texido, G., Dussel, R., Ehmke, H., and Zeller, R. (1998). Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. The EMBO journal *17*, 4213-4225.

Douglass, J., and Daoud, S. (1996). Characterization of the human cDNA and genomic DNA encoding CART: a cocaine- and amphetamine-regulated transcript. Gene *169*, 241-245.

Drew, L.J., Kheirbek, M.A., Luna, V.M., Denny, C.A., Cloidt, M.A., Wu, M.V., Jain, S., Scharfman, H.E., and Hen, R. (2016). Activation of local inhibitory circuits in the dentate gyrus by adult-born neurons. Hippocampus *26*, 763-778.

E

Eckenstein, F., Woodward, W.R., and Nishi, R. (1991). Differential localization and possible functions of aFGF and bFGF in the central and peripheral nervous systems. Annals of the New York Academy of Sciences *638*, 348-360.

Eckenstein, F.P. (1994). Fibroblast growth factors in the nervous system. Journal of neurobiology *25*, 1467-1480.

Ehirchiou, D., Xiong, Y., Xu, G., Chen, W., Shi, Y., and Zhang, L. (2007). CD11b facilitates the development of peripheral tolerance by suppressing Th17 differentiation. The Journal of experimental medicine *204*, 1519-1524.

Ehninger, D., and Kempermann, G. (2008). Neurogenesis in the adult hippocampus. Cell and tissue research *331*, 243-250.

Eidet, J.R., Pasovic, L., Maria, R., Jackson, C.J., and Utheim, T.P. (2014). Objective assessment of changes in nuclear morphology and cell distribution following induction of apoptosis. Diagnostic pathology *9*, 92.

Eiriz, M.F., Grade, S., Rosa, A., Xapelli, S., Bernardino, L., Agasse, F., and Malva, J.O. (2011). Functional evaluation of neural stem cell differentiation by single cell calcium imaging. Current stem cell research & therapy *6*, 288-296.

Ekdahl, C.T., Claasen, J.H., Bonde, S., Kokaia, Z., and Lindvall, O. (2003). Inflammation is detrimental for neurogenesis in adult brain. Proceedings of the National Academy of Sciences of the United States of America *100*, 13632-13637.

Elliott, M.R., Chekeni, F.B., Trampont, P.C., Lazarowski, E.R., Kadl, A., Walk, S.F., Park, D., Woodson, R.I., Ostankovich, M., Sharma, P*., et al.* (2009). Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature *461*, 282-286.

Emoto, N., Gonzalez, A.M., Walicke, P.A., Wada, E., Simmons, D.M., Shimasaki, S., and Baird, A. (1989). Basic fibroblast growth factor (FGF) in the central nervous system: identification of specific loci of basic FGF expression in the rat brain. Growth factors *2*, 21-29.

Encinas, J.M., and Enikolopov, G. (2008). Identifying and quantitating neural stem and progenitor cells in the adult brain. Methods in cell biology *85*, 243-272.

Encinas, J.M., Michurina, T.V., Peunova, N., Park, J.H., Tordo, J., Peterson, D.A., Fishell, G., Koulakov, A., and Enikolopov, G. (2011). Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. Cell stem cell *8*, 566-579.

Encinas, J.M., and Sierra, A. (2012). Neural stem cell deforestation as the main force driving the age-related decline in adult hippocampal neurogenesis. Behavioural brain research *227*, 433-439.

Encinas, J.M., Vaahtokari, A., and Enikolopov, G. (2006). Fluoxetine targets early progenitor cells in the adult brain. Proceedings of the National Academy of Sciences of the United States of America *103*, 8233-8238.

Enikolopov, G., Overstreet-Wadiche, L., and Ge, S. (2015). Viral and transgenic reporters and genetic analysis of adult neurogenesis. Cold Spring Harbor perspectives in biology *7*, a018804.

Erdman, L.K., Cosio, G., Helmers, A.J., Gowda, D.C., Grinstein, S., and Kain, K.C. (2009). CD36 and TLR interactions in inflammation and phagocytosis: implications for malaria. Journal of immunology *183*, 6452-6459.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A., and Gage, F.H. (1998). Neurogenesis in the adult human hippocampus. Nature medicine *4*, 1313-1317.

Ernfors, P., Lonnerberg, P., Ayer-LeLievre, C., and Persson, H. (1990). Developmental and regional expression of basic fibroblast growth factor mRNA in the rat central nervous system. Journal of neuroscience research *27*, 10-15.

Ethell, I.M., and Ethell, D.W. (2007). Matrix metalloproteinases in brain development and remodeling: synaptic functions and targets. Journal of neuroscience research *85*, 2813-2823.

Eyo, U.B., Peng, J., Murugan, M., Mo, M., Lalani, A., Xie, P., Xu, P., Margolis, D.J., and Wu, L.J. (2016). Regulation of Physical Microglia-Neuron Interactions by Fractalkine Signaling after Status Epilepticus. eNeuro *3*.

F

F. Chris Bennett, M.L.B., Fazeela Yaqoob, Sara B. Mulinyawe, Gerald A. Grant, Melanie Hayden Gephart, Edward D. Plowey, Ben A. Barres (2018). A Combination of Ontogeny and CNS Environment Establishes Microglial Identity. In Neuron.

Fadok, V.A., Bratton, D.L., Konowal, A., Freed, P.W., Westcott, J.Y., and Henson, P.M. (1998). Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. The Journal of clinical investigation *101*, 890-898.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., and Henson, P.M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. Journal of immunology *148*, 2207-2216.

Favaro, R., Valotta, M., Ferri, A.L., Latorre, E., Mariani, J., Giachino, C., Lancini, C., Tosetti, V., Ottolenghi, S., Taylor, V.*, et al.* (2009). Hippocampal development and neural stem cell maintenance require Sox2-dependent regulation of Shh. Nature neuroscience *12*, 1248-1256. Fensterl, V., and Sen, G.C. (2009). Interferons and viral infections. BioFactors *35*, 14-20.

Ferrara, N. (2009). VEGF-A: a critical regulator of blood vessel growth. European cytokine network *20*, 158-163.

Fielder, G.C., Yang, T.W., Razdan, M., Li, Y., Lu, J., Perry, J.K., Lobie, P.E., and Liu, D.X. (2018). The GDNF Family: A Role in Cancer? Neoplasia *20*, 99-117.

Foglesong, G.D., Huang, W., Liu, X., Slater, A.M., Siu, J., Yildiz, V., Salton, S.R., and Cao, L. (2016). Role of Hypothalamic VGF in Energy Balance and Metabolic Adaption to Environmental Enrichment in Mice. Endocrinology *157*, 983-996.

Fontainhas, A.M., Wang, M., Liang, K.J., Chen, S., Mettu, P., Damani, M., Fariss, R.N., Li, W., and Wong, W.T. (2011). Microglial morphology and dynamic behavior is regulated by ionotropic glutamatergic and GABAergic neurotransmission. PloS one *6*, e15973.

Fourgeaud, L., Traves, P.G., Tufail, Y., Leal-Bailey, H., Lew, E.D., Burrola, P.G., Callaway, P., Zagorska, A., Rothlin, C.V., Nimmerjahn, A.*, et al.* (2016). TAM receptors regulate multiple features of microglial physiology. Nature *532*, 240-244.

Fournier, N.M., and Duman, R.S. (2012). Role of vascular endothelial growth factor in adult hippocampal neurogenesis: implications for the pathophysiology and treatment of depression. Behavioural brain research *227*, 440-449.

Francis, K., Lewis, B.M., Akatsu, H., Monk, P.N., Cain, S.A., Scanlon, M.F., Morgan, B.P., Ham, J., and Gasque, P. (2003). Complement C3a receptors in the pituitary gland: a novel pathway by which an innate immune molecule releases hormones involved in the control of inflammation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *17*, 2266-2268.

Fraser, D.A., Laust, A.K., Nelson, E.L., and Tenner, A.J. (2009). C1q differentially modulates phagocytosis and cytokine responses during ingestion of apoptotic cells by human monocytes, macrophages, and dendritic cells. Journal of immunology *183*, 6175-6185.

Fraser, D.A., Pisalyaput, K., and Tenner, A.J. (2010). C1q enhances microglial clearance of apoptotic neurons and neuronal blebs, and modulates subsequent inflammatory cytokine production. J Neurochem *112*, 733-743.

Fraser, D.A., and Tenner, A.J. (2008). Directing an appropriate immune response: the role of defense collagens and other soluble pattern recognition molecules. Current drug targets *9*, 113-122.

Fricker, M., Neher, J.J., Zhao, J.W., Thery, C., Tolkovsky, A.M., and Brown, G.C. (2012). MFG-E8 mediates primary phagocytosis of viable neurons during neuroinflammation. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 2657-2666.

Fuller, A.D., and Van Eldik, L.J. (2008). MFG-E8 regulates microglial phagocytosis of apoptotic neurons. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology *3*, 246-256.

G

Gaiano, N., and Fishell, G. (2002). The role of notch in promoting glial and neural stem cell fates. Annual review of neuroscience *25*, 471-490.

Gardai, S.J., Bratton, D.L., Ogden, C.A., and Henson, P.M. (2006). Recognition ligands on apoptotic cells: a perspective. Journal of leukocyte biology *79*, 896-903.

Gardai, S.J., McPhillips, K.A., Frasch, S.C., Janssen, W.J., Starefeldt, A., Murphy-Ullrich, J.E., Bratton, D.L., Oldenborg, P.A., Michalak, M., and Henson, P.M. (2005). Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell *123*, 321-334.

Garden, G.A., and Moller, T. (2006). Microglia biology in health and disease. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology *1*, 127-137.

Garin, J., Diez, R., Kieffer, S., Dermine, J.F., Duclos, S., Gagnon, E., Sadoul, R., Rondeau, C., and Desjardins, M. (2001). The phagosome proteome: insight into phagosome functions. The Journal of cell biology *152*, 165-180.

Garthe, A., Behr, J., and Kempermann, G. (2009). Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. PloS one *4*, e5464.

Ge, S., Sailor, K.A., Ming, G.L., and Song, H. (2008). Synaptic integration and plasticity of new neurons in the adult hippocampus. The Journal of physiology *586*, 3759-3765.

Geissmann, F., Gordon, S., Hume, D.A., Mowat, A.M., and Randolph, G.J. (2010). Unravelling mononuclear phagocyte heterogeneity. Nature reviews Immunology *10*, 453-460.

Geloneze, B., de Lima-Junior, J.C., and Velloso, L.A. (2017). Glucagon-Like Peptide-1 Receptor Agonists (GLP-1RAs) in the Brain-Adipocyte Axis. Drugs *77*, 493-503.

Geraerts, M., Eggermont, K., Hernandez-Acosta, P., Garcia-Verdugo, J.M., Baekelandt, V., and Debyser, Z. (2006). Lentiviral vectors mediate efficient and stable gene transfer in adult neural stem cells in vivo. Human gene therapy *17*, 635-650.

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley, E.R.*, et al.* (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science *330*, 841-845.

Ginhoux, F., Lim, S., Hoeffel, G., Low, D., and Huber, T. (2013). Origin and differentiation of microglia. Frontiers in cellular neuroscience *7*, 45.

Ginhoux, F., and Prinz, M. (2015). Origin of microglia: current concepts and past controversies. Cold Spring Harbor perspectives in biology *7*, a020537.

Golpon, H.A., Fadok, V.A., Taraseviciene-Stewart, L., Scerbavicius, R., Sauer, C., Welte, T., Henson, P.M., and Voelkel, N.F. (2004). Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *18*, 1716-1718.

Gomez-Nicola, D., and Perry, V.H. (2015). Microglial dynamics and role in the healthy and diseased brain: a paradigm of functional plasticity. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry *21*, 169-184.

Gomez-Pinilla, F., Lee, J.W., and Cotman, C.W. (1994). Distribution of basic fibroblast growth factor in the developing rat brain. Neuroscience *61*, 911-923.

Goncalves, J.T., Bloyd, C.W., Shtrahman, M., Johnston, S.T., Schafer, S.T., Parylak, S.L., Tran, T., Chang, T., and Gage, F.H. (2016a). In vivo imaging of dendritic pruning in dentate granule cells. Nature neuroscience *19*, 788-791.

Goncalves, J.T., Schafer, S.T., and Gage, F.H. (2016b). Adult Neurogenesis in the Hippocampus: From Stem Cells to Behavior. Cell *167*, 897-914.

Gordon, S., and Pluddemann, A. (2018). Macrophage Clearance of Apoptotic Cells: A Critical Assessment. Frontiers in immunology *9*, 127.

Goshen, I., Kreisel, T., Ounallah-Saad, H., Renbaum, P., Zalzstein, Y., Ben-Hur, T., Levy-Lahad, E., and Yirmiya, R. (2007). A dual role for interleukin-1 in hippocampal-dependent memory processes. Psychoneuroendocrinology *32*, 1106-1115.

Grandbarbe, L., Bouissac, J., Rand, M., Hrabe de Angelis, M., Artavanis-Tsakonas, S., and Mohier, E. (2003). Delta-Notch signaling controls the generation of neurons/glia from neural stem cells in a stepwise process. Development *130*, 1391-1402.

Grathwohl, S.A., Kalin, R.E., Bolmont, T., Prokop, S., Winkelmann, G., Kaeser, S.A., Odenthal, J., Radde, R., Eldh, T., Gandy, S*., et al.* (2009). Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia. Nature neuroscience *12*, 1361-1363.

Grothe, C., Zachmann, K., and Unsicker, K. (1991). Basic FGF-like immunoreactivity in the developing and adult rat brainstem. The Journal of comparative neurology *305*, 328-336.

Gude, D.R., Alvarez, S.E., Paugh, S.W., Mitra, P., Yu, J., Griffiths, R., Barbour, S.E., Milstien, S., and Spiegel, S. (2008). Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *22*, 2629-2638.

H

Hack, M.A., Sugimori, M., Lundberg, C., Nakafuku, M., and Gotz, M. (2004). Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. Molecular and cellular neurosciences *25*, 664-678.

Haimon, Z., Volaski, A., Orthgiess, J., Boura-Halfon, S., Varol, D., Shemer, A., Yona, S., Zuckerman, B., David, E., Chappell-Maor, L*., et al.* (2018). Re-evaluating microglia expression profiles using RiboTag and cell isolation strategies. Nature immunology *19*, 636-644.

Haley, M.J., Brough, D., Quintin, J., and Allan, S.M. (2017). Microglial Priming as Trained Immunity in the Brain. Neuroscience.

Hamley, I.W. (2012). The amyloid beta peptide: a chemist's perspective. Role in Alzheimer's and fibrillization. Chemical reviews *112*, 5147-5192.

Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A., and Nagata, S. (2002). Identification of a factor that links apoptotic cells to phagocytes. Nature *417*, 182-187.

Hanisch, U.K. (2002). Microglia as a source and target of cytokines. Glia *40*, 140-155.

Hardy, J., Bogdanovic, N., Winblad, B., Portelius, E., Andreasen, N., Cedazo-Minguez, A., and Zetterberg, H. (2014). Pathways to Alzheimer's disease. Journal of internal medicine *275*, 296-303.

Hassan, M., Watari, H., AbuAlmaaty, A., Ohba, Y., and Sakuragi, N. (2014). Apoptosis and molecular targeting therapy in cancer. BioMed research international *2014*, 150845.

Heldin, C.H. (2012). Autocrine PDGF stimulation in malignancies. Upsala journal of medical sciences *117*, 83-91.

Hellwig, S., Masuch, A., Nestel, S., Katzmarski, N., Meyer-Luehmann, M., and Biber, K. (2015). Forebrain microglia from wild-type but not adult 5xFAD mice prevent amyloid-beta plaque formation in organotypic hippocampal slice cultures. Scientific reports *5*, 14624.

Henson, P.M. (2005). Engulfment: ingestion and migration with Rac, Rho and TRIO. Current biology : CB *15*, R29-30.

Henson, P.M., and Bratton, D.L. (2013). Antiinflammatory effects of apoptotic cells. The Journal of clinical investigation *123*, 2773-2774.

Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T., and Kishimoto, T. (1990). Molecular cloning and expression of an IL-6 signal transducer, gp130. Cell *63*, 1149-1157.

Hibi, Y., Nitta, A., Nabeshima, T., and Yamada, K. (2009). [Possible relation of BDNF and GDNF to neuropsychiatric disorders]. Nihon shinkei seishin yakurigaku zasshi = Japanese journal of psychopharmacology *29*, 85-89.

Hickman, S.E., Kingery, N.D., Ohsumi, T.K., Borowsky, M.L., Wang, L.C., Means, T.K., and El Khoury, J. (2013). The microglial sensome revealed by direct RNA sequencing. Nature neuroscience *16*, 1896-1905.

Hirbec, H., Marmai, C., Duroux-Richard, I., Roubert, C., Esclangon, A., Croze, S., Lachuer, J., Peyroutou, R., and Rassendren, F. (2018). The microglial reaction signature revealed by RNAseq from individual mice. Glia *66*, 971-986.

Holtman, I.R., Noback, M., Bijlsma, M., Duong, K.N., van der Geest, M.A., Ketelaars, P.T., Brouwer, N., Vainchtein, I.D., Eggen, B.J., and Boddeke, H.W. (2015). Glia Open Access Database (GOAD): A comprehensive gene expression encyclopedia of glia cells in health and disease. Glia *63*, 1495-1506.

Hong, S., Beja-Glasser, V.F., Nfonoyim, B.M., Frouin, A., Li, S., Ramakrishnan, S., Merry, K.M., Shi, Q., Rosenthal, A., Barres, B.A.*, et al.* (2016). Complement and microglia mediate early synapse loss in Alzheimer mouse models. Science *352*, 712-716.

Hoshiko, M., Arnoux, I., Avignone, E., Yamamoto, N., and Audinat, E. (2012). Deficiency of the microglial receptor CX3CR1 impairs postnatal functional development of thalamocortical synapses in the barrel cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 15106-15111.

Hosmane, S., Tegenge, M.A., Rajbhandari, L., Uapinyoying, P., Ganesh Kumar, N., Thakor, N., and Venkatesan, A. (2012). Toll/interleukin-1 receptor domain-containing adapter inducing interferon-beta mediates microglial phagocytosis of degenerating axons. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 7745-7757.

Hsieh, C.L., Koike, M., Spusta, S.C., Niemi, E.C., Yenari, M., Nakamura, M.C., and Seaman, W.E. (2009). A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. Journal of neurochemistry *109*, 1144-1156.

BIBLIOGRAPHY

Hsu, M.P., Frausto, R., Rose-John, S., and Campbell, I.L. (2015). Analysis of IL-6/gp130 family receptor expression reveals that in contrast to astroglia, microglia lack the oncostatin M receptor and functional responses to oncostatin M. Glia *63*, 132-141.

Hua, J.Y., and Smith, S.J. (2004). Neural activity and the dynamics of central nervous system development. Nature neuroscience *7*, 327-332.

Huat, T.J., Khan, A.A., Pati, S., Mustafa, Z., Abdullah, J.M., and Jaafar, H. (2014). IGF-1 enhances cell proliferation and survival during early differentiation of mesenchymal stem cells to neural progenitor-like cells. BMC neuroscience *15*, 91.

Hughes, C.E., and Nibbs, R.J.B. (2018). A Guide to Chemokines and Their Receptors. The FEBS journal.

Hunsberger, J.G., Newton, S.S., Bennett, A.H., Duman, C.H., Russell, D.S., Salton, S.R., and Duman, R.S. (2007). Antidepressant actions of the exercise-regulated gene VGF. Nature medicine *13*, 1476-1482.

I

Iosif, R.E., Ekdahl, C.T., Ahlenius, H., Pronk, C.J., Bonde, S., Kokaia, Z., Jacobsen, S.E., and Lindvall, O. (2006). Tumor necrosis factor receptor 1 is a negative regulator of progenitor proliferation in adult hippocampal neurogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience *26*, 9703-9712.

Iram, T., Ramirez-Ortiz, Z., Byrne, M.H., Coleman, U.A., Kingery, N.D., Means, T.K., Frenkel, D., and El Khoury, J. (2016). Megf10 Is a Receptor for C1Q That Mediates Clearance of Apoptotic Cells by Astrocytes. The Journal of neuroscience : the official journal of the Society for Neuroscience *36*, 5185-5192.

Irvin, D.K., Dhaka, A., Hicks, C., Weinmaster, G., and Kornblum, H.I. (2003). Extrinsic and intrinsic factors governing cell fate in cortical progenitor cultures. Developmental neuroscience *25*, 162-172.

Ishii, Y., Matsumoto, Y., Watanabe, R., Elmi, M., Fujimori, T., Nissen, J., Cao, Y., Nabeshima, Y., Sasahara, M., and Funa, K. (2008). Characterization of neuroprogenitor cells expressing the PDGF beta-receptor within the subventricular zone of postnatal mice. Molecular and cellular neurosciences *37*, 507-518.

Ishimoto, Y., Ohashi, K., Mizuno, K., and Nakano, T. (2000). Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6. Journal of biochemistry *127*, 411-417.

J

Jana, M., Palencia, C.A., and Pahan, K. (2008). Fibrillar amyloid-beta peptides activate microglia via TLR2: implications for Alzheimer's disease. Journal of immunology *181*, 7254-7262.

Jaye, M., Schlessinger, J., and Dionne, C.A. (1992). Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. Biochimica et biophysica acta *1135*, 185-199.

Jeannin, P., Paolini, L., Adam, C., and Delneste, Y. (2018). The roles of CSFs on the functional polarization of tumor-associated macrophages. The FEBS journal *285*, 680-699.

Jensen, J.B., and Parmar, M. (2006). Strengths and limitations of the neurosphere culture system. Molecular neurobiology *34*, 153-161.

Jeong, J., Paskus, J.D., and Roche, K.W. (2017). Posttranslational modifications of neuroligins regulate neuronal and glial signaling. Current opinion in neurobiology *45*, 130-138.

Jessberger, S., Clark, R.E., Broadbent, N.J., Clemenson, G.D., Jr., Consiglio, A., Lie, D.C., Squire, L.R., and Gage, F.H. (2009). Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. Learning & memory *16*, 147-154.

Jethwa, P.H., and Ebling, F.J. (2008). Role of VGF-derived peptides in the control of food intake, body weight and reproduction. Neuroendocrinology *88*, 80-87.

Jin, K., Li, B., Lou, L., Xu, Y., Ye, X., Yao, K., Ye, J., and Gao, C. (2016). In vivo vascularization of MSC-loaded porous hydroxyapatite constructs coated with VEGF-functionalized collagen/heparin multilayers. Scientific reports *6*, 19871.

Jin, X., and Yamashita, T. (2016). Microglia in central nervous system repair after injury. Journal of biochemistry *159*, 491-496.

Jones, R.S., Minogue, A.M., Connor, T.J., and Lynch, M.A. (2013). Amyloid-beta-induced astrocytic phagocytosis is mediated by CD36, CD47 and RAGE. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology *8*, 301-311.

Jung, K.H., Chu, K., Lee, S.T., Kang, L., Kim, S.U., Kim, M., and Roh, J.K. (2006). G-CSF protects human cerebral hybrid neurons against in vitro ischemia. Neuroscience letters *394*, 168-173.

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M.J., Kreutzberg, G.W., Sher, A., and Littman, D.R. (2000). Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Molecular and cellular biology *20*, 4106-4114.

K

Kaindl, A.M., Degos, V., Peineau, S., Gouadon, E., Chhor, V., Loron, G., Le Charpentier, T., Josserand, J., Ali, C., Vivien, D.*, et al.* (2012). Activation of microglial N-methyl-D-aspartate receptors triggers inflammation and neuronal cell death in the developing and mature brain. Annals of neurology *72*, 536-549.

Kempermann, G., Jessberger, S., Steiner, B., and Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. Trends in neurosciences *27*, 447-452.

Kettenmann, H., Kirchhoff, F., and Verkhratsky, A. (2013). Microglia: new roles for the synaptic stripper. Neuron *77*, 10-18.

Kinchen, J.M., Doukoumetzidis, K., Almendinger, J., Stergiou, L., Tosello-Trampont, A., Sifri, C.D., Hengartner, M.O., and Ravichandran, K.S. (2008). A pathway for phagosome maturation during engulfment of apoptotic cells. Nature cell biology *10*, 556-566.

BIBLIOGRAPHY

Kinchen, J.M., and Ravichandran, K.S. (2008). Phagosome maturation: going through the acid test. Nature reviews Molecular cell biology *9*, 781-795.

Kirik, D., Georgievska, B., and Bjorklund, A. (2004). Localized striatal delivery of GDNF as a treatment for Parkinson disease. Nature neuroscience *7*, 105-110.

Klein, C., Butt, S.J., Machold, R.P., Johnson, J.E., and Fishell, G. (2005). Cerebellum- and forebrain-derived stem cells possess intrinsic regional character. Development *132*, 4497-4508.
Klein, M.A., Moller, J.C., Jones, L.L., Bluethmann, H., Kreutzberg, G.W., and Raivich, G. (1997). Impaired neuroglial activation in interleukin-6 deficient mice. Glia *19*, 227-233.

Klintworth, H., Garden, G., and Xia, Z. (2009). Rotenone and paraquat do not directly activate microglia or induce inflammatory cytokine release. Neuroscience letters *462*, 1-5.

Kloss, C.U., Kreutzberg, G.W., and Raivich, G. (1997). Proliferation of ramified microglia on an astrocyte monolayer: characterization of stimulatory and inhibitory cytokines. Journal of neuroscience research *49*, 248-254.

Kocur, M., Schneider, R., Pulm, A.K., Bauer, J., Kropp, S., Gliem, M., Ingwersen, J., Goebels, N., Alferink, J., Prozorovski, T.*, et al.* (2015). IFNbeta secreted by microglia mediates clearance of myelin debris in CNS autoimmunity. Acta neuropathologica communications *3*, 20.

Kohyama, J., Sanosaka, T., Tokunaga, A., Takatsuka, E., Tsujimura, K., Okano, H., and Nakashima, K. (2010). BMP-induced REST regulates the establishment and maintenance of astrocytic identity. The Journal of cell biology *189*, 159-170.

Koivunen, P., Hirsila, M., Remes, A.M., Hassinen, I.E., Kivirikko, K.I., and Myllyharju, J. (2007). Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: possible links between cell metabolism and stabilization of HIF. The Journal of biological chemistry *282*, 4524-4532.

Komine-Kobayashi, M., Zhang, N., Liu, M., Tanaka, R., Hara, H., Osaka, A., Mochizuki, H., Mizuno, Y., and Urabe, T. (2006). Neuroprotective effect of recombinant human granulocyte colony-stimulating factor in transient focal ischemia of mice. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism *26*, 402-413.

Koo, J.W., and Duman, R.S. (2008). IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. Proceedings of the National Academy of Sciences of the United States of America *105*, 751-756.

Korb, L.C., and Ahearn, J.M. (1997). C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. Journal of immunology *158*, 4525-4528.

Kotter, M.R., Li, W.W., Zhao, C., and Franklin, R.J. (2006). Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience *26*, 328-332.

Krabbe, G., Halle, A., Matyash, V., Rinnenthal, J.L., Eom, G.D., Bernhardt, U., Miller, K.R., Prokop, S., Kettenmann, H., and Heppner, F.L. (2013). Functional impairment of microglia

coincides with Beta-amyloid deposition in mice with Alzheimer-like pathology. PloS one *8*, e60921.

Krause, T.A., Alex, A.F., Engel, D.R., Kurts, C., and Eter, N. (2014). VEGF-production by CCR2-dependent macrophages contributes to laser-induced choroidal neovascularization. PloS one *9*, e94313.

Krishan, A. (1975). Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. The Journal of cell biology *66*, 188-193.

Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N.*, et al.* (1998). Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature *393*, 72-76.

Kronenberg, G., Reuter, K., Steiner, B., Brandt, M.D., Jessberger, S., Yamaguchi, M., and Kempermann, G. (2003). Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. The Journal of comparative neurology *467*, 455-463.

Krupinski, J., Issa, R., Bujny, T., Slevin, M., Kumar, P., Kumar, S., and Kaluza, J. (1997). A putative role for platelet-derived growth factor in angiogenesis and neuroprotection after ischemic stroke in humans. Stroke *28*, 564-573.

Krysko, D.V., Vanden Berghe, T., D'Herde, K., and Vandenabeele, P. (2008). Apoptosis and necrosis: detection, discrimination and phagocytosis. Methods *44*, 205-221.

Kubota, Y., Takubo, K., Shimizu, T., Ohno, H., Kishi, K., Shibuya, M., Saya, H., and Suda, T. (2009). M-CSF inhibition selectively targets pathological angiogenesis and lymphangiogenesis. The Journal of experimental medicine *206*, 1089-1102.

Kuhn, H.G., Eisch, A.J., Spalding, K., and Peterson, D.A. (2016). Detection and Phenotypic Characterization of Adult Neurogenesis. Cold Spring Harbor perspectives in biology *8*, a025981.

Kwon, M.J., Oh, E., Lee, S., Roh, M.R., Kim, S.E., Lee, Y., Choi, Y.L., In, Y.H., Park, T., Koh, S.S.*, et al.* (2009). Identification of novel reference genes using multiplatform expression data and their validation for quantitative gene expression analysis. PloS one *4*, e6162.

L

Lagace, D.C., Whitman, M.C., Noonan, M.A., Ables, J.L., DeCarolis, N.A., Arguello, A.A., Donovan, M.H., Fischer, S.J., Farnbauch, L.A., Beech, R.D.*, et al.* (2007). Dynamic contribution of nestin-expressing stem cells to adult neurogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 12623-12629.

Lakatos, A., Prinster, S., Vicentic, A., Hall, R.A., and Kuhar, M.J. (2005). Cocaine- and amphetamine-regulated transcript (CART) peptide activates the extracellular signal-regulated kinase (ERK) pathway in AtT20 cells via putative G-protein coupled receptors. Neuroscience letters *384*, 198-202.

BIBLIOGRAPHY

Lampron, A., Larochelle, A., Laflamme, N., Prefontaine, P., Plante, M.M., Sanchez, M.G., Yong, V.W., Stys, P.K., Tremblay, M.E., and Rivest, S. (2015). Inefficient clearance of myelin debris by microglia impairs remyelinating processes. The Journal of experimental medicine *212*, 481-495.

Langfelder, P., and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. BMC bioinformatics *9*, 559.

Langfelder, P., and Horvath, S. (2012). Fast R Functions for Robust Correlations and Hierarchical Clustering. Journal of statistical software *46*.

Laplagne, D.A., Esposito, M.S., Piatti, V.C., Morgenstern, N.A., Zhao, C., van Praag, H., Gage, F.H., and Schinder, A.F. (2006). Functional convergence of neurons generated in the developing and adult hippocampus. PLoS biology *4*, e409.

Laron, Z. (2001). Insulin-like growth factor 1 (IGF-1): a growth hormone. Molecular pathology : MP *54*, 311-316.

Lau, L.T., and Yu, A.C. (2001). Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. Journal of neurotrauma *18*, 351-359.

Lauber, K., Bohn, E., Krober, S.M., Xiao, Y.J., Blumenthal, S.G., Lindemann, R.K., Marini, P., Wiedig, C., Zobywalski, A., Baksh, S.*, et al.* (2003). Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell *113*, 717-730.

Lawrie, A.M., Noble, M.E., Tunnah, P., Brown, N.R., Johnson, L.N., and Endicott, J.A. (1997). Protein kinase inhibition by staurosporine revealed in details of the molecular interaction with CDK2. Nature structural biology *4*, 796-801.

Learish, R.D., Bruss, M.D., and Haak-Frendscho, M. (2000). Inhibition of mitogen-activated protein kinase kinase blocks proliferation of neural progenitor cells. Brain research Developmental brain research *122*, 97-109.

Lee, B., Vouthounis, C., Stojadinovic, O., Brem, H., Im, M., and Tomic-Canic, M. (2005). From an enhanceosome to a repressosome: molecular antagonism between glucocorticoids and EGF leads to inhibition of wound healing. Journal of molecular biology *345*, 1083-1097.

Lee, C.G., Link, H., Baluk, P., Homer, R.J., Chapoval, S., Bhandari, V., Kang, M.J., Cohn, L., Kim, Y.K., McDonald, D.M.*, et al.* (2004a). Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. Nature medicine *10*, 1095-1103.

Lee, G.A., Lin, C.H., Jiang, H.H., Chao, H.J., Wu, C.L., and Hsueh, C.M. (2004b). Microglia-derived glial cell line-derived neurotrophic factor could protect Sprague-Dawley rat astrocyte from in vitro ischemia-induced damage. Neuroscience letters *356*, 111-114.

Lee, J., and Tansey, M.G. (2013). Microglia isolation from adult mouse brain. Microglia: Methods and Protocols, Methods in Molecular Biology *1041*.

Lee, J.W., Bae, S.H., Jeong, J.W., Kim, S.H., and Kim, K.W. (2004c). Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions. Experimental & molecular medicine *36*, 1-12.

Lehnardt, S. (2010). Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. Glia *58*, 253-263.

Lewis, J.E., Brameld, J.M., and Jethwa, P.H. (2015). Neuroendocrine Role for VGF. Frontiers in endocrinology *6*, 3.

Li, R.B., Guo, X.C., Liang, H.X., Wang, F.Y., and Zhu, B.L. (2009). Study on changes of MMP-3 expression after brain contusion in rats. Legal medicine *11 Suppl 1*, S176-179.

Lin, H.W., and Levison, S.W. (2009). Context-dependent IL-6 potentiation of interferon-gamma-induced IL-12 secretion and CD40 expression in murine microglia. Journal of neurochemistry *111*, 808-818.

Lin, L.F., Doherty, D.H., Lile, J.D., Bektesh, S., and Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science *260*, 1130-1132.

Linnartz, B., Kopatz, J., Tenner, A.J., and Neumann, H. (2012). Sialic acid on the neuronal glycocalyx prevents complement C1 binding and complement receptor-3-mediated removal by microglia. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 946-952.

Liu, H.K., Belz, T., Bock, D., Takacs, A., Wu, H., Lichter, P., Chai, M., and Schutz, G. (2008). The nuclear receptor tailless is required for neurogenesis in the adult subventricular zone. Genes & development *22*, 2473-2478.

Liu, S., Liu, Y., Hao, W., Wolf, L., Kiliaan, A.J., Penke, B., Rube, C.E., Walter, J., Heneka, M.T., Hartmann, T.*, et al.* (2012). TLR2 is a primary receptor for Alzheimer's amyloid beta peptide to trigger neuroinflammatory activation. Journal of immunology *188*, 1098-1107.

Liu, X., Mashour, G.A., Webster, H.F., and Kurtz, A. (1998). Basic FGF and FGF receptor 1 are expressed in microglia during experimental autoimmune encephalomyelitis: temporally distinct expression of midkine and pleiotrophin. Glia *24*, 390-397.

Liu, Y., Hao, W., Letiembre, M., Walter, S., Kulanga, M., Neumann, H., and Fassbender, K. (2006). Suppression of microglial inflammatory activity by myelin phagocytosis: role of p47-PHOX-mediated generation of reactive oxygen species. The Journal of neuroscience : the official journal of the Society for Neuroscience *26*, 12904-12913.

Liu, Y., Miao, Q., Yuan, J., Han, S., Zhang, P., Li, S., Rao, Z., Zhao, W., Ye, Q., Geng, J.*, et al.* (2015). Ascl1 Converts Dorsal Midbrain Astrocytes into Functional Neurons In Vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience *35*, 9336-9355.

Liu, Z., Condello, C., Schain, A., Harb, R., and Grutzendler, J. (2010). CX3CR1 in microglia regulates brain amyloid deposition through selective protofibrillar amyloid-beta phagocytosis. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 17091-17101.

Llorens-Martin, M., Torres-Aleman, I., and Trejo, J.L. (2009). Mechanisms mediating brain plasticity: IGF1 and adult hippocampal neurogenesis. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry *15*, 134-148.

Loftis, J.M., Huckans, M., and Morasco, B.J. (2010). Neuroimmune mechanisms of cytokine-induced depression: current theories and novel treatment strategies. Neurobiology of disease *37*, 519-533.

Lopez-Atalaya, J.P., Askew, K.E., Sierra, A., and Gomez-Nicola, D. (2017). Development and maintenance of the brain's immune toolkit: Microglia and non-parenchymal brain macrophages. Developmental neurobiology.

Lu, C.Z., and Xiao, B.G. (2006). G-CSF and neuroprotection: a therapeutic perspective in cerebral ischaemia. Biochemical Society transactions *34*, 1327-1333.

Lu, Z., Elliott, M.R., Chen, Y., Walsh, J.T., Klibanov, A.L., Ravichandran, K.S., and Kipnis, J. (2011). Phagocytic activity of neuronal progenitors regulates adult neurogenesis. Nature cell biology *13*, 1076-1083.

Lucas, S.M., Rothwell, N.J., and Gibson, R.M. (2006). The role of inflammation in CNS injury and disease. British journal of pharmacology *147 Suppl 1*, S232-240.

Lutolf, S., Radtke, F., Aguet, M., Suter, U., and Taylor, V. (2002). Notch1 is required for neuronal and glial differentiation in the cerebellum. Development *129*, 373-385.

Lynch, N.J., Willis, C.L., Nolan, C.C., Roscher, S., Fowler, M.J., Weihe, E., Ray, D.E., and Schwaeble, W.J. (2004). Microglial activation and increased synthesis of complement component C1q precedes blood-brain barrier dysfunction in rats. Molecular immunology *40*, 709-716.

M

MacLeod, A.S., and Mansbridge, J.N. (2016). The Innate Immune System in Acute and Chronic Wounds. Advances in wound care *5*, 65-78.

Magnus, T., Chan, A., Grauer, O., Toyka, K.V., and Gold, R. (2001). Microglial phagocytosis of apoptotic inflammatory T cells leads to down-regulation of microglial immune activation. Journal of immunology *167*, 5004-5010.

Magnus, T., Chan, A., Linker, R.A., Toyka, K.V., and Gold, R. (2002). Astrocytes are less efficient in the removal of apoptotic lymphocytes than microglia cells: implications for the role of glial cells in the inflamed central nervous system. Journal of neuropathology and experimental neurology *61*, 760-766.

Makide, K., Uwamizu, A., Shinjo, Y., Ishiguro, J., Okutani, M., Inoue, A., and Aoki, J. (2014). Novel lysophospholipid receptors: their structure and function. Journal of lipid research *55*, 1986-1995.

Masure, S., Geerts, H., Cik, M., Hoefnagel, E., Van Den Kieboom, G., Tuytelaars, A., Harris, S., Lesage, A.S., Leysen, J.E., Van Der Helm, L.*, et al.* (1999). Enovin, a member of the glial cell-line-derived neurotrophic factor (GDNF) family with growth promoting activity on neuronal cells. Existence and tissue-specific expression of different splice variants. European journal of biochemistry *266*, 892-902.

Mathieu, P., Adami, P.V., and Morelli, L. (2013). Notch signaling in the pathologic adult brain. Biomolecular concepts *4*, 465-476.

Mawuenyega, K.G., Sigurdson, W., Ovod, V., Munsell, L., Kasten, T., Morris, J.C., Yarasheski, K.E., and Bateman, R.J. (2010). Decreased clearance of CNS beta-amyloid in Alzheimer's disease. Science *330*, 1774.

McCoy, M.K., and Tansey, M.G. (2008). TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. Journal of neuroinflammation *5*, 45.

McGonigal, R., Cunningham, M.E., Yao, D., Barrie, J.A., Sankaranarayanan, S., Fewou, S.N., Furukawa, K., Yednock, T.A., and Willison, H.J. (2016). C1q-targeted inhibition of the classical complement pathway prevents injury in a novel mouse model of acute motor axonal neuropathy. Acta neuropathologica communications *4*, 23.

Medina, M.A., Andrade, V.M., Caracci, M.O., Avila, M.E., Verdugo, D.A., Vargas, M.F., Ugarte, G.D., Reyes, A.E., Opazo, C., and De Ferrari, G.V. (2018). Wnt/beta-catenin signaling stimulates the expression and synaptic clustering of the autism-associated Neuroligin 3 gene. Translational psychiatry *8*, 45.

Meyer-Luehmann, M., Spires-Jones, T.L., Prada, C., Garcia-Alloza, M., de Calignon, A., Rozkalne, A., Koenigsknecht-Talboo, J., Holtzman, D.M., Bacskai, B.J., and Hyman, B.T. (2008). Rapid appearance and local toxicity of amyloid-beta plaques in a mouse model of Alzheimer's disease. Nature *451*, 720-724.

Mignone, J.L., Kukekov, V., Chiang, A.S., Steindler, D., and Enikolopov, G. (2004). Neural stem and progenitor cells in nestin-GFP transgenic mice. The Journal of comparative neurology *469*, 311-324.

Miyake, A., and Itoh, N. (1996). Rat fibroblast growth factor receptor-4 mRNA in the brain is preferentially expressed in cholinergic neurons in the medial habenular nucleus. Neuroscience letters *203*, 101-104.

Mizutani, M., Pino, P.A., Saederup, N., Charo, I.F., Ransohoff, R.M., and Cardona, A.E. (2012). The fractalkine receptor but not CCR2 is present on microglia from embryonic development throughout adulthood. Journal of immunology *188*, 29-36.

Mlecnik, B., Galon, J., and Bindea, G. (2018). Comprehensive functional analysis of large lists of genes and proteins. Journal of proteomics *171*, 2-10.

Moeton, M., Stassen, O.M., Sluijs, J.A., van der Meer, V.W., Kluivers, L.J., van Hoorn, H., Schmidt, T., Reits, E.A., van Strien, M.E., and Hol, E.M. (2016). GFAP isoforms control intermediate filament network dynamics, cell morphology, and focal adhesions. Cellular and molecular life sciences : CMLS *73*, 4101-4120.

Monje, M.L., Toda, H., and Palmer, T.D. (2003). Inflammatory blockade restores adult hippocampal neurogenesis. Science *302*, 1760-1765.

Montgomery, S.L., and Bowers, W.J. (2012). Tumor necrosis factor-alpha and the roles it plays in homeostatic and degenerative processes within the central nervous system. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology *7*, 42-59.

Moorlag, S., Roring, R.J., Joosten, L.A.B., and Netea, M.G. (2018). The role of the interleukin-1 family in trained immunity. Immunological reviews *281*, 28-39.

Moreno-Sanchez, R., Rodriguez-Enriquez, S., Marin-Hernandez, A., and Saavedra, E. (2007). Energy metabolism in tumor cells. The FEBS journal *274*, 1393-1418.

Morgan, S.C., Taylor, D.L., and Pocock, J.M. (2004). Microglia release activators of neuronal proliferation mediated by activation of mitogen-activated protein kinase, phosphatidylinositol-3-kinase/Akt and delta-Notch signalling cascades. Journal of neurochemistry *90*, 89-101.

Mori, T., Tanaka, K., Buffo, A., Wurst, W., Kuhn, R., and Gotz, M. (2006). Inducible gene deletion in astroglia and radial glia--a valuable tool for functional and lineage analysis. Glia *54*, 21-34.

Morikawa, M., Derynck, R., and Miyazono, K. (2016). TGF-beta and the TGF-beta Family: Context-Dependent Roles in Cell and Tissue Physiology. Cold Spring Harbor perspectives in biology *8*.

Morland, C., Lauritzen, K.H., Puchades, M., Holm-Hansen, S., Andersson, K., Gjedde, A., Attramadal, H., Storm-Mathisen, J., and Bergersen, L.H. (2015). The lactate receptor, G-protein-coupled receptor 81/hydroxycarboxylic acid receptor 1: Expression and action in brain. Journal of neuroscience research *93*, 1045-1055.

Morrison, S.J., Perez, S.E., Qiao, Z., Verdi, J.M., Hicks, C., Weinmaster, G., and Anderson, D.J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. Cell *101*, 499-510.

Morshead, C.M., Benveniste, P., Iscove, N.N., and van der Kooy, D. (2002). Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. Nature medicine *8*, 268-273.

Mukherjee, S., Ghosh, R.N., and Maxfield, F.R. (1997). Endocytosis. Physiological reviews *77*, 759-803.

N

Nagata, S., Hanayama, R., and Kawane, K. (2010). Autoimmunity and the clearance of dead cells. Cell *140*, 619-630.

Nait Oumesmar, B., Vignais, L., and Baron-Van Evercooren, A. (1997). Developmental expression of platelet-derived growth factor alpha-receptor in neurons and glial cells of the mouse CNS. The Journal of neuroscience : the official journal of the Society for Neuroscience *17*, 125-139.

Nakashiba, T., Cushman, J.D., Pelkey, K.A., Renaudineau, S., Buhl, D.L., McHugh, T.J., Rodriguez Barrera, V., Chittajallu, R., Iwamoto, K.S., McBain, C.J.*, et al.* (2012). Young dentate granule cells mediate pattern separation, whereas old granule cells facilitate pattern completion. Cell *149*, 188-201.

Nauta, A.J., Trouw, L.A., Daha, M.R., Tijsma, O., Nieuwland, R., Schwaeble, W.J., Gingras, A.R., Mantovani, A., Hack, E.C., and Roos, A. (2002). Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. European journal of immunology *32*, 1726-1736.

Nayak, D., Roth, T.L., and McGavern, D.B. (2014). Microglia development and function. Annual review of immunology *32*, 367-402.

Netea, M.G., Latz, E., Mills, K.H., and O'Neill, L.A. (2015). Innate immune memory: a paradigm shift in understanding host defense. Nature immunology *16*, 675-679.

Neumann, H., and Takahashi, K. (2007). Essential role of the microglial triggering receptor expressed on myeloid cells-2 (TREM2) for central nervous tissue immune homeostasis. Journal of neuroimmunology *184*, 92-99.

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science *308*, 1314-1318.

Nistico, R., Salter, E., Nicolas, C., Feligioni, M., Mango, D., Bortolotto, Z.A., Gressens, P., Collingridge, G.L., and Peineau, S. (2017). Synaptoimmunology - roles in health and disease. Molecular brain *10*, 26.

Nizet, V., and Johnson, R.S. (2009). Interdependence of hypoxic and innate immune responses. Nature reviews Immunology *9*, 609-617.

Nowacka, M.M., and Obuchowicz, E. (2012). Vascular endothelial growth factor (VEGF) and its role in the central nervous system: a new element in the neurotrophic hypothesis of antidepressant drug action. Neuropeptides *46*, 1-10.

O

O'Neill, L.A., and Hardie, D.G. (2013). Metabolism of inflammation limited by AMPK and pseudo-starvation. Nature *493*, 346-355.

O'Sullivan, S.A., O'Sullivan, C., Healy, L.M., Dev, K.K., and Sheridan, G.K. (2018). Sphingosine 1-phosphate receptors regulate TLR4-induced CXCL5 release from astrocytes and microglia. Journal of neurochemistry.

Ogden, C.A., deCathelineau, A., Hoffmann, P.R., Bratton, D., Ghebrehiwet, B., Fadok, V.A., and Henson, P.M. (2001). C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. The Journal of experimental medicine *194*, 781-795.

Ogden, C.A., and Elkon, K.B. (2006). Role of complement and other innate immune mechanisms in the removal of apoptotic cells. Current directions in autoimmunity *9*, 120-142.

Opal, S.M., and DePalo, V.A. (2000). Anti-inflammatory cytokines. Chest *117*, 1162-1172.

Opstal-van Winden, A.W., Beijnen, J.H., Loof, A., van Heerde, W.L., Vermeulen, R., Peeters, P.H., and van Gils, C.H. (2012). Search for breast cancer biomarkers in fractionated serum samples by protein profiling with SELDI-TOF MS. Journal of clinical laboratory analysis *26*, 1-9.

Orihuela, R., McPherson, C.A., and Harry, G.J. (2016). Microglial M1/M2 polarization and metabolic states. British journal of pharmacology *173*, 649-665.

Overall, R.W., Paszkowski-Rogacz, M., and Kempermann, G. (2012). The mammalian adult neurogenesis gene ontology (MANGO) provides a structural framework for published information on genes regulating adult hippocampal neurogenesis. PloS one *7*, e48527.

Ozdemir, D., Baykara, B., Aksu, I., Kiray, M., Sisman, A.R., Cetin, F., Dayi, A., Gurpinar, T., Uysal, N., and Arda, M.N. (2012). Relationship between circulating IGF-1 levels and traumatic brain injury-induced hippocampal damage and cognitive dysfunction in immature rats. Neuroscience letters *507*, 84-89.

P

Paidassi, H., Tacnet-Delorme, P., Garlatti, V., Darnault, C., Ghebrehiwet, B., Gaboriaud, C., Arlaud, G.J., and Frachet, P. (2008). C1q binds phosphatidylserine and likely acts as a multiligand-bridging molecule in apoptotic cell recognition. Journal of immunology *180*, 2329-2338.

Paloneva, J., Manninen, T., Christman, G., Hovanes, K., Mandelin, J., Adolfsson, R., Bianchin, M., Bird, T., Miranda, R., Salmaggi, A.*, et al.* (2002). Mutations in two genes encoding different subunits of a receptor signaling complex result in an identical disease phenotype. American journal of human genetics *71*, 656-662.

Palsson-McDermott, E.M., Curtis, A.M., Goel, G., Lauterbach, M.A., Sheedy, F.J., Gleeson, L.E., van den Bosch, M.W., Quinn, S.R., Domingo-Fernandez, R., Johnston, D.G.*, et al.* (2015). Pyruvate kinase M2 regulates Hif-1alpha activity and IL-1beta induction and is a critical determinant of the warburg effect in LPS-activated macrophages. Cell metabolism *21*, 65-80.

Panaretakis, T., Kepp, O., Brockmeier, U., Tesniere, A., Bjorklund, A.C., Chapman, D.C., Durchschlag, M., Joza, N., Pierron, G., van Endert, P.*, et al.* (2009). Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. The EMBO journal *28*, 578-590.

Pang, Y., Cai, Z., and Rhodes, P.G. (2000). Effects of lipopolysaccharide on oligodendrocyte progenitor cells are mediated by astrocytes and microglia. Journal of neuroscience research *62*, 510-520.

Pang, Y., Fan, L.W., Tien, L.T., Dai, X., Zheng, B., Cai, Z., Lin, R.C., and Bhatt, A. (2013). Differential roles of astrocyte and microglia in supporting oligodendrocyte development and myelination in vitro. Brain and behavior *3*, 503-514.

Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T.A., Guiducci, E., Dumas, L.*, et al.* (2011). Synaptic pruning by microglia is necessary for normal brain development. Science *333*, 1456-1458.

Parakalan, R., Jiang, B., Nimmi, B., Janani, M., Jayapal, M., Lu, J., Tay, S.S., Ling, E.A., and Dheen, S.T. (2012). Transcriptome analysis of amoeboid and ramified microglia isolated from the corpus callosum of rat brain. BMC neuroscience *13*, 64.

Park, D., Han, C.Z., Elliott, M.R., Kinchen, J.M., Trampont, P.C., Das, S., Collins, S., Lysiak, J.J., Hoehn, K.L., and Ravichandran, K.S. (2011). Continued clearance of apoptotic cells critically depends on the phagocyte Ucp2 protein. Nature *477*, 220-224.

Parmar, M., Skogh, C., Bjorklund, A., and Campbell, K. (2002). Regional specification of neurosphere cultures derived from subregions of the embryonic telencephalon. Molecular and cellular neurosciences *21*, 645-656.

Parnaik, R., Raff, M.C., and Scholes, J. (2000). Differences between the clearance of apoptotic cells by professional and non-professional phagocytes. Current biology : CB *10*, 857-860.

Penkowa, M., Moos, T., Carrasco, J., Hadberg, H., Molinero, A., Bluethmann, H., and Hidalgo, J. (1999). Strongly compromised inflammatory response to brain injury in interleukin-6-deficient mice. Glia *25*, 343-357.

Pennisi, E. (2014). Immunology. Metabolic shift may train immune cells. Science *345*, 1550-1551.

Perego, C., Fumagalli, S., and De Simoni, M.G. (2011). Temporal pattern of expression and colocalization of microglia/macrophage phenotype markers following brain ischemic injury in mice. Journal of neuroinflammation *8*, 174.

Peri, F., and Nusslein-Volhard, C. (2008). Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion in vivo. Cell *133*, 916-927.

Perry, V.H., Nicoll, J.A., and Holmes, C. (2010). Microglia in neurodegenerative disease. Nature reviews Neurology *6*, 193-201.

Peter, C., Waibel, M., Radu, C.G., Yang, L.V., Witte, O.N., Schulze-Osthoff, K., Wesselborg, S., and Lauber, K. (2008). Migration to apoptotic "find-me" signals is mediated via the phagocyte receptor G2A. The Journal of biological chemistry *283*, 5296-5305.

Peter, C., Wesselborg, S., and Lauber, K. (2010). Molecular suicide notes: last call from apoptosing cells. Journal of molecular cell biology *2*, 78-80.

Phypers, M., Harris, T., and Power, C. (2006). CNS tuberculosis: a longitudinal analysis of epidemiological and clinical features. The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease *10*, 99-103.

Piatti, V.C., Davies-Sala, M.G., Esposito, M.S., Mongiat, L.A., Trinchero, M.F., and Schinder, A.F. (2011). The timing for neuronal maturation in the adult hippocampus is modulated by local network activity. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 7715-7728.

Piatti, V.C., Esposito, M.S., and Schinder, A.F. (2006). The timing of neuronal development in adult hippocampal neurogenesis. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry *12*, 463-468.

Pitossi, F., del Rey, A., Kabiersch, A., and Besedovsky, H. (1997). Induction of cytokine transcripts in the central nervous system and pituitary following peripheral administration of endotoxin to mice. Journal of neuroscience research *48*, 287-298.

Plumpe, T., Ehninger, D., Steiner, B., Klempin, F., Jessberger, S., Brandt, M., Romer, B., Rodriguez, G.R., Kronenberg, G., and Kempermann, G. (2006). Variability of doublecortin-associated dendrite maturation in adult hippocampal neurogenesis is independent of the regulation of precursor cell proliferation. BMC neuroscience *7*, 77.

Pomilio, C., Pavia, P., Gorojod, R.M., Vinuesa, A., Alaimo, A., Galvan, V., Kotler, M.L., Beauquis, J., and Saravia, F. (2016). Glial alterations from early to late stages in a model of Alzheimer's disease: Evidence of autophagy involvement in Abeta internalization. Hippocampus *26*, 194-210.

Poon, I.K., Chiu, Y.H., Armstrong, A.J., Kinchen, J.M., Juncadella, I.J., Bayliss, D.A., and Ravichandran, K.S. (2014). Unexpected link between an antibiotic, pannexin channels and apoptosis. Nature *507*, 329-334.

Preissler, J., Grosche, A., Lede, V., Le Duc, D., Krugel, K., Matyash, V., Szulzewsky, F., Kallendrusch, S., Immig, K., Kettenmann, H.*, et al.* (2015). Altered microglial phagocytosis in GPR34-deficient mice. Glia *63*, 206-215.

Proia, P., Di Liegro, C.M., Schiera, G., Fricano, A., and Di Liegro, I. (2016). Lactate as a Metabolite and a Regulator in the Central Nervous System. International journal of molecular sciences *17*.

Prokop, S., Miller, K.R., Drost, N., Handrick, S., Mathur, V., Luo, J., Wegner, A., Wyss-Coray, T., and Heppner, F.L. (2015). Impact of peripheral myeloid cells on amyloid-beta pathology in Alzheimer's disease-like mice. The Journal of experimental medicine *212*, 1811-1818.

Pul, R., Chittappen, K., and Stangel, M. (2013). Quantification of microglial phagocytosis by a flow cytometer-based assay. Microglia: Methods and Protocols, Methods in Molecular Biology *1041*.

R

Rai, K.S., Hattiangady, B., and Shetty, A.K. (2007). Enhanced production and dendritic growth of new dentate granule cells in the middle-aged hippocampus following intracerebroventricular FGF-2 infusions. The European journal of neuroscience *26*, 1765-1779.
Ramakers, C., Ruijter, J.M., Deprez, R.H., and Moorman, A.F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neuroscience letters *339*, 62-66.

Rangel, L.M., Alexander, A.S., Aimone, J.B., Wiles, J., Gage, F.H., Chiba, A.A., and Quinn, L.K. (2014). Temporally selective contextual encoding in the dentate gyrus of the hippocampus. Nature communications *5*, 3181.

Ransohoff, R.M. (2016). A polarizing question: do M1 and M2 microglia exist? Nature neuroscience *19*, 987-991.

Raponi, E., Agenes, F., Delphin, C., Assard, N., Baudier, J., Legraverend, C., and Deloulme, J.C. (2007). S100B expression defines a state in which GFAP-expressing cells lose their neural stem cell potential and acquire a more mature developmental stage. Glia *55*, 165-177.

Rawji, K.S., Kappen, J., Tang, W., Teo, W., Plemel, J.R., Stys, P.K., and Yong, V.W. (2018). Deficient Surveillance and Phagocytic Activity of Myeloid Cells Within Demyelinated Lesions in Aging Mice Visualized by Ex Vivo Live Multiphoton Imaging. The Journal of neuroscience : the official journal of the Society for Neuroscience *38*, 1973-1988.

Rawji, K.S., and Yong, V.W. (2013). The benefits and detriments of macrophages/microglia in models of multiple sclerosis. Clinical & developmental immunology *2013*, 948976.

Razzoli, M., Bo, E., Pascucci, T., Pavone, F., D'Amato, F.R., Cero, C., Sanghez, V., Dadomo, H., Palanza, P., Parmigiani, S.*, et al.* (2012). Implication of the VGF-derived peptide TLQP-21 in mouse acute and chronic stress responses. Behavioural brain research *229*, 333-339.

Ren, Y., and Savill, J. (1998). Apoptosis: the importance of being eaten. Cell death and differentiation *5*, 563-568.

Ribes, S., Ebert, S., Regen, T., Agarwal, A., Tauber, S.C., Czesnik, D., Spreer, A., Bunkowski, S., Eiffert, H., Hanisch, U.K.*, et al.* (2010). Toll-like receptor stimulation enhances phagocytosis and intracellular killing of nonencapsulated and encapsulated Streptococcus pneumoniae by murine microglia. Infection and immunity *78*, 865-871.

Riedl, M.S., Braun, P.D., Kitto, K.F., Roiko, S.A., Anderson, L.B., Honda, C.N., Fairbanks, C.A., and Vulchanova, L. (2009). Proteomic analysis uncovers novel actions of the neurosecretory protein VGF in nociceptive processing. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 13377-13388.

Rietze, R.L., and Reynolds, B.A. (2006). Neural stem cell isolation and characterization. Methods in enzymology *419*, 3-23.

Rocha, N.P., Teixeira, A.L., Coelho, F.M., Caramelli, P., Guimaraes, H.C., Barbosa, I.G., da Silva, T.A., Mukhamedyarov, M.A., Zefirov, A.L., Rizvanov, A.A.*, et al.* (2012). Peripheral blood mono-nuclear cells derived from Alzheimer's disease patients show elevated baseline levels of secreted cytokines but resist stimulation with beta-amyloid peptide. Molecular and cellular neurosciences *49*, 77-84.

Rogge, G., Jones, D., Hubert, G.W., Lin, Y., and Kuhar, M.J. (2008). CART peptides: regulators of body weight, reward and other functions. Nature reviews Neuroscience *9*, 747-758.

Rolls, A., Shechter, R., London, A., Ziv, Y., Ronen, A., Levy, R., and Schwartz, M. (2007). Toll-like receptors modulate adult hippocampal neurogenesis. Nat Cell Biol *9*, 1081-1088.

Rose-John, S., and Heinrich, P.C. (1994). Soluble receptors for cytokines and growth factors: generation and biological function. The Biochemical journal *300 (Pt 2)*, 281-290.

Rothaug, M., Becker-Pauly, C., and Rose-John, S. (2016). The role of interleukin-6 signaling in nervous tissue. Biochimica et biophysica acta *1863*, 1218-1227.

Rotheneichner, P., Romanelli, P., Bieler, L., Pagitsch, S., Zaunmair, P., Kreutzer, C., Konig, R., Marschallinger, J., Aigner, L., and Couillard-Despres, S. (2017). Tamoxifen Activation of Cre-Recombinase Has No Persisting Effects on Adult Neurogenesis or Learning and Anxiety. Frontiers in neuroscience *11*, 27.

Ruijter, J.M., Ramakers, C., Hoogaars, W.M., Karlen, Y., Bakker, O., van den Hoff, M.J., and Moorman, A.F. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic acids research *37*, e45.

S

Safaiyan, S., Kannaiyan, N., Snaidero, N., Brioschi, S., Biber, K., Yona, S., Edinger, A.L., Jung, S., Rossner, M.J., and Simons, M. (2016). Age-related myelin degradation burdens the clearance function of microglia during aging. Nature neuroscience *19*, 995-998.

Sahay, A., Scobie, K.N., Hill, A.S., O'Carroll, C.M., Kheirbek, M.A., Burghardt, N.S., Fenton, A.A., Dranovsky, A., and Hen, R. (2011). Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature *472*, 466-470.

Sarno, E., and Robison, A.J. (2018). Emerging role of viral vectors for circuit-specific gene interrogation and manipulation in rodent brain. Pharmacology, biochemistry, and behavior.

Sasmono, R.T., Oceandy, D., Pollard, J.W., Tong, W., Pavli, P., Wainwright, B.J., Ostrowski, M.C., Himes, S.R., and Hume, D.A. (2003). A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. Blood *101*, 1155-1163.

Sastre, M., Klockgether, T., and Heneka, M.T. (2006). Contribution of inflammatory processes to Alzheimer's disease: molecular mechanisms. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience *24*, 167-176.

Saxe, M.D., Battaglia, F., Wang, J.W., Malleret, G., David, D.J., Monckton, J.E., Garcia, A.D., Sofroniew, M.V., Kandel, E.R., Santarelli, L.*, et al.* (2006). Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. Proceedings of the National Academy of Sciences of the United States of America *103*, 17501-17506.

Schabitz, W.R., Kollmar, R., Schwaninger, M., Juettler, E., Bardutzky, J., Scholzke, M.N., Sommer, C., and Schwab, S. (2003). Neuroprotective effect of granulocyte colony-stimulating factor after focal cerebral ischemia. Stroke *34*, 745-751.

Schafer, D.P., Lehrman, E.K., Kautzman, A.G., Koyama, R., Mardinly, A.R., Yamasaki, R., Ransohoff, R.M., Greenberg, M.E., Barres, B.A., and Stevens, B. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron *74*, 691-705.

Schafer, M.K., Schwaeble, W.J., Post, C., Salvati, P., Calabresi, M., Sim, R.B., Petry, F., Loos, M., and Weihe, E. (2000). Complement C1q is dramatically up-regulated in brain microglia in response to transient global cerebral ischemia. Journal of immunology *164*, 5446-5452.

Schlitzer, A., Sivakamasundari, V., Chen, J., Sumatoh, H.R., Schreuder, J., Lum, J., Malleret, B., Zhang, S., Larbi, A., Zolezzi, F.*, et al.* (2015). Identification of cDC1- and cDC2-committed DC progenitors reveals early lineage priming at the common DC progenitor stage in the bone marrow. Nature immunology *16*, 718-728.

Schmid, R.S., McGrath, B., Berechid, B.E., Boyles, B., Marchionni, M., Sestan, N., and Anton, E.S. (2003). Neuregulin 1-erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex. Proceedings of the National Academy of Sciences of the United States of America *100*, 4251-4256.

Schneider, A., Kuhn, H.G., and Schabitz, W.R. (2005). A role for G-CSF (granulocyte-colony stimulating factor) in the central nervous system. Cell cycle *4*, 1753-1757.

Scott, R.S., McMahon, E.J., Pop, S.M., Reap, E.A., Caricchio, R., Cohen, P.L., Earp, H.S., and Matsushima, G.K. (2001). Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature *411*, 207-211.

Segawa, K., Kurata, S., Yanagihashi, Y., Brummelkamp, T.R., Matsuda, F., and Nagata, S. (2014). Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. Science *344*, 1164-1168.

Seiki, M. (2002). The cell surface: the stage for matrix metalloproteinase regulation of migration. Current opinion in cell biology *14*, 624-632.

Sell, C. (2015). Minireview: The Complexities of IGF/Insulin Signaling in Aging: Why Flies and Worms Are Not Humans. Molecular endocrinology *29*, 1107-1113.

Seri, B., Garcia-Verdugo, J.M., Collado-Morente, L., McEwen, B.S., and Alvarez-Buylla, A. (2004). Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. The Journal of comparative neurology *478*, 359-378.

Severini, C., Ciotti, M.T., Biondini, L., Quaresima, S., Rinaldi, A.M., Levi, A., Frank, C., and Possenti, R. (2008). TLQP-21, a neuroendocrine VGF-derived peptide, prevents cerebellar granule cells death induced by serum and potassium deprivation. Journal of neurochemistry *104*, 534-544.

Shimojo, M., Nakajima, K., Takei, N., Hamanoue, M., and Kohsaka, S. (1991). Production of basic fibroblast growth factor in cultured rat brain microglia. Neuroscience letters *123*, 229-231.

Sierra, A., Abiega, O., Shahraz, A., and Neumann, H. (2013). Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis. Front Cell Neurosci *7*, 6.

Sierra, A., Beccari, S., Diaz-Aparicio, I., Encinas, J.M., Comeau, S., and Tremblay, M.E. (2014a). Surveillance, phagocytosis, and inflammation: how never-resting microglia influence adult hippocampal neurogenesis. Neural Plast *2014*, 610343.

Sierra, A., Encinas, J.M., Deudero, J.J., Chancey, J.H., Enikolopov, G., Overstreet-Wadiche, L.S., Tsirka, S.E., and Maletic-Savatic, M. (2010). Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. Cell stem cell *7*, 483-495.

Sierra, A., Gottfried-Blackmore, A., Milner, T.A., McEwen, B.S., and Bulloch, K. (2008). Steroid hormone receptor expression and function in microglia. Glia *56*, 659-674.

Sierra, A., Gottfried-Blackmore, A.C., McEwen, B.S., and Bulloch, K. (2007). Microglia derived from aging mice exhibit an altered inflammatory profile. Glia *55*, 412-424.

BIBLIOGRAPHY

Sierra, A., Martin-Suarez, S., Valcarcel-Martin, R., Pascual-Brazo, J., Aelvoet, S.A., Abiega, O., Deudero, J.J., Brewster, A.L., Bernales, I., Anderson, A.E*., et al.* (2015). Neuronal hyperactivity accelerates depletion of neural stem cells and impairs hippocampal neurogenesis. Cell stem cell *16*, 488-503.

Sierra, A., Tremblay, M.E., and Wake, H. (2014b). Never-resting microglia: physiological roles in the healthy brain and pathological implications. Frontiers in cellular neuroscience *8*, 240.

Sil, S., Periyasamy, P., Thangaraj, A., Chivero, E.T., and Buch, S. (2018). PDGF/PDGFR axis in the neural systems. Molecular aspects of medicine.

Singer, B.H., Gamelli, A.E., Fuller, C.L., Temme, S.J., Parent, J.M., and Murphy, G.G. (2011). Compensatory network changes in the dentate gyrus restore long-term potentiation following ablation of neurogenesis in young-adult mice. Proceedings of the National Academy of Sciences of the United States of America *108*, 5437-5442.

Singh, R.P., Dhanalakshmi, S., Mohan, S., Agarwal, C., and Agarwal, R. (2006). Silibinin inhibits UVB- and epidermal growth factor-induced mitogenic and cell survival signaling involving activator protein-1 and nuclear factor-kappaB in mouse epidermal JB6 cells. Molecular cancer therapeutics *5*, 1145-1153.

Skriver, K., Roig, M., Lundbye-Jensen, J., Pingel, J., Helge, J.W., Kiens, B., and Nielsen, J.B. (2014). Acute exercise improves motor memory: exploring potential biomarkers. Neurobiology of learning and memory *116*, 46-58.

Smith, J.A., Das, A., Ray, S.K., and Banik, N.L. (2012). Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. Brain research bulletin *87*, 10-20.

Sorrells, S.F., Paredes, M.F., Cebrian-Silla, A., Sandoval, K., Qi, D., Kelley, K.W., James, D., Mayer, S., Chang, J., Auguste, K.I*., et al.* (2018). Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. Nature *555*, 377-381.

Spalding, K.L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H.B., Bostrom, E., Westerlund, I., Vial, C., Buchholz, B.A*., et al.* (2013). Dynamics of hippocampal neurogenesis in adult humans. Cell *153*, 1219-1227.

Spangenberg, E.E., and Green, K.N. (2017). Inflammation in Alzheimer's disease: Lessons learned from microglia-depletion models. Brain, behavior, and immunity *61*, 1-11.

Spanos, J.P., Hsu, N.J., and Jacobs, M. (2015). Microglia are crucial regulators of neuro-immunity during central nervous system tuberculosis. Frontiers in cellular neuroscience *9*, 182.

Spulber, S., Oprica, M., Bartfai, T., Winblad, B., and Schultzberg, M. (2008). Blunted neurogenesis and gliosis due to transgenic overexpression of human soluble IL-1ra in the mouse. The European journal of neuroscience *27*, 549-558.

Stamenkovic, I. (2003). Extracellular matrix remodelling: the role of matrix metalloproteinases. The Journal of pathology *200*, 448-464.

Stefani, J., Tschesnokowa, O., Parrilla, M., Robaye, B., Boeynaems, J.M., Acker-Palmer, A., Zimmermann, H., and Gampe, K. (2018). Disruption of the Microglial ADP Receptor P2Y13 Enhances Adult Hippocampal Neurogenesis. Frontiers in cellular neuroscience *12*, 134.

Steiner, B., Kronenberg, G., Jessberger, S., Brandt, M.D., Reuter, K., and Kempermann, G. (2004). Differential regulation of gliogenesis in the context of adult hippocampal neurogenesis in mice. Glia *46*, 41-52.

Stern, M., Savill, J., and Haslett, C. (1996). Human monocyte-derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis. Mediation by alpha v beta 3/CD36/thrombospondin recognition mechanism and lack of phlogistic response. The American journal of pathology *149*, 911-921.

Stevens, B., Allen, N.J., Vazquez, L.E., Howell, G.R., Christopherson, K.S., Nouri, N., Micheva, K.D., Mehalow, A.K., Huberman, A.D., Stafford, B.*, et al.* (2007). The classical complement cascade mediates CNS synapse elimination. Cell *131*, 1164-1178.

Stump, G., Durrer, A., Klein, A.L., Lutolf, S., Suter, U., and Taylor, V. (2002). Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain. Mechanisms of development *114*, 153-159.

Su, E.J., Cao, C., Fredriksson, L., Nilsson, I., Stefanitsch, C., Stevenson, T.K., Zhao, J., Ragsdale, M., Sun, Y.Y., Yepes, M.*, et al.* (2017). Microglial-mediated PDGF-CC activation increases cerebrovascular permeability during ischemic stroke. Acta neuropathologica *134*, 585-604.

Su, F., Bai, F., and Zhang, Z. (2016). Inflammatory Cytokines and Alzheimer's Disease: A Review from the Perspective of Genetic Polymorphisms. Neuroscience bulletin *32*, 469-480.

Sugo, T., Tachimoto, H., Chikatsu, T., Murakami, Y., Kikukawa, Y., Sato, S., Kikuchi, K., Nagi, T., Harada, M., Ogi, K.*, et al.* (2006). Identification of a lysophosphatidylserine receptor on mast cells. Biochemical and biophysical research communications *341*, 1078-1087.

Suh, Y.A., Kim, J.H., Sung, M.A., Boo, H.J., Yun, H.J., Lee, S.H., Lee, H.J., Min, H.Y., Suh, Y.G., Kim, K.W.*, et al.* (2013). A novel antitumor activity of deguelin targeting the insulin-like growth factor (IGF) receptor pathway via up-regulation of IGF-binding protein-3 expression in breast cancer. Cancer letters *332*, 102-109.

Sun, M., and Fink, P.J. (2007). A new class of reverse signaling costimulators belongs to the TNF family. Journal of immunology *179*, 4307-4312.

Suzuki, J., Imanishi, E., and Nagata, S. (2016). Xkr8 phospholipid scrambling complex in apoptotic phosphatidylserine exposure. Proceedings of the National Academy of Sciences of the United States of America *113*, 9509-9514.

T

Tahara, K., Kim, H.D., Jin, J.J., Maxwell, J.A., Li, L., and Fukuchi, K. (2006). Role of toll-like receptor signalling in Abeta uptake and clearance. Brain : a journal of neurology *129*, 3006-3019.

Takeda, K., Kaisho, T., and Akira, S. (2003). Toll-like receptors. Annual review of immunology *21*, 335-376.

Takizawa, F., Tsuji, S., and Nagasawa, S. (1996). Enhancement of macrophage phagocytosis upon iC3b deposition on apoptotic cells. FEBS letters *397*, 269-272.

Tanaka, T., Ueno, M., and Yamashita, T. (2009). Engulfment of axon debris by microglia requires p38 MAPK activity. The Journal of biological chemistry *284*, 21626-21636.

Tannahill, G.M., Curtis, A.M., Adamik, J., Palsson-McDermott, E.M., McGettrick, A.F., Goel, G., Frezza, C., Bernard, N.J., Kelly, B., Foley, N.H.*, et al.* (2013). Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. Nature *496*, 238-242.

Tashiro, A., Makino, H., and Gage, F.H. (2007). Experience-specific functional modification of the dentate gyrus through adult neurogenesis: a critical period during an immature stage. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 3252-3259.

Taylor, R.C., Cullen, S.P., and Martin, S.J. (2008). Apoptosis: controlled demolition at the cellular level. Nature reviews Molecular cell biology *9*, 231-241.

ten Dijke, P., and Arthur, H.M. (2007). Extracellular control of TGFbeta signalling in vascular development and disease. Nature reviews Molecular cell biology *8*, 857-869.

Thakker-Varia, S., Behnke, J., Doobin, D., Dalal, V., Thakkar, K., Khadim, F., Wilson, E., Palmieri, A., Antila, H., Rantamaki, T.*, et al.* (2014). VGF (TLQP-62)-induced neurogenesis targets early phase neural progenitor cells in the adult hippocampus and requires glutamate and BDNF signaling. Stem cell research *12*, 762-777.

Thakker-Varia, S., Krol, J.J., Nettleton, J., Bilimoria, P.M., Bangasser, D.A., Shors, T.J., Black, I.B., and Alder, J. (2007). The neuropeptide VGF produces antidepressant-like behavioral effects and enhances proliferation in the hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 12156-12167.

Ting, A.K., Chen, Y., Wen, L., Yin, D.M., Shen, C., Tao, Y., Liu, X., Xiong, W.C., and Mei, L. (2011). Neuregulin 1 promotes excitatory synapse development and function in GABAergic interneurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 15-25.

Tisoncik, J.R., Korth, M.J., Simmons, C.P., Farrar, J., Martin, T.R., and Katze, M.G. (2012). Into the eye of the cytokine storm. Microbiology and molecular biology reviews : MMBR *76*, 16-32.
Tizard, I. (2008). Sickness behavior, its mechanisms and significance. Animal health research reviews *9*, 87-99.

Torres-Aleman, I. (1999). Insulin-like growth factors as mediators of functional plasticity in the adult brain. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme *31*, 114-119.

Trani, E., Giorgi, A., Canu, N., Amadoro, G., Rinaldi, A.M., Halban, P.A., Ferri, G.L., Possenti, R., Schinina, M.E., and Levi, A. (2002). Isolation and characterization of VGF peptides in rat brain. Role of PC1/3 and PC2 in the maturation of VGF precursor. Journal of neurochemistry *81*, 565-574.

Tremblay, M.E., Lowery, R.L., and Majewska, A.K. (2010). Microglial interactions with synapses are modulated by visual experience. PLoS biology *8*, e1000527.

Tropepe, V., Sibilia, M., Ciruna, B.G., Rossant, J., Wagner, E.F., and van der Kooy, D. (1999). Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Developmental biology *208*, 166-188.

Trouw, L.A., Blom, A.M., and Gasque, P. (2008). Role of complement and complement regulators in the removal of apoptotic cells. Molecular immunology *45*, 1199-1207.

Truman, L.A., Ford, C.A., Pasikowska, M., Pound, J.D., Wilkinson, S.J., Dumitriu, I.E., Melville, L., Melrose, L.A., Ogden, C.A., Nibbs, R.*, et al.* (2008). CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. Blood *112*, 5026-5036.

V

Vaillant, C., Meissirel, C., Mutin, M., Belin, M.F., Lund, L.R., and Thomasset, N. (2003). MMP-9 deficiency affects axonal outgrowth, migration, and apoptosis in the developing cerebellum. Molecular and cellular neurosciences *24*, 395-408.

Valero, J., Bernardino, L., Cardoso, F.L., Silva, A.P., Fontes-Ribeiro, C., Ambrosio, A.F., and Malva, J.O. (2017). Impact of Neuroinflammation on Hippocampal Neurogenesis: Relevance to Aging and Alzheimer's Disease. Journal of Alzheimer's disease : JAD *60*, S161-S168.

Valero, J., Mastrella, G., Neiva, I., Sanchez, S., and Malva, J.O. (2014). Long-term effects of an acute and systemic administration of LPS on adult neurogenesis and spatial memory. Frontiers in neuroscience *8*, 83.

van de Moosdijk, A.A., and van Amerongen, R. (2016). Identification of reliable reference genes for qRT-PCR studies of the developing mouse mammary gland. Scientific reports *6*, 35595.

van der Meer, J.W., Joosten, L.A., Riksen, N., and Netea, M.G. (2015). Trained immunity: A smart way to enhance innate immune defence. Molecular immunology *68*, 40-44.

Van Hove, I., Lemmens, K., Van de Velde, S., Verslegers, M., and Moons, L. (2012). Matrix metalloproteinase-3 in the central nervous system: a look on the bright side. Journal of neurochemistry *123*, 203-216.

van Praag, H., Schinder, A.F., Christie, B.R., Toni, N., Palmer, T.D., and Gage, F.H. (2002). Functional neurogenesis in the adult hippocampus. Nature *415*, 1030-1034.

Vega, C.J., and Peterson, D.A. (2005). Stem cell proliferative history in tissue revealed by temporal halogenated thymidine analog discrimination. Nature methods *2*, 167-169.

Veldhoen, E.S., Wolfs, T.F., and van Vught, A.J. (2007). Two cases of fatal meningitis due to Fusobacterium necrophorum. Pediatric neurology *36*, 261-263.

Vezzani, A., French, J., Bartfai, T., and Baram, T.Z. (2011). The role of inflammation in epilepsy. Nature reviews Neurology *7*, 31-40.

Vilalta, A., and Brown, G.C. (2017). Neurophagy, the phagocytosis of live neurons and synapses by glia, contributes to brain development and disease. The FEBS journal.

Voll, R.E., Herrmann, M., Roth, E.A., Stach, C., Kalden, J.R., and Girkontaite, I. (1997). Immunosuppressive effects of apoptotic cells. Nature *390*, 350-351.

W

BIBLIOGRAPHY

Wachs, F.P., Winner, B., Couillard-Despres, S., Schiller, T., Aigner, R., Winkler, J., Bogdahn, U., and Aigner, L. (2006). Transforming growth factor-beta1 is a negative modulator of adult neurogenesis. Journal of neuropathology and experimental neurology *65*, 358-370.

Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003). Tumor necrosis factor signaling. Cell death and differentiation *10*, 45-65.

Wake, H., Moorhouse, A.J., Jinno, S., Kohsaka, S., and Nabekura, J. (2009). Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 3974-3980.

Wallner, S., Peters, S., Pitzer, C., Resch, H., Bogdahn, U., and Schneider, A. (2015). The Granulocyte-colony stimulating factor has a dual role in neuronal and vascular plasticity. Frontiers in cell and developmental biology *3*, 48.

Walton, N.M., Sutter, B.M., Laywell, E.D., Levkoff, L.H., Kearns, S.M., Marshall, G.P., 2nd, Scheffler, B., and Steindler, D.A. (2006). Microglia instruct subventricular zone neurogenesis. Glia *54*, 815-825.

Wang, D.D., and Bordey, A. (2008). The astrocyte odyssey. Progress in neurobiology *86*, 342-367.

Warburg, O. (1956). On the origin of cancer cells. Science *123*, 309-314.

Weber, A., Wasiliew, P., and Kracht, M. (2010). Interleukin-1 (IL-1) pathway. Science signaling *3*, cm1.

Weidemann, A., and Johnson, R.S. (2008). Biology of HIF-1alpha. Cell death and differentiation *15*, 621-627.

Weinhard, L., di Bartolomei, G., Bolasco, G., Machado, P., Schieber, N.L., Neniskyte, U., Exiga, M., Vadisiute, A., Raggioli, A., Schertel, A*., et al.* (2018). Microglia remodel synapses by presynaptic trogocytosis and spine head filopodia induction. Nature communications *9*, 1228.

Wendeln, A.C., Degenhardt, K., Kaurani, L., Gertig, M., Ulas, T., Jain, G., Wagner, J., Hasler, L.M., Wild, K., Skodras, A*., et al.* (2018). Innate immune memory in the brain shapes neurological disease hallmarks. Nature *556*, 332-338.

Werner, H., and LeRoith, D. (2014). Insulin and insulin-like growth factor receptors in the brain: physiological and pathological aspects. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology *24*, 1947-1953.

Werner, S., Unsicker, K., and von Bohlen und Halbach, O. (2011). Fibroblast growth factor-2 deficiency causes defects in adult hippocampal neurogenesis, which are not rescued by exogenous fibroblast growth factor-2. Journal of neuroscience research *89*, 1605-1617.

Werther, G.A., Abate, M., Hogg, A., Cheesman, H., Oldfield, B., Hards, D., Hudson, P., Power, B., Freed, K., and Herington, A.C. (1990). Localization of insulin-like growth factor-I mRNA in rat brain by in situ hybridization--relationship to IGF-I receptors. Molecular endocrinology *4*, 773-778.

Wes, P.D., Holtman, I.R., Boddeke, E.W., Moller, T., and Eggen, B.J. (2016). Next generation transcriptomics and genomics elucidate biological complexity of microglia in health and disease. Glia *64*, 197-213.

Wierup, N., Richards, W.G., Bannon, A.W., Kuhar, M.J., Ahren, B., and Sundler, F. (2005). CART knock out mice have impaired insulin secretion and glucose intolerance, altered beta cell morphology and increased body weight. Regulatory peptides *129*, 203-211.

Wijeyesakere, S.J., Bedi, S.K., Huynh, D., and Raghavan, M. (2016). The C-Terminal Acidic Region of Calreticulin Mediates Phosphatidylserine Binding and Apoptotic Cell Phagocytosis. Journal of immunology *196*, 3896-3909.

Williams, P.A., Tribble, J.R., Pepper, K.W., Cross, S.D., Morgan, B.P., Morgan, J.E., John, S.W., and Howell, G.R. (2016). Inhibition of the classical pathway of the complement cascade prevents early dendritic and synaptic degeneration in glaucoma. Molecular neurodegeneration *11*, 26.

Witting, A., Muller, P., Herrmann, A., Kettenmann, H., and Nolte, C. (2000). Phagocytic clearance of apoptotic neurons by Microglia/Brain macrophages in vitro: involvement of lectin-, integrin-, and phosphatidylserine-mediated recognition. Journal of neurochemistry *75*, 1060-1070.

Wlodkowic, D., Skommer, J., and Darzynkiewicz, Z. (2012). Cytometry of apoptosis. Historical perspective and new advances. Experimental oncology *34*, 255-262.

Wong, R.W., and Guillaud, L. (2004). The role of epidermal growth factor and its receptors in mammalian CNS. Cytokine & growth factor reviews *15*, 147-156.

Woodbury, M.E., and Ikezu, T. (2014). Fibroblast growth factor-2 signaling in neurogenesis and neurodegeneration. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology *9*, 92-101.

Wrigley, S., Arafa, D., and Tropea, D. (2017). Insulin-Like Growth Factor 1: At the Crossroads of Brain Development and Aging. Frontiers in cellular neuroscience *11*, 14.

Wu, Q., Combs, C., Cannady, S.B., Geldmacher, D.S., and Herrup, K. (2000). Beta-amyloid activated microglia induce cell cycling and cell death in cultured cortical neurons. Neurobiology of aging *21*, 797-806.

Wu, Y., Singh, S., Georgescu, M.M., and Birge, R.B. (2005). A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. Journal of cell science *118*, 539-553.

X

Xapelli, S., Agasse, F., Sarda-Arroyo, L., Bernardino, L., Santos, T., Ribeiro, F.F., Valero, J., Braganca, J., Schitine, C., de Melo Reis, R.A.*, et al.* (2013). Activation of type 1 cannabinoid receptor (CB1R) promotes neurogenesis in murine subventricular zone cell cultures. PloS one *8*, e63529.

Xing, F., Kobayashi, A., Okuda, H., Watabe, M., Pai, S.K., Pandey, P.R., Hirota, S., Wilber, A., Mo, Y.Y., Moore, B.E.*, et al.* (2013). Reactive astrocytes promote the metastatic growth of breast cancer stem-like cells by activating Notch signalling in brain. EMBO molecular medicine *5*, 384-396.

Xu, J., Wang, T., Wu, Y., Jin, W., and Wen, Z. (2016). Microglia Colonization of Developing Zebrafish Midbrain Is Promoted by Apoptotic Neuron and Lysophosphatidylcholine. Developmental cell *38*, 214-222.

Y

Yamada, M., Ikeuchi, T., and Hatanaka, H. (1997). The neurotrophic action and signalling of epidermal growth factor. Progress in neurobiology *51*, 19-37.

Yamaguchi, H., Sasaki, K., Satomi, Y., Shimbara, T., Kageyama, H., Mondal, M.S., Toshinai, K., Date, Y., Gonzalez, L.J., Shioda, S.*, et al.* (2007). Peptidomic identification and biological validation of neuroendocrine regulatory peptide-1 and -2. The Journal of biological chemistry *282*, 26354-26360.

Yang, D., Zhang, W., Padhiar, A., Yue, Y., Shi, Y., Zheng, T., Davis, K., Zhang, Y., Huang, M., Li, Y.*, et al.* (2016). NPAS3 Regulates Transcription and Expression of VGF: Implications for Neurogenesis and Psychiatric Disorders. Frontiers in molecular neuroscience *9*, 109.

Yang, P., Wen, H., Ou, S., Cui, J., and Fan, D. (2012). IL-6 promotes regeneration and functional recovery after cortical spinal tract injury by reactivating intrinsic growth program of neurons and enhancing synapse formation. Experimental neurology *236*, 19-27.

Ye, L., Huang, Y., Zhao, L., Li, Y., Sun, L., Zhou, Y., Qian, G., and Zheng, J.C. (2013). IL-1beta and TNF-alpha induce neurotoxicity through glutamate production: a potential role for neuronal glutaminase. Journal of neurochemistry *125*, 897-908.

Ye, P., and D'Ercole, A.J. (2006). Insulin-like growth factor actions during development of neural stem cells and progenitors in the central nervous system. Journal of neuroscience research *83*, 1-6.

Z

Zagorska, A., Traves, P.G., Lew, E.D., Dransfield, I., and Lemke, G. (2014). Diversification of TAM receptor tyrosine kinase function. Nature immunology *15*, 920-928.

Zaman, S., Wang, R., and Gandhi, V. (2014). Targeting the apoptosis pathway in hematologic malignancies. Leukemia & lymphoma *55*, 1980-1992.

Zhan, Y., Paolicelli, R.C., Sforazzini, F., Weinhard, L., Bolasco, G., Pagani, F., Vyssotski, A.L., Bifone, A., Gozzi, A., Ragozzino, D.*, et al.* (2014). Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. Nature neuroscience *17*, 400-406.

Zhang, J., Wang, S., Yuan, L., Yang, Y., Zhang, B., Liu, Q., Chen, L., Yue, W., Li, Y., and Pei, X. (2012). Neuron-restrictive silencer factor (NRSF) represses cocaine- and amphetamine-regulated transcript (CART) transcription and antagonizes cAMP-response element-binding

protein signaling through a dual NRSE mechanism. The Journal of biological chemistry *287*, 42574-42587.

Zhang, J., Xiao, X., Liu, W., Demirci, G., and Li, X.C. (2009). Inhibitory receptors of the immune system: functions and therapeutic implications. Cellular & molecular immunology *6*, 407-414.

Zhang, Y., Chen, K., Sloan, S.A., Bennett, M.L., Scholze, A.R., O'Keeffe, S., Phatnani, H.P., Guarnieri, P., Caneda, C., Ruderisch, N.*, et al.* (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience *34*, 11929-11947.

Zhao, C., Teng, E.M., Summers, R.G., Jr., Ming, G.L., and Gage, F.H. (2006). Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience *26*, 3-11.

Zhao, R., Hu, W., Tsai, J., Li, W., and Gan, W.B. (2017). Microglia limit the expansion of beta-amyloid plaques in a mouse model of Alzheimer's disease. Molecular neurodegeneration *12*, 47.

Zhao, X., Eyo, U.B., Murugan, M., and Wu, L.J. (2018). Microglial interactions with the neurovascular system in physiology and pathology. Developmental neurobiology.

Zhao, X., Zhang, L., Ting, S.M., and Aronowski, J. (2016). Phagocytosis Assay of Microglia for Dead Neurons in Primary Rat Brain Cell Cultures. Bio-protocol *6*.

Zheng, C., Zhou, X.W., and Wang, J.Z. (2016). The dual roles of cytokines in Alzheimer's disease: update on interleukins, TNF-alpha, TGF-beta and IFN-gamma. Translational neurodegeneration *5*, 7.

Zheng, X.Z., Du, L.F., and Wang, H.P. (2010). An immunohistochemical analysis of a rat model of proliferative vitreoretinopathy and a comparison of the expression of TGF-beta and PDGF among the induction methods. Bosnian journal of basic medical sciences *10*, 204-209.

Zhou, Z.D., Kumari, U., Xiao, Z.C., and Tan, E.K. (2010). Notch as a molecular switch in neural stem cells. IUBMB life *62*, 618-623.

Ziv, Y., and Schwartz, M. (2008). Orchestrating brain-cell renewal: the role of immune cells in adult neurogenesis in health and disease. Trends in molecular medicine *14*, 471-478.

Zuchero, J.B., and Barres, B.A. (2015). Glia in mammalian development and disease. Development *142*, 3805-3809.