Universitat Autònoma de Barcelona

Effects of astrocyte-targeted production of either IL-6 or IL-10 after facial nerve axotomy in the adult mouse

Nàdia Villacampa Pérez

Effects of astrocyte-targeted production of either **IL-6 or IL-10** after facial nerve axotomy in the adult mouse

Nàdia Villacampa Pérez

PhD Dissertation

Universitat Autònoma de Barcelona

INc Institut de Neurociències

Bellaterra, 2016

A mi padre

A mi mami y mis yayos

No te rindas, aún estás a tiempo
De alcanzar y comenzar de nuevo,
Aceptar tus sombras,
Enterrar tus miedos,
Liberar el lastre,
Retomar el vuelo.
No te rindas que la vida es eso,
Continuar el viaje,
Perseguir tus sueños,
Destrabar el tiempo,
Correr los escombros,
Y destapar el cielo.
No te rindas, por favor no cedas,
Aunque el frío queme,
Aunque el miedo muerda,
Aunque el sol se esconda,
Y se calle el viento,
Aún hay fuego en tu alma
Aún hay vida en tus sueños.
Porque la vida es tuya y tuyo también el deseo
Porque lo has querido y porque te quiero
Porque existe el vino y el amor, es cierto.
Porque no hay heridas que no cure el tiempo.
Abrir las puertas,
Quitar los cerrojos,
Abandonar las murallas que te protegieron,
Vivir la vida y aceptar el reto,
Recuperar la risa,
Ensayar un canto,
Bajar la guardia y extender las manos
Desplegar las alas
E intentar de nuevo,
Celebrar la vida y retomar los cielos.
No te rindas, por favor no cedas,
Aunque el frío queme,
Aunque el miedo muerda,
Aunque el sol se ponga y se calle el viento,
Aún hay fuego en tu alma,
Aún hay vida en tus sueños
Porque cada día es un comienzo nuevo,
Porque esta es la hora y el mejor momento.
Porque no estás solo, porque yo te quiero.

Mario Benedetti

No te rindas

AGRAÏMENTS

M'agradaria començar aquests agraïments recordant a tots els investigadors que han ajudat al desenvolupament del coneixement científic al llarg dels segles. Sense ells, encara potser ara no sabríem com és una cèl·lula, quina és l'estructura de l'ADN o no coneixeríem la teoria de l'evolució ni la penicil·lina. Les seves investigacions han aportat coneixement a la humanitat i han permès que estigui avui aquí, defensant una tesis en un tema molt específic i tot perquè ells van donar els petits grans passos molts anys enrere. Aquesta immensa quantitat de coneixement no hagués estat possible sense els milions d'animals de laboratori que s'han utilitzat; els hi vull donar les gràcies a tots i cadascun d'aquests petits éssers vius eternament oblidats en tantes i tantes seccions d'agraïments.

Ni la tesis más tocho tendría suficientes páginas para agradecerles a Bernardo y Berta todo lo que han hecho por mí. Yo que llegué casi por casualidad a este laboratorio y ahora puedo decir que soy afortunada de pertenecer a la familia BBG. Paciencia han tenido y mucha para domar a esta cabezona medio-aragonesa; no sólo me han formado en el campo científico, sino que me han hecho una mejor persona, más tranquila, más abierta a nuevas ideas. Gracias por darme la oportunidad de viajar tanto, conocer a tanta gente y vivir innumerables buenos momentos: la cabra en llamas, las pintas en la Guinness de Dublín, ostras y "scallops" en casa de Sue Ling, la interminable escalera de la catedral de Köln, el carrito de golf en Isla Mujeres, las tiendas de los museos en Boston, y las múltiples cenas con nuestros amigos internacionales allí dónde nos llevase el congreso de turno. Millones de gracias por animarme y ayudarme a conseguir la estancia en Australia, que como sabéis, ha cambiado mi vida en muchos sentidos. Y no sólo eso, por ser allí los mejores guías turísticos que un lugar pueda desear, descubrir ese país a vuestro lado hizo que lo apreciase muchísimo más.

Gracias a la gente que forma parte de la familia BBG, en especial, gracias a Bea por todo: por esta etapa que ha sido de aprendizaje y crecimiento para ambas, por los momentos compartidos de risas y también de lágrimas; gracias por tu paciencia. Miguel e Isabella, grandes sufridores de mis peripecias por el lab y de mis peticiones de ayuda. Perdón por andar descalza y por romper algún que otro envase de vidrio, sobretodo uno que no sobrevivió al intento de convertirlo en un baño maría, llevándose por delante a un termómetro como daño colateral. I suposo que aquesta història et sona, Mireia, gràcies per ser la meva companya d'experiments, malifetes i riures al lab i companya de glamour absolut a la 080. Gemma, com deia aquell: "ojalá te hubiera conocido antes", m'ha encantat tenir una compi d'experiments del facial, encara que hagi estat poc temps; les teves paraules d'ànim i de calma m'han ajudat en molts moments, gràcies de veritat. Khvala Filip, alias "El Serbio", your pragmatic point of view and your Serbian sense of humor. Anna, gràcies per escoltar-me, merci. Mahsa, I wish you luck, Insha'allah. Gracias a las nueva prometedoras incorporaciones a la familia: Paula y Olaia i Paula de Lleide. No me puedo olvidar de las grandes personas que han pasado por el lab. Laia, m'hagués agradat que ho haguessis pogut veure i també haver gaudit de més temps al teu costat; la teva pèrdua va recol·locar la meva escala de valors. Carmencita, mi galleguiña, no tuve frío escribiendo esto pero viniéronme muchos buenos momentos a la cabeza. Ili, sin palabras, my darling, gracias por ayudarme y escucharme. Kalpanita, what can I say? My rock star and a model in so many ways, giving me really useful advices. Anto, grazie mile, hai un grande cuore italiano, grazie per l'ascolto e mi tengono nella vostra vita. Mariela, mi

profesora de argentino, che gracias loca. César, mi corderito, espero que el fucking toxic no decaiga y que esta ciencia loca nos vuelva a juntar en alguna otra parte del mundo.

I am also very grateful to Professor Iain L. Campbell for hosting me in the Psychokines lab and giving me the opportunity to discover this mesmerizing, amazing, wonderful country. I think Australia's people opened my mind, which I needed so badly, I know. A big thank you and a sangria toast for all the people in the lab: Sue Ling, Vicki, Meng and Laura. Special thanks to Sue Ling for letting me stay in your house, and all the things you and Jimmy did for me; as required, I tried to just bring my smile. Also, traveling to end of the world allowed me to meet there "mi vecina de abajo" Maria and her husband Jordi, who showed me the lab and the city during my first week in my beloved Sydney. I hope life gather us again in some awesome city for a meeting and enjoy a nice dinner together.

Vull agrair enormement la teva insistència, Mar. Sabem molt bé les dues que sense tu, jo no seria ara mateix on sóc, tu que no vas llençar mai la tovallola quan jo ja la tenia ben plegadeta en el fons d'un armari. Gràcies a tu vaig demanar aquesta beca que m'ha canviat la vida en tots els sentits. Vull fer extensible aquest agraïment a la resta de dinos: Glòria, Laura i Marisa pels grans moments viscuts durant i després de la carrera i els que encara ens queden per viure. No crec que per molts anys que passin, puguem algun dia deixar de riure en pensar en la gravació dels "Dinos al vacío", en l'actuació del gato makinero o en les majestuoses imitacions del Gran Colombo que ens amenitzaven les llargues tardes i nits d'estudi a les biblioteques i sales de fluorescentes enfermos, a on sempre, sempre i sempre serem les més guapes, verdad, Marisa? Gracias Isa, mi hermana del alma, por apoyarme siempre, por comprenderme y desearme siempre lo mejor, aunque lo "mejor" para mí no corresponda a veces con lo que tú preferirías y gracias por haber puesto a Ian en mi vida; you can count on me. Gràcies Núria, per escoltar-me, per les cerveses i per "colar-me" al confo de tant en tant. Gràcies Aleix, tu vas patir-me en la primera part d'aquesta aventura i em vas ajudar moltíssim, moltes gràcies. Gracias Laura, por el english check *in extremis*.

Gracias a mis hermanos Yaiza y Erick, a Pili y a mi padre, por enseñarme a ser independiente y a tomar iniciativa por mí misma y por entender mi voluntad de querer hacer carrera en un mundo tan difícil como este. Quisiera que hubieras podido verme en este día, pero no ha podido ser; sé que hubieras estado muy orgulloso. Gracias a mi familia aragonesa, y también a mi familia navassenca-salmantina.

Gracias a mi Cachi, por entender que esto no es un trabajo ni una profesión, que es un estilo de vida; por entender los horarios, por comprender las ausencias y los viajes a la otra punta del mundo. Gracias por cuidarme, hacerme masajes, llevarme a la uni, traerme cenita rica, escucharme y quererme. Gracias por aceptar ser mi compañero de esta gran aventura que recién comienza. Gracias, mi amor, porque me quieres y porque te quiero.

It wasn't always easy, it wasn't always fun, but it was always worth it

TABLE OF CONTENTS

1. ABBREVIATIONS

ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
CCL	Chemokine (c-c motif) ligand
CCR	C-C chemokine receptor type
CGRP	Calcitonin gene-related peptide
ChAT	Choline acetyltransferase
CNS	Central Nervous System
CNTF	Ciliary neurotrophic factor
CSIF	Cytokine synthesis inhibitory factor
CXCL	Chemokine (C-X-C motif) ligand
CX3CR1	CX3C chemokine receptor 1
DAP12	DNAX activation protein of 12 kDa
DUSP1	Dual specificity phosphatase 1
EAE	Experimental autoimmune encephalomyelitis
eNO	Endothelial nitric oxide
FG	Fluorogold
FGF	Fibroblast growth factor
FMN	Facial motor neuron
FN	Facial nucleus
FNA	Facial nerve axotomy
GABA	Gamma-aminobutyric acid
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ICAM	Intercellular adhesion molecules
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KO	Knock-out
LPS	Lipopolysaccharide
m2MAchR	M2 muscarinic acetylcholine receptor
MAC-1	Macrophage-1 antigen
MCAO	Middle cerebral artery occlusion
MCSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NGF	Nerve growth factor
NHD	Nasu-Hakola disease
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NT-3	Neurotrophin-3
NTPDase	Nucleotide triphosphate diphosphohydrolase
OPN	Osteopontin

PACAP	Pituitary adenyl cyclase-activating polypeptide
PH3	Phosphohistone-3
RAG	Regeneration-associated genes
RAG2-KO	Recombination activating gene 2-knock out
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SCID	Severe combined immunodeficiency
sIL-6R	soluble interleukin-6 receptor
SR	Scavenger receptors
STAT3	Signal transducer and activator of transcription-3
TBI	Traumatic brain injury
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TREM2	Triggering receptor expressed on myeloid cells 2
TSP-1	Thrombospondin 1
TTP	Tristetraprolin
UTP	Uridine-5'-triphosphate
VAchT	Vesicular acetylcholine transporter
VCAM	Vascular cell adhesion molecule-1
VGAT	Vesicular GABA Transporter
VGLUT	Vesicular glutamate transporter
WT	Wild-type

2. ABSTRACT

In the CNS, neuroinflammation is a process triggered by a variety of circumstances including infection, toxicity, traumatic injury or autoimmunity and as hallmark always involves the quick activation of glial cells, in particular astrocytes and microglia. As the prime component of the CNS immune system, microglia plays a major role regulating neuroinflammation. Once activated, microglial cells transform, proliferate, migrate and release immunomodulatory molecules like cytokines. In turn, cytokines can strongly influence microglial phenotype. In this sense, IL-6 can be a potent inducer and modulator of microglial activation while IL-10 can downregulate the pro-inflammatory phenotype of microglia. One of the most-well described models of peripheral nerve injury is facial nerve axotomy (FNA), characterized by retrograde neuronal degeneration, glial activation and lymphocyte recruitment. To understand the role of IL-6 and IL-10 in the orchestration of the inflammatory response after FNA, we used two transgenic mice, GFAP-IL6Tg and GFAP-IL10Tg, which produce the cytokines IL-6 and IL-10, respectively, under the GFAP promoter in astrocytes. Our results showed that after FNA, IL-6 elicited a slight effect on attenuating astroglial activation, but induced intensive effects in terms of microglial activation that correlated with a higher neuronal death. Thus, we found that IL-6 increased microglia density and modify the pattern of expression of several molecules related with cellular cross-talk. IL-6 produced a drop in microglial adhesion molecules, which may affect how microglia communicate with the lesioned motorneurons. Importantly, IL-6 led to less microglial cluster formation, calling in question the assumption that these clusters correspond just to places of dying neurons and phagocytic microglia. Also, our findings showed that IL-6 produced an increase in the number of infiltrated T-cell within the lesioned facial nucleus. Although infiltration of T-cells, specifically Th2 lymphocytes, has been reported to play a neuroprotective role in WT, we can speculate that IL-6 induced the recruitment of non-neuroprotective T-cells and therefore modify the outcome of lesioned motor neurons. Despite its detrimental effects in neuronal survival at 21 days, IL-6 did not continue increasing neuronal death 7 weeks after axotomy but seemed to impair effective functional regeneration at this time-point. On the other hand IL-10 promoted an increase in neuronal survival, in front to WT, correlating with changes in microglial and immune responses. Remarkably, IL-10 led to increased microglial clusters formation, showing again no correlation between the number of microglial clusters and the amount of neuronal death. Moreover, IL-10 decreased the expression of molecules associated with microglial phagocytosis during early time-points. These findings, together with the observations in GFAP-IL6Tg animals, lead us to propose that microglial clusters may have another functions rather than phagocytosis of neuronal debris. In this sense, IL-10 increased co-stimulatory molecule expression in clustering microglia, pointing to these clusters as locations of interaction with recruited lymphocytes. We showed that also IL-10 promoted the recruitment of T-cells into the facial nucleus, yet the mechanism by which infiltrated T-cells

contribute to enhanced neuronal survival has not been yet elucidated. Despite its beneficial effects on neuronal survival at 21 days, IL-10 was not able to increase neither neuronal survival nor functional regeneration 7 weeks after FNA.

In summary, the results obtained in the present thesis show that astrocyte-targeted production of IL-6 and IL-10 modulates the neuroinflammatory response orchestrated after FNA and influence the neuronal survival and nerve regeneration.

Supported by: PhD fellowship of the "Ministerio de Ciencia e Innovación"(BES-2009-027175), PhD fellowship of the "Universitat Autònoma de Barcelona"(456-04-2/2012), Research Grant of the "Ministerio de Ciencia e Innovación (BFU2008-04407) and Research Grants of the "Ministerio de Economía y Competitividad" (BFU2011-27400) and (BFU2014-55456).

2. RESUM

Al SNC, la neuroinflamació és un procés desencadenat per varies circumstàncies incloent infeccions, toxicitat, lesió traumàtica o autoimmunitat i sempre implica la ràpida activació de les cèl·lules glials, en particular astròcits i micròglia. Com a major component del sistema immunitari al SNC, la micròglia juga un paper fonamental regulant la neuroinflamació. Un cop activada, la micròglia es transforma, prolifera, migra i secreta molècules immunomoduladores com les citoquines. A la mateixa vegada, les citoquines poden influenciar el fenotip microglial. Així, la IL-6 pot ser un potent inductor i modulador de l'activació microglial, mentre que la IL-10 pot reprimir el fenotip pro-inflamatori de la micròglia. Un dels models millor caracteritzats de lesió de nervi perifèric es la lesió del nervi facial (FNA), caracteritzada per la degeneració neuronal retrògrada, l'activació glial i el reclutament limfocític. Per entendre el paper de la IL-6 i la IL-10 en la regulació de la resposta inflamatòria després de FNA, utilitzem dos soques de ratolins transgènics, GFAP-IL6Tg i GFAP-IL10Tg, que produeixen les citoquines IL-6 i IL-10, respectivament, sota el promotor de GFAP en astròcits. Els nostres resultats demostren que després de FNA, la IL-6 té un lleu efecte atenuant l'activació astroglial, però indueix importants efectes en termes d'activació micròglia, que correlacionen amb una major mort neuronal. Així, trobem que la IL-6 augmenta la densitat microglial i modifica l'expressió de moltes molècules relacionades amb la comunicació cel·lular. La IL-6 produeix una baixada en les molècules d'adhesió microglial, les quals poden afectar la manera com la micròglia es comunica amb les neurones axotomitzades. Remarcablement, la IL-6 indueix menor formació de clústers microglials, qüestionant la noció de que aquests clústers corresponen a llocs de neurones degenerant-se i micròglia fagocítica. També, demostrem que la IL-6 provoca un augment en el nombre de limfòcits-T infiltrats al nucli facial. Tot i que la infiltració de limfòcits-T, especialment els Th2, es considera neuroprotectora en WT, podem especular que la IL-6 indueix el reclutament de limfòcits-T no-neuroprotectors i, per tant, es modificaria la supervivència neuronal. Malgrat els efectes detrimentals en supervivència neuronal als 21 dies, la IL-6 no continua augmentant la mort neuronal 7 setmanes després de FNA però sembla que empitjora la regeneració funcional efectiva a aquest temps. Per una altra banda, la IL-10 augmenta la supervivència neuronal comparada amb el WT, correlacionant amb canvis en la resposta microglial i immunitària. Remarcablement, la IL-10 provoca una major formació de clústers de micròglia, demostrant de nou que no hi ha correlació entre el nombre de clústers i la quantitat de mort neuronal. A més, la IL-10 disminueix l'expressió de molècules associades amb la fagocitosis microglial al principi de la lesió. Aquests resultats, conjuntament amb l'observat amb els animals GFAP-IL6Tg, ens duen a proposar que els clústers de micròglia poden tenir unes altres funcions, més enllà de la fagocitosis de residus neuronals. En aquest sentit, la IL-10 incrementa l'expressió de molècules coestimuladores en la micròglia dels clústers, senyalant aquestes estructures com a llocs d'interacció amb els limfòcits-T. Demostrem també que la IL-10 provoca un major reclutament de limfòcits-T al nucli facial,

tot i que el mecanisme pel qual els limfòcits-T promouen la supervivència neuronal no ha estat encara aclarit. Malgrat els efectes beneficiosos en supervivència neuronal a 21 dies, la IL-10 no té efectes en aquesta ni en la regeneració funcional efectiva 7 setmanes després de FNA.

En resum, els resultats obtinguts en la present tesis mostren que la producció dirigida en astròcits de IL-6 i IL-10 modula la resposta neuroinflamatòria orquestrada després de FNA i influencien la supervivència neuronal i la regeneració del nervi.

Amb el suport de: Beca FPI del "Ministerio de Ciencia e Innovación"(BES-2009-027175), Beca PIF de la "Universitat Autònoma de Barcelona"(456-04-2/2012), Projecte del "Ministerio de Ciencia e Innovación (BFU2008-04407)i Projectes del "Ministerio de Economía y Competitividad" (BFU2011-27400) and (BFU2014-55456).

3. INTRODUCTION

Inflammation in the central nervous system (CNS) is a common hallmark in a wide range of neurological disorders including acute insults such as stroke, trauma and infections, and chronic diseases such as Alzheimer's, Parkinson's and multiple sclerosis (Allan and Rothwell 2003). Although classically seen as deleterious for the brain (Eikelenboom et al. 2000; McGeer and McGeer 2001; Mun-Bryce and Rosenberg 1998), nowadays it is clear that neuroinflammation can have both beneficial and detrimental roles that depend on multiple factors, including the nature of the insult and the immune response generated, the time in which these responses occurs as well as how they are regulated within the CNS (Stoll et al. 2002). Likewise, an acute response may content tissue damage in some circumstances but a prolonged activation can lead to a chronic inflammatory environment that increases neurodegeneration (Chang et al. 2009; Griffiths et al. 2007). The neuroinflammatory response involves the activation of local glial cells (Cherry et al. 2014; Olson and Miller 2004; Pekny and Nilsson 2005; Streit et al. 1999; Wu et al. 2014), the recruitment of peripheral immune cells (Gonzalez and Pacheco 2014; Schwartz and Moalem 2001; Weller et al. 1996), and the upregulation and secretion of several inflammatory mediators such as cytokines and chemokines (Cacquevel et al. 2004; Merrill and Benveniste 1996; Mracsko and Veltkamp 2014; Rodgers and Miller 2012). In this context, one of the cells playing a major role in the regulation and orchestration of the neuroinflammatory response is microglia.

3.1 Microglia

Microglia was first described as a distinct cell type with long and branched processes in 1920 by Pio del Rio-Hortega using a modified silver carbonate impregnation method (Castellano et al. 1991; Del Rio-Hortega 1920a; Del Rio-Hortega 1920b; Del Rio-Hortega 1921). Del Rio-Hortega also postulated that these cells, with an amoeboid morphology invade the brain during development, transform into ramified cells in the postnatal brain and occupy a defined territory in the adult healthy CNS. Del Rio Hortega also described that microglia undergo a transformation, migrate and proliferate after a pathological event (del Rio-Hortega 1932). These findings have been widely validated with almost a century of research on microglial cells. Despite its early discovery, a search in Pubmed reveals that it was not until the early 90's when microglia started to be in the spotlight of neuroscience research and it was in the last ten years that the amount of references about microglia have exponentially increased achieving more than thousand publications per year.

3.1.1 Origin, differentiation and functions of microglia in the healthy CNS

Although the origin of microglia is still a matter of an intense debate, emerging evidence indicate that microglial precursors come from the yolk salk and invade the CNS parenchyma through blood vessels around embryonic day 8.5-9 (Ginhoux et al. 2010) (Figure 1). However, alternative routes of entry for microglial precursors, including the meninges and the ventricular space, were described (Ginhoux et al. 2010; Ginhoux et al. 2013; Ginhoux and Prinz 2015; Navascues et al. 2000). Once microglial precursors reach the CNS parenchyma, they adopt an amoeboid morphology, proliferate, aggregate in the periventricular areas and the white matter (Ling and Wong 1993; Perry et al. 1985; Zusso et al. 2012), display a high phagocytic activity (Mallat et al. 2005) and undergo changes in the expression

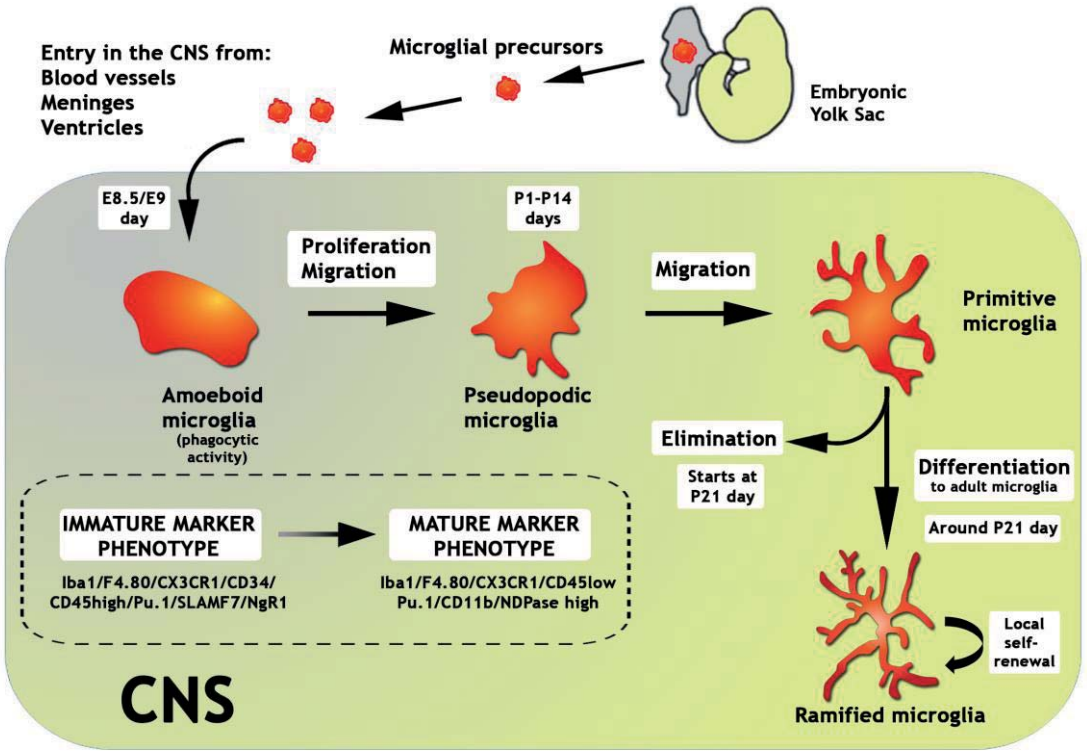

Figure 1. Origin and differentiation of microglia. Schematic representation illustrating the origin, entry to the CNS, migration and differentiation of microglia from embryonic day (E8.5/9) to adult age. The important morphological changes observed in microglia ranging from amoeboid to ramified are depicted. A summary of the most important phenotypic markers expressed by immature or mature microglia is shown in the image.

of several markers from immature to mature phenotype (Christensen et al. 2014; Harry 2013; Liu et al. 2015). Amoeboid microglia progressively adopt a pseudopodic morphology, migrate (Navascues et al. 1995; Orlowski et al. 2003) and start to distribute first into white matter areas and later into the grey matter (Hristova et al. 2010; Ling and Wong 1993), and differentiate into primitive microglia, i.e. cells with short and sparse ramifications. After an initial increase during the first two weeks of the postnatal period in mice (Dalmau et al. 2003), the number of microglia start declining on the third postnatal week and only a third of the initial amoeboid population will differentiate into adult ramified microglia (Cuadros et al. 1994; Harry 2013).

In the normal brain, adult ramified microglia are far from being considered "dormant soldiers", simply waiting for a CNS challenge, they are rather "sentinels" with very motile branches that constantly survey their microenvironment (Hanisch and Kettenmann 2007). Over the years, microglia have emerged as a key cell to maintain CNS homeostasis due to the expression of numerous membrane receptors that allows them to detect slight disturbances in the microenvironment (Kettenmann et al. 2011). Thus, subtle changes in the extracellular concentration of adenosine and its phosphorylated compounds like ADP or ATP, for example, will be detected by purinergic receptors located in the plasma membrane of microglia leading to specific responses (Castellano et al. 2016; Davalos et al. 2005; Haynes et al. 2006). As part of the continuous screening of the microenvironment, microglia is in physical cell-to-cell contact and in constant molecular cross-talk

with the other components of the CNS parenchyma, including neurons, astrocytes, oligodendrocytes and blood vessels (see next section). Although these cell interactions are not totally understood it is thought that they might contribute to the maintenance of microglia in a non-inflammatory/deactivated state in the healthy brain. Accumulating evidence indicate that this deactivated state of microglia is driven by the so-called "off-signals", see Figure 2. Among the known off-signaling systems, the best characterized so far include:

1) binding of inhibitory receptors, like CD200R, CX3C chemokine receptor 1 (CX3CR1), CD172a and CD45 on the membrane of microglia with their corresponding ligands CD200, CX3CL1, CD47 and CD22 located on neurons (Biber et al. 2007; Dentesano et al. 2014; Eyo and Wu 2013; Kierdorf and Prinz 2013)

2) neuronal release of soluble molecules, such as transforming growth factor (TGF)-ß and CD22, and neurotransmitters and neurotrophins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-3 into the extracellular space (Biber et al. 2007)

3) astrocytic release of TGF-ß, glial-derived neurotrophic factor (GDNF) and gamma-aminobutyric acid (GABA) (Herrera-Molina and von Bernhardi 2005; Lee et al. 2011; Rocha et al. 2012)

4) release of endothelial nitric oxide (eNO) from endothelial cells (Katusic and Austin 2014)

In addition to their role in surveillance, microglia are efficient phagocytes of apoptotic cells during physiological conditions in both the developing and the adult brain (Dalmau et al. 2003; Neumann et al. 2009; Schafer et al. 2012; Sierra et al. 2010). They can also eliminate synapses, either by pruning the supernumerary synapses during brain development (Marin-Teva et al. 2011; Miyamoto et al. 2013; Stevens et al. 2007) or remodeling the synapses in processes of neuronal plasticity in the adult brain (Salter and Beggs 2014; Schafer and Stevens 2013; Siskova and Tremblay 2013; Tremblay 2011; Wake et al. 2013).

3.1.2 Microglial activation in the injured CNS

As the prime component of the brain immune system, microglia are considered the resident macrophages of the CNS (Gordon and Taylor 2005; Ransohoff and Cardona 2010). They become activated when the homeostasis of the CNS is disturbed as occurs during infections (Hong and Banks 2015; Kaushik et al. 2011), acute injuries (Chio et al. 2015; Loane et al. 2015; Raposo and Schwartz 2014), ischemia (Benakis et al. 2014) or chronic neurodegenerative diseases such as Alzheimer's disease (Heppner et al. 2015; Mhatre et al. 2015), Parkinson's disease (Moehle and West 2015; Wang et al. 2015a) and multiple sclerosis (Almolda et al. 2011; Bogie et al. 2014).

In injured CNS, a plethora of signals arising from neurons, glial cells or infiltrated peripheral cells could trigger microglial activation (Figure 3). One of the most studied are the signals from over-activated, impaired or endangered neurons. These signals can be classified into three principal categories:

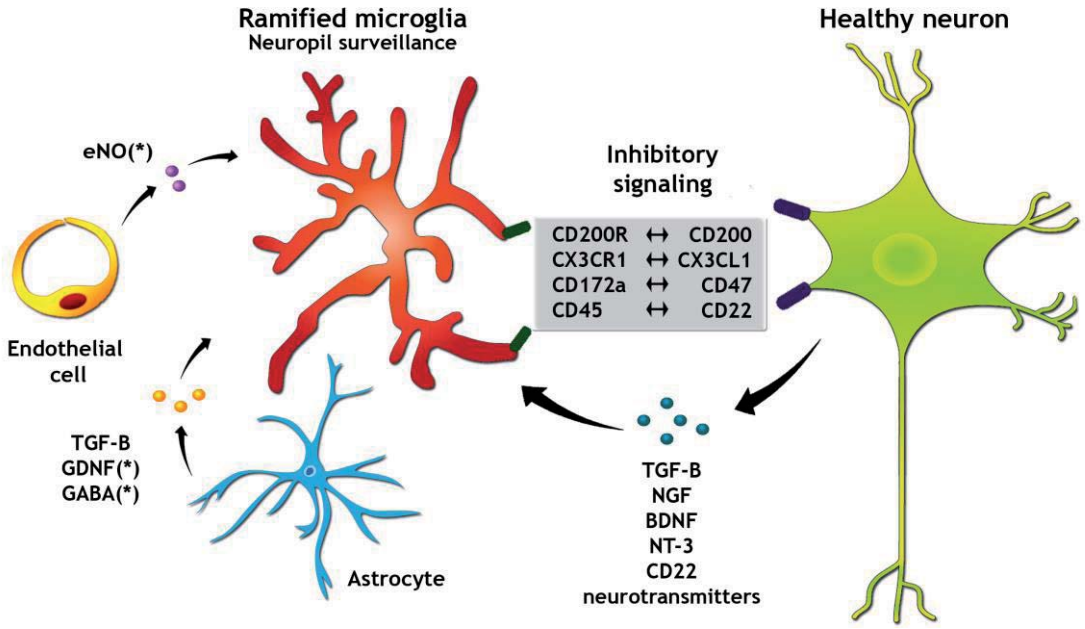

Figure 2. Non-inflammatory microglia maintained through inhibitory or "Off" signaling. Schematic representation of the "off-signals" either expressed on the surface (grey box) or secreted (colored dots) by neurons, astrocytes and endothelial cells that contribute to the maintenance of microglia in a non-inflammatory/deactivated state in basal conditions. Molecules marked with (*) have downregulatory effects on microglia playing a role as off-signals.

1) loss of calming inputs, i.e. disrupted signaling of the above-mentioned "off-signals",

2) release of a broad amount of different "help-me/find-me" signals including purines such as ATP and UTP (Sperlagh and Illes 2007); chemokines such as C-C motif ligand (CCL)-21 and C-X-C motif ligand (CXCL)10 (de Jong et al. 2005; Rappert et al. 2004); cytokines like interleukin (IL-)1β (Cartier et al. 2005) and (Mizuno et al. 2011); neuropeptides such as bradykinin, endothelin, galanin and neurotensin (Ifuku et al. 2011) (Filipovich-Rimon and Fleisher-Berkovich 2010; Grillner et al. 2005; Ifuku et al. 2007); neurotransmitters such as glutamate, adrenaline and dopamine (Farber et al. 2005; Liu et al. 2009); cannabinoids (Walter et al. 2003), morphine (Takayama and Ueda 2005) and lipocalin-2 (Xing et al. 2014).

3) up-regulation of the so-called "eat-me signals", like the expression of "triggering receptor expressed on myeloid cells 2" (TREM2) on the microglial membrane that recognizes a still undefined ligand in damaged neurons (Hanisch and Kettenmann 2007; Linnartz and Neumann 2013; Neumann and Takahashi 2007; Takahashi et al. 2005).

In addition to the cross-talk with neurons (Cardona et al. 2006; Suzumura 2013), the regulation of microglial activation during pathological conditions also involves the interaction with the rest of glial cells, the blood vessels and the recruited peripheral immune cells (Figure 3). Activated astrocytes are known to produce ATP and chemokines such as CCL2, CCL5, CXCL10 and CXCL12 to recruit microglia to the lesion site (Dong and Benveniste 2001; Huang et al. 2012; Sofroniew 2014). It has

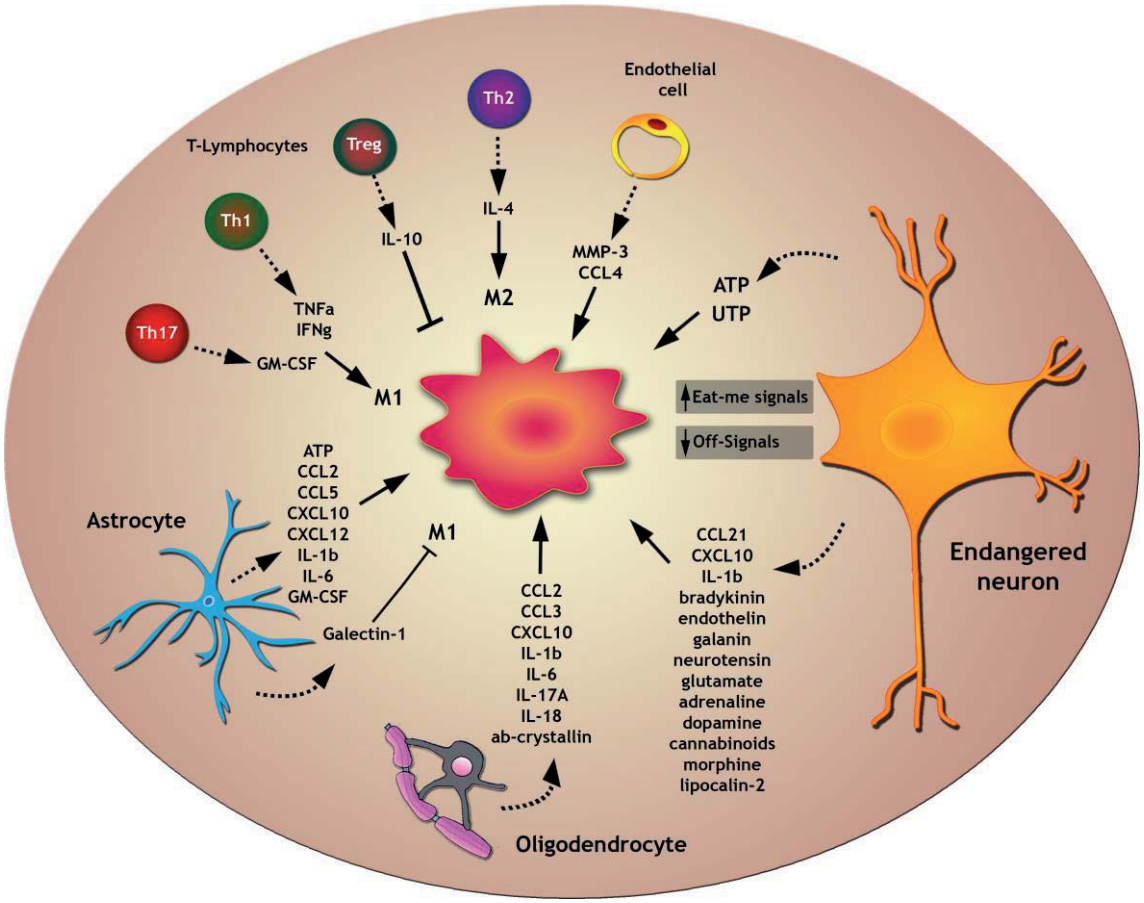

Figure 3. Crosstalk of CNS resident and recruited cells with activated microglia. Schematic representation of the "on-signals" secreted by damaged neurons, activated astrocytes, oligodendrocytes, different subtypes of recruited T-lymphocytes (Th1, Th2, Th17 and Treg) and endothelial cells that could modulate microglial activation after a CNS challenge. Importantly, both loss of "off-signals" and upregulation of "eat-me" signals from neurons may lead to microglial activation. Pointed arrows indicate activating stimuli, perpendicular lined arrows indicate deactivating stimuli and dotted arrows indicate secreted molecules.

also been reported that activated astrocytes are able to either promote microglial activation via granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1β and IL-6 (Mayo et al. 2014; Sofroniew 2014; Zamanian et al. 2012) or inhibit the microglial inflammatory M1 phenotype via galectin-1 (Burda et al. 2016; Correale and Farez 2015; Han et al. 2011; Liu et al. 2011). In response to stress, oligodendrocytes produce several mediators known to modulate the activation state of microglia such as the chemokines CXCL10, CCL2 and CCL3 (Balabanov et al. 2007; Ramesh et al. 2012), cytokines like IL-1β, IL-6, IL-17A and IL-18 (Cannella and Raine 2004; Moyon et al. 2015; Peferoen et al. 2014; Ramesh et al. 2012; Tzartos et al. 2008; Zeis et al. 2015) and the heat-shock protein αβ-crystallin (van Noort et al. 2010). Only a few number of molecules involved in the cross-talk between endothelial cells and microglia have been reported (da Fonseca et al. 2014). For instance, endothelial cell production of matrix metalloproteinase (MMP)-3 and CCL4 has been found to promote microglial activation (Lee et al. 2015; Wang et al. 2011). Important interactions between infiltrated T lymphocytes and microglia have also been described, mainly through the expression of

cytokines (reviewed in (Gonzalez et al. 2014)). On one hand, Th1 cells can promote microglial activation through TNF-α and IFN-γ (Murphy et al. 2010), whereas Th17 cells activate microglia via GM-CSF (Codarri et al. 2011). On the other hand, IL-4 secretion by Th2 cells promotes a more neuroprotective phenotype of microglia (Chiu et al. 2008) and T-regulatory cells are known to restrain microglial activation via IL-10 secretion (Xie et al. 2015).

Once activated, microglia experience profound changes in their morphology (Graeber and Streit 2010; Karperien et al. 2013), proliferate to achieve sufficient number of cells to overcome CNS challenge (Gomez-Nicola and Perry 2015; Kreutzberg 1996a), and can actively migrate towards the focus of the damage if necessary (Beamer et al. 2015; Bechmann and Nitsch 2000; Davalos et al. 2005; Scheiblich and Bicker 2015). Concomitantly, activated microglia display changes in their phenotype expressing a wide range of surface molecules for cell-cell and cell-matrix interactions (Kettenmann et al. 2011) and, in some circumstances, can upregulate the cellular machinery required for phagocytosis such as Toll-like receptors (TLRs), Fc receptors, complement receptors and scavenger receptors (SR), among others (Aderem and Underhill 1999; Fu et al. 2014; Lucin and Wyss-Coray 2009). In turn, activated microglia can release important immunomodulatory molecules like cytokines (Hanisch 2002), T-cell chemoattractants including CXCL10, CCL19 and CCL2 (Aloisi et al. 2000; Aschner et al. 1999; Gyoneva and Ransohoff 2015; Ransohoff 1999; Streit et al. 2000; Sun et al. 1997) and act as antigen presenting cell (Carson 2002; Strachan-Whaley et al. 2014), due to their ability to express major histocompatibility complex-II (MHC-II) and costimulatory molecules (Almolda et al. 2015; Aloisi et al. 2000; Benveniste et al. 2004).

For some years, microglial responses have been attempted to be categorized into the macrophage M1/M2 classification composed of the so-called 'M1 or classically activated phenotype', with a highly pro-inflammatory profile, or the 'M2 or alternatively activated phenotype' associated with a less inflammatory and more neuroprotective profile (See Table 1) (Cherry et al. 2014; Czeh et al. 2011; Eggen et al. 2013; Ransohoff and Cardona 2010; Schwartz et al. 2006). Although this M1/M2 classification results precise and useful to systematize peripheral macrophage polarization (Gordon 2003), an increasing number of studies highlighted that it does not translate accurately to microglial activation, as these cells are able to express a variable mishmash of M1 and M2 markers at the same time depending on the different experimental conditions, changes in the microenvironment, etc. (Crain et al. 2013; Olah et al. 2012). These evidences have contributed to the regard of microglia as a highly versatile and plastic population of cells with a broad spectrum of functions and phenotypes that cannot just fit into a M1 or a M2 category (Biber et al. 2014; Shechter and Schwartz 2013).

3.1.3 The dual beneficial and detrimental role of activated microglia

Over the years, it has become evident that microglial activation involves not only harmful and neurotoxic functions but can also be neuroprotective in some cases (Hanisch and Kettenmann 2007). In fact, numerous reviews discussing about the “good” and the “evil” features of microglia in both acute and chronic CNS disease models are available (Cherry et al. 2014; Czeh et al. 2011; Doring and Yong 2011; Henkel et al. 2009; Karve et al. 2016; Loane and Kumar 2016; Luo and Chen 2012).

Phagocytic activity is believed to be one of the putative neuroprotective features of microglia since implies efficient removal of apoptotic cells and clearance of tissue debris at the lesion site (Napoli and

Table 1. Macrophage/microglia and their attributed functions

	INDUCERS	MARKERS AND PRODUCTS		SIGNALING FACTORS	FUNCTION IN THE PERIPHERY	EFFECTS IN THE CNS
Classical Activation M1 	LPS IFNγ TNFα GM-CSF Th1 cytokines	CD14 ↑FcR CD86 ↑ROS IFN IL-1β IL-6 ↓IL-10	↑IL-12 IL-23 iNOS MCP-1 MHC-II TLR TNF	NFκβ STAT1 IRF5 AP-1 IRF7	Proinflammatory. Removal of cellular debris, apoptotic cells and pathogen agents.	Inhibition of NSC differentiation. Promotion of neuronal death. Increase EAE severity. Disruption of BBB. White matter injury.
Alternative activation						
M2a 	IL-4 IL-13 PPARγ agonists	Arg-1 ↓ROS CD200R Fizz-1 IGF-1 IL1RN Lectins MHCII	P2Y12 P2Y13 CD206 SCR SOCS3 Ym-1	STAT6 IRF4 PPARs	Profibrotic. Antiinflammatory. Neuroprotective. Phagocytosis of apoptotic cells. Proliferation and cellular migration. Growth factors synthesis. Th2 activation.	Remyelination, axonal regeneration and reduction of cell death.
M2b	LPS and TLR agonists Fc fraction agonists IL-1R agonists	c/EBPβ ↓ROS ↑IL-10 ↓IL-12 PD-L1	CD64 CD86 CD200R IGF-1 IL-6 CCL2 ↑MHC-II TNFα VEGF	NFκβ PI3K/Akt	Immunoregulation. Activation of Treg and inhibition of Th1. Cellular maduration. Tissue homeostasis. Angiogenesis. ECM synthesis.	Axonal regeneration.
M2c	IL-10 TGF-β Glucocorticoids		Arg-1 CD14 CD163 IL-4R IL-10R ↓MHC-II P2Y14 CD206 SCR	STAT3	Activation of Treg and inhibition of Th1. Resolution of the inflammation. Tissue repair. ECM synthesis.	Glial deactivation. Enhanced NSC proliferation. Oligodendrogenesis. Neurogenesis

Table 1. Classification of macrophages/microglia phenotypes and their attributed functions. A summary of the phenotypic markers of M1/M2 classification applied to macrophages/microglia. Besides the general role played by each phenotype in the periphery, a detailed list of their functions within the CNS has been included. **Abbreviations:** NSC, neural stem cell; EAE, experimental autoimmune encephalomyelitis; BBB, blood brain barrier; ECM, extracellular matrix, SCR, scavenger receptors. **References:** *(Stein et al 1992, Bagasra et al 1995, Skeen et al 1996, MacMicking et al 1997, Munder et al 1999, Mantovani et al 2004, Taylor et al 2005, Bouhlel et al 2008, Martinez et al 2008, King et al 2009, Mildner et al 2009, Anders & Ryu et al 2011, Manrique et al 2011, Liao et al 2012, Miron et al 2013, Shechter et al 2013, Tang et al 2014, Pepe et al 2014, Zhao et al 2014, Tanaka et al 2015, Moehle et al 2015, Moore et al 2015, Gensel & Zhang 2015).*

Neumann 2009) generating an appropriate microenvironment for regeneration and repair (Kotter et al. 2006). Furthermore, microglia have the capacity to remove β-amyloid deposits via the secretion of proteolytic enzymes (Walter and Neumann 2009) and malfunction in this clearance has been related with the development and progression of Alzheimer's disease (Malm et al. 2015; Schlachetzki and Hull 2009). Microglia can also display neuroprotective effects through the secretion of anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and TGF-β (Colton 2009; Kawabori and Yenari 2015; Lambertsen et al. 2009; Loane and Byrnes 2010; Streit 2002), the production of anti-oxidant enzymes such as heme oxygenase-1 (Noda et al. 2011) and the release of neurotrophic factors, including NGF, BDNF, NT-3, ciliary neurotrophic factor (CNTF), GDNF, insulin-like growth factor (IGF)-1 and fibroblast growth factor (FGF)-2 (Kim and de Vellis 2005; Nakajima and Kohsaka 2004; Nakajima et al. 2007; Napoli and Neumann 2009; Neumann et al. 2009; Polazzi and Monti 2010; Suzumura 2013; Voss et al. 2012) that promote further CNS repair.

In addition to their potential neuroprotective effects, microglia can display a harmful and neurotoxic phenotype, characterized by the secretion of pro-inflammatory cytokines such as IL-1β (Davies et al. 1999; Lee et al. 1993) and TNF-α (Sawada et al. 1999) both of which are known to produce neuronal cell death (Chao et al. 1995; Hu et al. 1997; Probert 2015; Ramesh et al. 2013; Smith et al. 2012) and to disrupt blood brain barrier (BBB) through upregulation of adhesion molecules (Brabers and Nottet 2006; Fiala et al. 1997; Nottet 1999). Activated microglia can also produce molecules such as: 1) reactive oxygen species (ROS), as hydrogen peroxide, superoxide anion and the hydroxyl radical, which lead to neuronal toxicity and degeneration (Kennedy et al. 2012; Loh et al. 2006; Navarro-Yepes et al. 2014); 2) nitric oxide (NO) (Moss and Bates 2001) that causes oxidative and nitrosative stress and activates mitochondrial apoptosis by several pathways (reviewed in (Brown and Neher 2010; Yuste et al. 2015)) and 3) glutamate (Yawata et al. 2008) that, in excess, can be directly toxic for depolarized neurons (Brown and Vilalta 2015) and can damage dendrites and synapses *in vitro* (Maezawa and Jin 2010).

Whether microglia would display a neurotoxic or neuroprotective role after a CNS challenge will depend on the balance between the different types of molecules they will release, as well as the microenvironmental context where this microglial activation takes place (Colton 2009; Marin-Teva et al. 2011; Ramesh et al. 2013) (Walter and Neumann 2009).

3.2 Cytokines in the CNS

Mounting evidence indicates that microglial activation is not a linear process, but rather a continuum event depending on the triggering stimuli and the local microenvironment (Boche et al. 2013; Henkel et al. 2009; Moehle and West 2015; Taylor and Sansing 2013; Varnum and Ikezu 2012). One of the most important regulatory molecules that can influence this local microenvironment and hence microglial phenotype are cytokines (Colton 2009; Ramesh et al. 2013). Cytokines are an extensive category of small proteins (~5–50 kDa) with pleiotropic functions that participate in cellular communication. They are rapidly upregulated and released in response to pathological challenges, orchestrating the inflammatory response (Allan and Rothwell 2001). Cytokine production was firstly described in immune cells but can also be produced by neurons and glial cells, among others (Mosmann and Coffman 1989; Pineau and Lacroix 2007; Sei et al. 1995; Vitkovic et al. 2000). Cytokines can be classified by their structural characteristics and also according to the receptor types

through they play their biological function (Akdis et al. 2011; Dinarello 2007; Lata and Raghava 2008; Oppenheim 2001). According to their biological function, cytokines are classified in two broad categories:

1) pro-inflammatory cytokines, such as IL-1, TNF, IFN-γ, IL-6, IL-12, IL-18 and GM-CSF.

2) anti-inflammatory cytokines, which include IL-4, IL-10, IL-13 and TGF-ß.

In the CNS, pro-inflammatory cytokines have been typically associated with neurotoxic and harmful effects, inducing the microglial production of toxic mediators, such as prostaglandins and ROS (Basu et al. 2004), the upregulation of adhesion molecules involved in leukocyte infiltration within the CNS and the microglial expression of MHC-II (Hosomi et al. 2005; Probert 2015; Sethna and Lampson 1991). By contrast, the production of anti-inflammatory cytokines has been linked with neuroprotective actions, as they are known to dampen the harmful effects of the pro-inflammatory cytokines mentioned above and are related with recovery in different CNS injuries (Al-Amin and Reza 2014; Falcone et al. 1998; McGeachy and Anderton 2005; Vidal et al. 2013). In this sense, many therapeutic approaches for CNS injuries and diseases have attempted to reverse the production of pro-inflammatory cytokines and to increase the presence of anti-inflammatory mediators in the milieu (Kleinig and Vink 2009; Laveti et al. 2013; Muller 2013; Owens 2002; Rivest 2011). However, this classification of cytokines in the categories of pro- and anti-inflammatories is a simplistic approach, giving the fact that cytokines have pleiotropic biological functions depending on the secretor cell, the target cell, the receptor they bind to and the intracellular signaling triggered (Aloisi et al. 1999; Ding et al. 2015; Milner and Campbell 2003). Moreover, the action of one specific cytokine may depend on presence of other cytokines with synergistic, additive or opposite actions (Du et al. 2010; Heinemann et al. 2014). Therefore, multiple factors must be considered before classifying the actions of a specific cytokine such as purely harmful or protective/beneficial. Among the variety of cytokines with proven important effects within the CNS, there are two that have focused the attention of neuroscientists over the years. The first is the cytokine IL-6, which is one of the main regulatory mediators of neuroinflammation, and the second the cytokine IL-10, which is considered as a counter-regulatory cytokine involved in the termination of the inflammatory process.

3.2.1 Interleukin-6 in the CNS and the microglial response

Discovered three decades ago, IL-6 was firstly characterized as a B-cell differentiation factor (Kishimoto 1985) and cloned just few years later, when IL-6 nomenclature was adopted (Tanabe et al. 1988; Yasukawa et al. 1987). IL-6 is a single-chain glycoprotein of 21-30 kDa with a four-helix bundle structure (Kishimoto et al. 1995a) that binds to its specific receptor IL-6R. However, the complex itself does not lead intracellular signaling and requires the association with a second transmembrane protein, gp130, which acts as a signal transducer of IL-6 (Hibi et al. 1990). Signaling of IL-6 occurs via two different pathways: via the membrane bound IL-6R, the so-called "classic signaling" (Kishimoto et al. 1995b) or via the soluble IL-6R (sIL-6R), known as "trans-signaling", implying that cells expressing gp130 but not IL-6R on their membrane can be stimulated by IL-6 (Campbell et al. 2014; Rose-John 2012; Rose-John and Heinrich 1994). Along 30 years of intensive research, this pleiotropic and multifunctional cytokine has been shown to play important functions

on behavior and metabolism control, in addition to its key role as regulator of inflammatory responses (Kishimoto 2006; Kishimoto et al. 1995a; Taga and Kishimoto 1997).

In the CNS, a key role for IL-6 was soon suspected as neurons, glial cells and endothelial cells express moderate levels of IL-6 and IL-6R in the healthy CNS (Cornfield and Sills 1991; Gadient and Otten 1994; Reyes et al. 1999; Schobitz et al. 1993; Schobitz et al. 1992) that substantially increase after several kinds of acute and chronic injuries (Benveniste 1998; Boche et al. 2013; Gadient and Otten 1997; Gruol and Nelson 1997; Nakamura et al. 2005; Spooren et al. 2011; Suzuki et al. 2009; Van Wagoner and Benveniste 1999). One of the main reasons for the interest shown by neuroscientists in this molecule is that despite the numerous studies in the field, the role played by IL-6 in harmful circumstances is still not yet well established, and both detrimental as well as beneficial functions have been reported. A noxious role has been classically attributed to IL-6 production as, along with TNF-α and IL-1β, this interleukin is considered one of the major inducers of the inflammatory response in the CNS (Benveniste 1998; Boche et al. 2013; Gadient and Otten 1997) leading to glial activation and the consequent production of deleterious ROS, which, among others, induce BBB disruption, lymphocyte recruitment and neurodegeneration (Brett et al. 1995; Brunello et al. 2000; Campbell et al. 1993; Chiang et al. 1994; Fisher et al. 2001; Krady et al. 2008; Tilgner et al. 2001). On the bright side, anti-inflammatory and neuroprotective functions have also been attributed to IL-6. *In vitro,* IL-6 has been shown to enhance neuronal survival (D'Arcangelo et al. 2000; Hama et al. 1989; Mendonca Torres and de Araujo 2001; Thier et al. 1999) and *in vivo* approaches demonstrated the ability of IL-6 to increase axonal regeneration and to improve neuronal survival after dorsal root ganglion (Cao et al. 2006) (Murphy et al. 1999) (Zhong et al. 1999) and sciatic nerve (Ikeda et al. 1996) axotomies as well as after aseptic cerebral injury (Swartz et al. 2001).

Specifically on microglia, IL-6 is known to activate these cells both *in vitro* and *in vivo* (Krady et al. 2008; Tilgner et al. 2001). For instance, microglia in transgenic mice with astrocyte targeted IL-6 production presented increased expression of macrophage-1 antigen (MAC-1) (Campbell et al. 1993; Chiang et al. 1994; Heyser et al. 1997). Administration of IL-6 induces microglial CX3CR1 expression after constriction of sciatic nerve (Lee et al. 2010b) and overproduction of IL-6 in a mice model of Alzheimer's disease has been shown to increase CD11b, class A Scavenger Receptor (SRA), CD68/macrosialin and Ym-1 expression in activated microglia and promotes Aβ42 uptake by these cells (Chakrabarty et al. 2010). More recently, it has been demonstrated that administration of anti-IL-6R antibody decreases CD68-positive microglial cells following olfactory system injury (Kobayashi et al. 2013) and promotes an alternative activation of microglia/macrophage after spinal cord injury (Guerrero et al. 2012). In addition, a role of IL-6 in promoting microglial proliferation has been demonstrated *in vitro* (Kloss et al. 1999; Streit et al. 2000) as well as *in vivo*, using either CNS-targeted production of IL-6 (Campbell et al. 1993; Chiang et al. 1994; Fattori et al. 1995; Heyser et al. 1997) or continuous IL-6 application (Tilgner et al. 2001). In agreement, a reduction of microglial proliferation was observed in IL-6 deficient mice after peripheral nerve injury. Finally, it has been reported that treatment with IL-6 has an effect on microglial survival protecting microglia from methamphetamine-induced cell death (Coelho-Santos et al. 2012).

3.2.2 Interleukin-10 in the CNS and the microglial response

In 1989, Fiorentino et al, described a novel immunoregulatory molecule, secreted by type-2 T-helper cells which was able to inhibit the synthesis of IL-2 and IFN-γ in Th1 cells (Fiorentino et al. 1989). Formerly known as "cytokine synthesis inhibitory factor" (CSIF), this molecule was accepted afterwards as "IL-10" in the cytokine nomenclature (Mosmann 1991). IL-10 is a 35 kDa homodimer composed of two, non-covalently bonded monomers (Windsor et al. 1993) that signals through a receptor complex consisting of two IL-10 receptor-1 and two IL-10 receptor-2 proteins (Moore et al. 2001). Cellular sources of IL-10 are found in almost all immune cells (Blanco et al. 2008; Chomarat et al. 1993; Fillatreau et al. 2002; Grant et al. 2008; Mehrotra et al. 1998; Murai et al. 2009; Rhodes et al. 2008; Roers et al. 2004; Seki et al. 1998; Speiran et al. 2009). IL-10 controls inflammation via inhibition of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8 and IL-12 (Strle et al. 2001) and modulates adaptive immune responses that lead to tissue damage (Asadullah et al. 2003; Mosmann 1991; Sabat 2010; Trinchieri 2007). Relevance of IL-10 becomes obvious when we take into account the numerous studies that assess its role in various peripheral immune-mediated diseases as systemic lupus erythematosus (Maini et al. 1994; McCarthy et al. 2014; Peng et al. 2013), among others (Asadullah et al. 2004; Chung 2001; Emilie et al. 1995; Glocker et al. 2009; Kuhn et al. 1993; Redford et al. 2011; Shouval et al. 2014; Trifunovic et al. 2015).

In the CNS, IL-10 is upregulated after acute injury, including traumatic brain injury (Kamm et al. 2006), excitotoxicity (Gonzalez et al. 2009) and middle cerebral artery occlusion (MCAO) (Zhai et al. 1997) and also in chronic neurodegenerative diseases such as Alzheimer's disease (Apelt and Schliebs 2001) and multiple sclerosis (Hulshof et al. 2002). Remarkably, an increase in IL-10 expression has been found in the recovery phase of EAE (Issazadeh et al. 1995; Kennedy et al. 1992; Ledeboer et al. 2003; Samoilova et al. 1998) or at later time-points after excitotoxic injury in the postnatal rat brain (Apelt and Schliebs 2001; Gonzalez et al. 2009). IL-10 can be synthesized mainly by activated microglia and astrocytes (Hulshof et al. 2002; Ledeboer et al. 2002; Park et al. 2007), which in turn are known to express IL-10 receptor (IL-10R) under determined circumstances (Mizuno et al. 1994; Pousset et al. 2001; Strle et al. 2002; Xin et al. 2011). The fact that IL-10R can also be expressed by oligodendrocytes (Molina-Holgado et al. 2001a) and neurons (Xin et al. 2011; Zhou et al. 2009) is indicative of the wide range of cellular targets for this cytokine in the CNS.

Several of the neuroprotective effects of IL-10 reported after different kinds of injury such as stroke (Spera et al. 1998), excitotoxic (Brewer et al. 1999) and traumatic (Bethea et al. 1999) spinal cord injuries, and peripheral sciatic nerve transection (Atkins et al. 2007) have been linked to the ability of this cytokine to downregulate microglial activation. For instance, *in vitro* studies demonstrated that IL-10 dampens the microglial production of lipopolysaccharide(LPS)-induced pro-inflammatory mediators such as IL-1β, IL-6, IL-12p70, IL-23 and TNF-α (Aloisi et al. 1997; Balasingam and Yong 1996; Chao et al. 1995; Correa et al. 2011; Frei et al. 1994a; Kremlev and Palmer 2005; Lodge and Sriram 1996; Molina-Holgado et al. 2001b; Norden et al. 2014; Pang et al. 2005; Park et al. 2007; Rasley et al. 2006; Sawada et al. 1999; van Strien et al. 2010) and chemokines such as CCL5 (Marques et al. 2004) as well as reduces the expression of the intracellular adhesion molecule-1 (ICAM-1) (Wirjatijasa et al. 2002), MHC-II and costimulatory molecules (Frei et al. 1994b; Menendez Iglesias et al. 1997; Mizuno et al. 1994; Wei and Jonakait 1999; Williams et al. 1996) and the microglial

production of NO (Ledeboer et al. 2000; Rozenfeld et al. 2003). Moreover, also *in vitro*, IL-10 administration increases microglial ramification, migration, ATP-induced chemotaxis, invasion, podosome formation and survival (Lam and Schlichter 2015; Siddiqui et al. 2014; Siddiqui et al. 2012; Strle et al. 2002; Wirjatijasa et al. 2002) but not proliferation (Lam and Schlichter 2015; Sawada et al. 1999; Strle et al. 2002). *In vivo* approaches using the IL-10 deficient mice showed a sustained activation of microglia after coronavirus-induced white matter lesion (Puntambekar et al. 2015) and an increased expression of TNF-α and IL-6 after intracerebroventricular LPS injection (Agnello et al. 2000). Similarly, IL-10 delivery within the CNS resulted in a decrease in microglial activation, characterized by less expression of CD45, CD68, CD11c, IB-4, TNF-α and inducible nitric oxide synthase (iNOS) and a predominance of ramified over amoeboid morphology (Jackson et al. 2003; Ren et al. 2011; Shechter et al. 2009; van Strien et al. 2010; Yang et al. 2009).

In front to IL-6, which may be considered as a potent inducer and modulator of microglial activation, IL-10 may be considered rather as a key molecule involved in the downregulation of the pro-inflammatory phenotype of microglia. Despite the available studies, it has not been clarified how these two cytokines specifically influence the activation/deactivation of microglia and their neurotoxic or neuroprotective role after a CNS injury.

3.3 Facial nerve axotomy model

Facial nerve axotomy (FNA) is one of the most well-characterized models of peripheral nerve injury, widely used to study retrograde neuronal degeneration in the CNS without disruption of the BBB (Moran and Graeber 2004). In addition, FNA is not only a useful model to study the molecular mechanisms involved in motor neuron death, but also in axonal damage and regeneration induced by axotomy (Moran and Graeber 2004). In fact, accumulating evidence suggested that early events in sporadic amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease with an unknown etiology characterized by the selective degeneration of upper and lower motor neurons, might also include axonal suffering. Similarly to found in ALS patients and in ALS mutant mice models, FNA in rodents produces an important glial reaction that precedes motor neuron death (Aldskogius and Kozlova 1998; Moran and Graeber 2004). In both cases, glial reaction mainly consists in an accumulation of activated microglial cells around injured motor neurons wrapping the neuronal bodies and dendrites (Ajami et al. 2007; Almolda et al. 2014; Sanagi et al. 2010). Also in both ALS and FNA, a significant accumulation of T-cells around the perikarya of neurons has been reported (Kawamura et al. 2012; Raivich et al. 1998b). Thus, it has been suggested that FNA may provide a valuable tool to study some of the features observed in motor neuron diseases including ALS and SMA (spinal muscular atrophy) and to test the ability of new drugs to promote motor neuron survival.

The facial nerves emerge through the stylomastoid foramen from the bilateral facial nuclei (FN) located in the ventral part of the brainstem. In adult mice, each nucleus harbors around 2000 facial motor neurons (FMNs), and is divided into seven subnuclei: lateral, dorsolateral, dorsal intermediate, ventral intermediate, dorsomedial, ventromedial and dorsal accessory (Figure 4). The lateral portions of FN innervate facial musculature in the nasolabial regions whereas the medial portions innervate the auricular musculature (Ashwell 1982; Komiyama et al. 1984). FMNs receive both excitatory (2% VGLUT1+/ 49% VGLUT2+) and inhibitory (49% VGAT+) inputs from neurons located in the red nucleus of the midbrain and in the pontine and the medullary reticular formation (Faunes et al. 2015;

Grillner et al. 2005; Pong et al. 2008; Stanek et al. 2014; Takatoh et al. 2013; Travers and Norgren 1983). Compared to other orofacial motor nuclei such as the trigeminal, the ambiguous or the hypoglossal, the density of terminals projecting to the FN is five to ten-fold higher, making evident the wide range of complex behaviors to which this nucleus contributes (Faunes et al. 2015). It is well known that disruption of facial nerve axons quickly triggers a complex response in the soma of the FMNs leading to structural, metabolic, electrophysiological and molecular alterations (Figure 5.1) (Aldskogius and Kozlova 1998; Aldskogius et al. 1999; Gonzalez-Forero and Moreno-Lopez 2014; Kreutzberg et al. 1989; Lieberman 1971; Nishimura et al. 1992). For instance, axotomized FMNs are known to up-regulate a wide range of molecules (reviewed in (Moran and Graeber 2004)) including: 1) cell-adhesion molecules such as CD44 (Jones et al. 1997; Moneta et al. 1993); 2) neuropeptides such as galanin and calcitonin gene-related peptide (CGRP) (Armstrong et al. 2008; Armstrong et al. 2003; Flugel et al. 2001; Haas et al. 1994; Saika et al. 1991); 3) transcription factors such as c-Jun and signal transducer and activator of transcription-3 (STAT3) (Haas et al. 1993; Raivich et al. 2004; Schwaiger et al. 2000), 4) chemokines such as C-C chemokine receptor type 3 (CCR3), CCL2 and CXCL11 (Flugel et al. 2001; Wainwright et al. 2009a; Wainwright et al. 2009b) and 5) Parkinson-related protein α-synuclein (Moran et al. 2001). Concomitantly, down-regulation of several molecules such as the neuronal marker NeuN (McPhail et al. 2004), fractalkine (Harrison et al. 1998), the extracellular matrix protein tenascin (Angelov et al. 1998) and neurotransmission-related molecules such as VAchT, ChAT and m2MAchR (Ichimiya et al. 2013) also occurs.

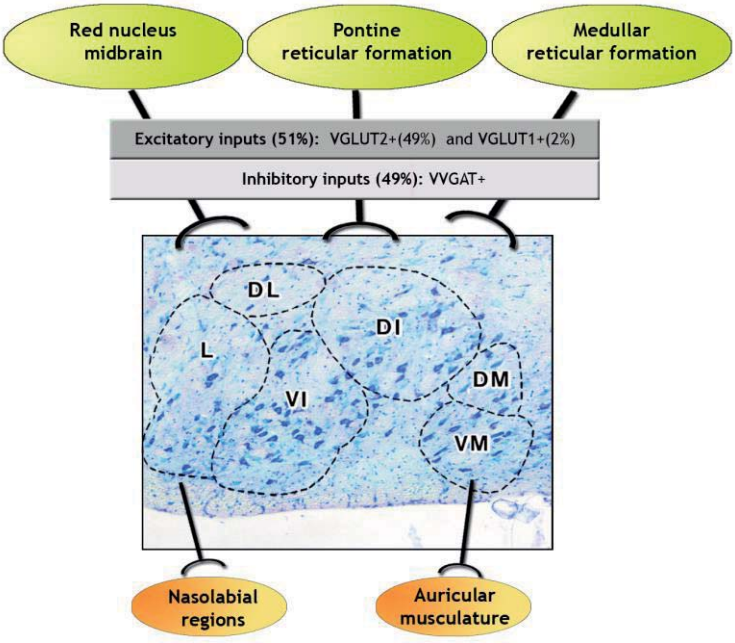

Figure 4. Facial nucleus inputs and innervation. Schematic representation of inputs and outputs of facial motor neurons (FMNs). FMNs receive both excitatory and inhibitory inputs from neurons located in the red nucleus of the midbrain, the pontine and the medullary reticular formation. Microphotograph of the right facial nucleus stained with toluidine blue where the distribution of the different subnuclei that compose this nucleus has been delimited with a dotted line: L, lateral; DL, dorsolateral, DI, dorsal intermediate; VI, ventral intermediate; DM, dorsomedial VM, ventromedial and dorsal accessory (not shown). The lateral portions of the nuclei supply facial musculature in the nasolabial regions whereas the medial portions supply the auricular musculature.

In order to survive, FMNs need to regenerate their axon and reconnect with muscle target (Raivich and Makwana 2007) which may take up to few weeks (Kamijo et al. 2003). It is believed that a small proportion of axotomized FMNs die irretrievably, despite their efforts to regenerate or the beneficial treatments they receive (Moller et al. 1996). The majority of neuronal death in axotomized FN has been reported to occur around 2-3 weeks post-injury and thus, major part of studies analyzed this survival time-point to assess possible alterations in different paradigms of FNA (Dauer et al. 2011; Fendrick et al. 2005; Mignini et al. 2012). Some studies have shown however that death of axotomized FMNs is not restricted to the first weeks but continues gradually until at least 10 weeks after axotomy (Haulcomb et al. 2014; Serpe et al. 2000). In addition, it should be taken into account that substantial differences regarding the amount of neuronal death can be found depending on the type of axotomy performed (Ha et al. 2008) and the specie, strain and age of animals (Dauer et al. 2011; Ha et al. 2006). For example, facial nerve axotomy in neonatal rats results in over 90% FMNs death (Baumgartner and Shine 1998; Garrah et al. 1998), whereas in adult rats, most FMNs survive (Egami et al. 2005; Mattsson et al. 1999; Streit and Kreutzberg 1988). By contrast, a loss of around 20-40% of axotomized FMNs is found in the majority of studies performed in adult mice (Castelnau et al. 1998; Sendtner et al. 1996).

Simultaneously to the fast and seemingly inevitable degenerative process affecting part of the axotomized FMN population, an important number of axotomized FMNs enter into a regenerative state (Figure 5.2) by: 1) quick induction of transcription factors such as STAT3 (Bareyre et al. 2011) and c-Jun (Keramaris et al. 2005; Ruff et al. 2012) and 2) upregulation of the so-called regeneration-associated genes (RAGs) that include molecules related with cell-cell signaling, axonal growth and sprouting such as CD44, CD29, galanin, CGRP, GAP-43, βII-tubulin and CX3CR1 (Haulcomb et al. 2014; Makwana and Raivich 2005; Patodia and Raivich 2012). Once the regenerative state is engaged, the axotomized FMN starts to regrow its axon; culminating in the re-innervation of target muscles and a partial functional recovery around 4 weeks after axotomy (Kamijo et al. 2003; Makwana et al. 2010; Patodia and Raivich 2012). However, some of the regenerative axons may fail to establish correct connections with their distal targets resulting into a disrupted somatotopic organization of the FN leading to synkinesis, i.e. abnormal involuntary facial movement that occurs with voluntary movement of a different facial muscle group (Aldskogius and Thomander 1986; Asahara et al. 1999; Choi and Raisman 2002; Dohm et al. 2000; Ito et al. 1994; Popratiloff et al. 2001). Successful regeneration depends on the activation of a targeted program for neurite outgrowth but also of a permissive environment (Chen et al. 2007). It is important to mention that axonal growth is highly affected by immune factors and we must take into consideration that FMN regeneration has to take place in the context of a disturbed immune environment both in the surroundings of the FMN as into the soma where the growth cone is being formed (Klimaschewski et al. 2013; Vidal et al. 2013).

If the conditions that favor the regeneration are not present or do not persist over time, FMN death may occur through activation of caspases, in the case of neonatal mice (de Bilbao et al. 2000; Vanderluit et al. 2000; Yaginuma et al. 2001). In adult mice, a slow degenerating and non-apoptotic FMN death occurs and it is characterized by absence of significant chromatin condensation and nuclear fragmentation (Graeber and Moran 2002). Also, some studies revealed a decline in the

expression of anti-apoptotic Bcl-2 (Wang et al. 2002) and an upregulation of α-synuclein in slow degenerating FMN (Moran et al. 2001) (Figure 5.3).

Although the exact mechanisms mediating either neuronal survival and axonal regeneration or neuronal death remain unclear, microglia and lymphocytes are thought to play a role in the maintenance of FMNs viability and thereby in supporting their ability to regenerate.

3.3.1 Microglial and astroglial reaction after FNA

Microglial activation in the FNA paradigm has been extensively investigated (Ha et al. 2006; Jinno and Yamada 2011; Kalla et al. 2001; Schoen et al. 1992). Within a few days after nerve transection, microglia become activated, proliferate and migrate, wrapping the axotomized FMNs (Svensson et al. 1994). Around 28 dpi, when the functionality of regenerated FMNs has been recovered, supernumerary activated microglia are gradually eliminated (Jones et al. 1997) to achieve a steady state, and remaining microglia start to adopt a resting-ramified morphology (Almolda et al. 2014).

In the earlier stages, wrapping microglia place their processes between the synaptic terminals and the somata and proximal dendrites of injured FMNs, a phenomenon described as "synaptic stripping" (Blinzinger and Kreutzberg 1968) and considered neuroprotective (Kreutzberg 1996a), as active disconnection of synaptic inputs by microglia may allow further axonal regeneration of axotomized FMNs (Perry and O'Connor 2010). In fact, after nerve axotomy in rats, excitatory glutamatergic synapses are preferably disconnected by microglia (Raslan et al. 2014). Considering that glutamate has excitotoxic effects for nerve cells (Mehta et al. 2013) and that blocking of the N-methyl-D-aspartate (NMDA) type glutamate receptor increases motor neuron survival after neonatal axotomy in rats (Mentis et al. 1993), this phenomenon of disconnection may be regarded as relevant for neuronal survival. It should be emphasized that disconnected terminals are not immediately phagocytosed by microglia but remain in the surroundings and, once axotomized FMNs regenerate the axon, these terminals can establish new connections with the FMNs (Navarro et al. 2007). However, this reconnection might be incomplete or present certain problems (Raslan et al. 2014).

In addition to synaptic stripping, microglial wrapping may play a function facilitating contact-dependent neuron–glia interactions that prevent neuron death and promote regeneration through the continuous supply of growth factors and other required molecules (Trapp et al. 2007). In fact, a neuroprotective role associated with this function of microglia after FNA is suspected as microglial cathepsin deficient mice (Hao et al. 2007) and TGF-β1 deficient animals (Makwana et al. 2007) have impaired microglial attachment to FMNs and subsequent higher neuronal death. However, in studies using hypoglossal nerve axotomy, other authors suggested that microglial wrapping may play a deleterious role since reduction of microglial activation by minocycline leads to increased motor neuron survival (Jinno and Yamada 2011; Yamada et al. 2011), (see the review from (Castellano et al. 2016), for a detailed discussion of the topic).

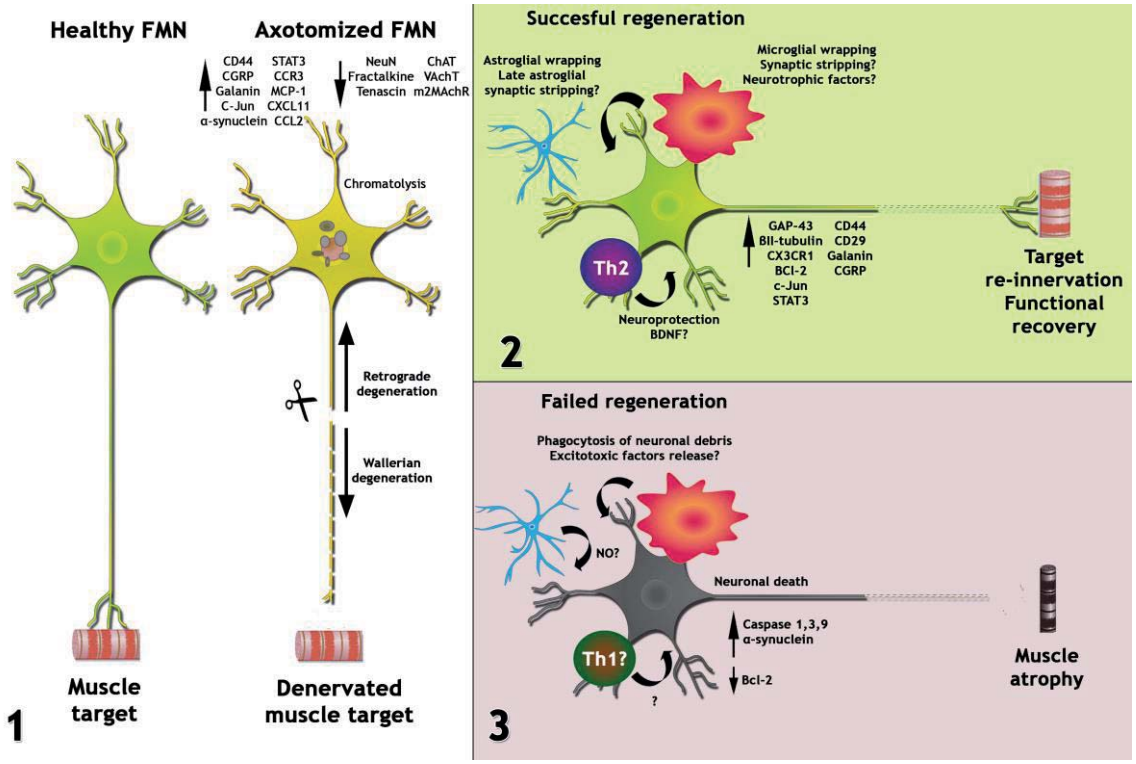

Figure 5. FMN responses and nerve regeneration after FNA. 1) Schematic representation of the main changes observed in the soma of healthy FMN (green) *versus* axotomized (yellow). **2 and 3)** Summary of the events and molecules described that might lead to successful (2) or failed (3) nerve regeneration after FNA are depicted.

Throughout all these processes, changes in morphology and distribution of microglial cells are accompanied by alterations in the levels of a broad range of factors, including increased expression of several cytokines (Streit et al. 2000), chemokines such as CX3CR1 (Harrison et al. 1998), cell adhesion molecules such as ICAM-1 (Werner et al. 1998; Werner et al. 2001), integrins such as CD18, CD11b, CD49d, CD49e, CD49f and CD11c (Galiano et al. 2001; Graeber et al. 1988a; Kloss et al. 1999; Moneta et al. 1993) and other pan markers of microglial activation, such as Iba1 (Raivich et al. 1998b) and CR3 complement receptors (Graeber et al. 1988a). Also, upregulation of the stem cell marker CD34 has been reported in axotomy-induced activated microglia (Ladeby et al. 2005). The fact that microglial cells up-regulate molecules related with both innate and adaptive immunity such as TLR2 (Wainwright et al. 2010), MHC-I and II (Ha et al. 2006; Petitto et al. 2003; Streit et al. 1989) Streit 1989, Ha 2006, (Bohatschek et al. 2004a) and the costimulatory molecule CD86 (Bohatschek et al. 2004b) points to their active role in the cross-talk with infiltrating leukocytes after FNA.

Along with microglia, also astrocytes surrounding the damaged FMN become reactive and hypertrophic (Aldskogius and Kozlova 1998; Graeber and Kreutzberg 1986) without undergo proliferation (Graeber et al. 1988b). As soon as 24h after injury, astrocytes synthetize *de novo* glial fibrillary acidic protein (GFAP) and reorganize their cytoskeleton (Tetzlaff et al. 2006). Importantly, GFAP expression can remain elevated for several weeks or months, depending on the severity of the lesion (Graeber and Kreutzberg 1986; Guntinas-Lichius et al. 1997; Laskawi and Wolff 1996).

Although, it has been shown that, in response to axotomy, reactive astrocytes have the potential for neural toxicity through NO (McElhaney et al. 1994), a role in neural repair and neuroprotection may also be expected (Sofroniew 2015). In fact, between 2-3 weeks after axotomy, activated astrocytes place stacks of thin astroglial lamellae on the surface of the axotomized FMNs, replacing the previously described wrapping microglia. Thus, the synaptic deafferentation of regenerating motor neurons is a long-lasting phenomenon, maintained by astrocytes at later time-points after injury (Graeber and Kreutzberg 1986; Kreutzberg 1996b). Moreover, it has been recently described that after axotomy, reactive astrocytes promote neuronal integrity and synapse recovery via STAT3-regulated thrombospondin-1 (TSP-1) expression (Tyzack et al. 2014).

3.3.2 Role of immune infiltrated cells after FNA

In contrast to other neuroinflammatory conditions, such as Alzheimer's disease, ischemia or EAE, that trigger invasion of circulating monocytes to the CNS parenchyma (Benakis et al. 2014; Greter et al. 2015; Mildner et al. 2009), infiltration of these leukocytes is absent in the FNA paradigm (Ajami et al. 2007).

Regarding lymphocytes, Raivich et al described in 1998 that infiltration of T-cells after FNA in mice occurs without BBB rupture in two different waves, one as early as 3 days post-injury (dpi) and a second at later time-points, i.e. 14 dpi. Infiltrated T-cells aggregate around axotomized motor neurons and activate microglia (Bohatschek et al. 2004b; Olsson et al. 1992; Raivich et al. 1998b). Although the lymphocyte recruitment after CNS injuries or diseases could be a double-edged sword, as both neuroprotective and neurotoxic actions are described (Baruch and Schwartz 2013; Rezai-Zadeh et al. 2009; Vanderlocht et al. 2007), specifically in the FNA paradigm T-cells seems to play a neuroprotective role. In fact, mice lacking functional mature T and B cells, in either the severe combined immunodeficiency (SCID) mutant mice or the recombination activation gene 2 knock-out (RAG2-KO) mice, have a dramatic increase in FMN death (Byram et al. 2004) that is rescued by reconstitution of these mice with functional T and B cells (Serpe et al. 1999). In 2003, the cells responsible for this neuroprotective effect were identified as CD4+ T-cells (Serpe et al. 2003) and later on, were accurately restricted to the Th2 subtype (Deboy et al. 2006; Xin et al. 2008). Although brain derived neurotrophic factor (BDNF) has been proposed as a candidate for Th2-secreted molecule playing a role in this immune-mediate neuroprotection (Xin et al. 2012), the mechanism by which Th2 cells confer neuroprotection to the FMN is still poorly unknown. Moreover, the specific subtypes of T-helper lymphocytes infiltrating the parenchyma of the facial nucleus and their specific contribution to the evolution of the lesion are still not well characterized.

3.3.3 Formation of cell clustering after FNA

One of the most relevant features observed after FNA is the formation of cell clusters around axotomized FMNs between 2-3 weeks after injury. The main part of authors assume that these clusters are composed by activated microglial cells that accumulate around damaged neurons and phagocytose neuronal debris (Moller et al. 1996; Raivich et al. 1998b). Some authors have even used the number of clusters as an indirect way to measure FMN death (Petitto et al. 2003). Nonetheless, the specific role of cluster formation in this paradigm has not yet been clarified. Considering that in addition to microglia, other cells such as recruited T-lymphocytes seem to form part of these clusters

(Raivich et al. 1998b), these cell accumulations have been proposed as the location of close interaction between T-lymphocytes, microglia and axotomized motor neurons (Byram et al. 2004; Raivich et al. 1998b). Supporting this theory, after FNA, clustering microglia has the ability to express MHC-II and costimulatory factors (Bohatschek et al. 2004b; Streit et al. 1999), two important family of molecules involved in the communication with T-cells. Microglial clusters have also been described in other experimental models of injury and disease where they have been associated with a great variety of functions. For example, after entorhinal cortex lesion, reactive microglia form clusters, which have been associated with both microglial proliferation (Dissing-Olesen et al. 2007) and with microglial interaction with myelin specific T-cells recruited into zones of axonal degeneration (Grebing et al. 2016). In multiple sclerosis, microglial clusters in pre-active lesions are known to express on one hand MHC-II, CD40 and CD86; suggesting that they are places of interaction with lymphocytes (Peferoen et al. 2015), and on the other hand high levels of IL-10, pointing them as regulators of inflammation (van Horssen et al. 2012).

3.3.4 Cytokines involved in the FNA outcome

As detailed in previous sections, cytokines play a key role in the cellular communication between microglia and other neural and immune cells after a wide range of CNS insults (Appel et al. 2011; Finsen and Owens 2011; Gao and Ji 2010; Ransohoff and Brown 2012; Suzumura 2013) and therefore, an important role could be expected to be played after FNA. In fact, concomitant to the neuronal degenerative changes, glial activation and lymphocyte recruitment described above, FNA in mice leads to a biphasic induction of pro-inflammatory cytokines:

1) a rapid, around 1-3 dpi, up-regulation of IL-6, IL-1β, TNF-α, IFN-γ and macrophage colony-stimulating factor (MCSF) receptor (Bohatschek et al. 2004b; Jones et al. 2000; Raivich et al. 1998a)

2) a second phase, around 14 dpi, coinciding with the peak of neuronal cell death and lymphocyte infiltration, with increased production of IL-1β, TNF-α and IFN-γ (Raivich et al. 1998b).

Regarding production of anti-inflammatory cytokines after FNA in mice, available information is very sparse; IL-10 does not change after axotomy (Xin et al. 2011) and IL-4 decreases at 7 dpi after facial nerve crush (Armstrong et al. 2008).

Over the years, researchers have attempted to unravel the specific role of all these cytokines after FNA using knock-out mice. In some of these studies, pro-inflammatory cytokines are thought to have a neurodegenerative function and to increase glial reaction and lymphocyte recruitment. For instance, IL-6 deficient mice show weak astroglial and microglial activation (Klein et al. 1997), an important decrease in lymphocyte recruitment and impairment in nerve regeneration (Galiano et al. 2001). In IL-15 deficient animals there are decreased MHC-II+ microglial cells and less number of infiltrated T-cells (Huang et al. 2007). Moreover, absence of IL1R1, TNFR1 or TNFR2 all caused a reduction in microglial activation and lymphocyte infiltration and combined deletion of TNFR1 and TNFR2 prevented FMN death (Bohatschek et al. 2004a; Raivich et al. 2003; Raivich et al. 2002). Importantly, absence of IFN-γ signaling in both IFNγR1KO and IFNγKO mice did not have an

effect on neuronal survival (Bohatschek et al. 2004b; Deboy et al. 2006; Lidman et al. 2002). Anti-inflammatory cytokines such as TGF-β, IL-4 and IL-10 have been proposed to play a neuroprotective role after FNA since their absence leads to increased neuronal death (Deboy et al. 2006; Makwana et al. 2007; Xin et al. 2011).

Altogether, these studies highlighted the important role that both pro-inflammatory and anti-inflammatory cytokines may play in regulating the evolution and the outcome of FNA. However, as already mentioned most of the knowledge emerges either from KO mice models or from transgenic mice for a specific cytokine in the whole organism making very difficult to discriminate whether the changes observed are due to direct effects in the CNS or to the functions played by the cytokine in the periphery. In this context, the use of transgenic animals producing (either IL-6 or IL-10) within the CNS will be a very useful tool to elucidate the role played by these molecules in regulating the neuronal, glial and immune responses associated with a peripheral nerve injury.