Maria Guedes Vaz Pinto

Licenciada em Biologia

BASHY dyes for characterization of demyelination and possible microglia targeting

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Adelaide Maria Afonso Fernandes Borralho,
Professora Doutora, Faculdade de Farmácia da
Universidade de Lisboa

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Characterization of demyelination and possible microglia targeting using BASHY dyes

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To R. Mayer

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RESUMO

O processo de desmielinização, que se caracteriza pela perda das camadas lipídicas da mielina que envolvem o axónio, é uma das características patológicas de muitas doenças neurodegenerativas como a Esclerose Múltipla. Este processo inicia-se com a descompactação inicial das camadas de mielina seguida de intensa vesiculação desta e culminando na degradação total da membrana sob a forma de detritos de mielina, estes que serão depois removidos e fagocitados pela microglia. Assim, estudar as alterações que ocorrem na mielina bem como todo o processo de desmielinização é essencial para compreender as vias de processamento da mielina numa tentativa de promover a remielinização. Recentemente, estudos demostraram que moléculas fluorescentes conjugadas com o ácido borónico, as sondas BASHY, apresentavam especificidade apenas para agregados lipídicos não polares, tendo sido por isso consideradas potenciais ferramentas para a caracterização da desmielinização e para o *targeting* específico de células envolvidas neste processo.

Este trabalho teve como objetivo avaliar a capacidade de marcação das sondas BASHY num modelo *ex vivo* de desmielinização. Para além disto, fomos igualmente estudar o seu potencial como veículo de entrega de fármacos diretamente para a microglia. Tendo sido utilizada no contexto deste trabalho a molécula anti-inflamatória galantamina, sendo esta conjugada com a sonda BASHY (FFS2B).

Inicialmente, observámos que a sonda BASHY marcava detritos de mielina ricos em colesterol, que se acumulam ao longo do processo de desmielinização. Seguidamente, devido à especificidade para estes agregados de mielina, verificámos que a sonda BASHY era preferencialmente internalizada pela microglia. Verificámos, também, que apesar do composto FFS2B apresentar a mesma capacidade de *targeting* para a microglia que a sonda BASHY, nas amostras onde induzimos a desmielinização não observámos o efeito esperado da galantamina. Contudo, a incubação com a galantamina não ligada mostrou efeitos mais benéficos do que quando ligada à sonda BASHY, melhorando a sinaptogénese e prevenindo a perda de oligodendrócitos.

Em suma, os nossos resultados permitem-nos concluir que a sonda BASHY poderá vir a ter um papel importante como biomarcador de estados tardios do processo de desmielinização bem como da microglia ativada com elevada capacidade fagocítica. Mais ainda, embora não tenhamos observado os resultados esperados com a molécula FFS2B, a bioconjugação das BASHY com outras moléculas poderá ser uma nova estratégia terapêutica não só para impedir a desmielinização mas também para potenciar o processo de remielinização, através da modelação da microglia.

Palavras Chave: Sondas BASHY, Desmielinização, “Targeting” da microglia, Neuroinflamação, Marcador de microglia ativada, Galantamina

ABSTRACT

Demyelination or the loss of lipid-rich myelin layers surrounding the axons, is a pathological feature of many neurodegenerative disorders like Multiple Sclerosis. Myelin degradation starts with an initial membrane decompaction followed by intense vesiculation and culminating in full destruction in the form of vesiculated myelin debris, which will be posteriorly cleared by microglial cells. Indeed, studying myelin alterations and understanding the demyelinating process is crucial to comprehend myelin processing pathway and promote remyelinating-based therapies. Recently, fluorescent Boronic acid-based complexes - BASHY dyes – showed high specificity for the nonpolar lipid aggregates, therefore envisioned to be a promising tool to characterize demyelination and possibly target specific cells that play a critical role on ongoing demyelination. Here, we aimed to address BASHY labeling in the context of demyelination using an *ex vivo* cerebellar slice model. Moreover, we also asses BASHY potential as a targeting delivery system for myelin-phagocytes as microglia, using BASHY conjugated with the anti-inflammatory molecule galantamine (FFS2B).

Firstly, we observed that BASHY dye stains cholesterol-rich myelin debris that accumulate over ongoing demyelination. Moreover, due to this specificity for myelin vesiculated aggregates, BASHY was preferentially staining microglial cells, the major phagocytes of myelin debris. Furthermore, although FFS2B showed an equal capacity as BASHY alone to target microglia, in demyelinated slices the potential action of galantamine was not observed. Instead galantamine alone showed a more beneficial effects than if bound to BASHY dye, improving synaptogenesis and preventing OL's loss.

Overall, our results suggest that BASHY molecule may become a useful biomarker for later stages of demyelination and even for activated phagocytic microglia. Additionally, although FFS2B did not showed the expected effects, our study open new therapeutic strategies, by bioconjugation of BASHY with other molecules, to prevent demyelination and enhance remyelination through microglial modulation.

Keywords: BASHY dyes, Demyelination, Microglia targeting, Neuroinflammation, Microglia activated biomarker, Galantamine

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Abbreviations

AChE	Acetylcholinesterase
AP	Action Potentials
ApoD	Apolipoprotein D
Arg1	Arginase 1
ATP	Adenosine triphosphate
BASHY	Boronic acid Salicylidenehydrazone
BBB	Blood brain barrier
CNP	2',3'-cyclic nucleotide phosphodiesterase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CR3	Complement receptor 3
FGF	Fibroblast growth factor
FIZZ1	Found in inflammatory zone 1
GFAP	Glial fibrillary acidic protein
Iba1	Ionized calcium binding-adaptor molecule-1
IFN	Interferon
IGF-2	Insulin growth factor-2
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPL	Intraperiod line
K^+	Potassium
LIF	Leukemia inhibitory factor
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MCT	Monocarboxylate transporters
MDL	Major dense line
MHC	Major histocompatibility complex
MOG	Myelin-oligodendrocyte glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis

Na^+	Sodium
NF	Neurofilaments
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG2	Neuron-glia antigen 2
NLRP3	NLR family pyrin domain containing 3
NR	Nile red
Nr-CAMs	Neuronal cell-adhesion molecules
OCSC	Organotypic cerebellar slice cultures
OL	Oligodendrocytes
OPC	Oligodendrocyte progenitor cells
ORO	Oil red O
PBS	Phosphate buffer
PDGF	Platelet-derived growth factor
PET	Positron Emission Tomography
PFA	Paraformadehyde
PIP2	Phosphatidylinositol-4,5-bisphosphate
PLP	Proteolipid protein
PPAR	Peroxisome proliferator-activated receptor
PSA-NCAM	Polysialylated-neural cell adhesion molecule
PSD	Postsynaptic protein density
qRT-PCR	Quantitative Real-Time PCR
ROS	Reactive oxygen species
RT	Room Temperature
SVZ	Subventricular zone
SYP	Synaptophysin
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TREM2	Triggering Receptor Expressed On Myeloid Cells 2
TSPO	Translocator protein 18
WT	Wild Type

I- Introduction

1. Myelin: an overview

The term myelin was early introduced by Virchow, even though little or nothing was known about its origin, structure, and function at the time. Only around 1954, based on Betty Ben Geren studies, myelination was seen as a result of complex neuron-glia interactions rather than a lipid structure secreted by the axons to the extracellular space, as initially postulated (Simons and Nave, 2015;Lee et al., 2012a). Myelin was then characterized as a specialized extended membrane of either Schwann cells or Oligodendrocytes (OLs) – in the Peripheral or Central Nervous System (CNS), respectively. By encircling the majority of the axon, myelin develops into a highly stable multilamellar layer mostly compact yet alternating with fewer uncompacted areas disposed preferentially at the edges of the myelin-covered axonal segment (internode) (Bunge, 1968;Hildebrand. C, 1993;Aggarwal et al., 2011a;Paz Soldan and Pirko, 2012). This fascinating biological acquisition was firstly described in the cartilaginous fishes and quickly evolved to become essential in the formation and function of the Nervous System (reviewed by(de Bellard, 2016). Indeed, once enveloping the axons, myelin allowed a marked increase in nerve conduction speed, while becoming, at the same time, vital in maintaining axonal metabolic support, integrity, and stability. Notably, its importance is further highlighted by myelin-associated disorders in which demyelination or the complete loss of myelin sheaths besides disrupting neuronal conduction, it also leads to axonal degeneration and neurotoxicity - the leading cause of permanent neurological disability in demyelinating diseases (Trapp and Nave, 2008). Therefore, myelin function preservation is key to the maintenance of a healthy nervous system, which is primarily dependent on the presence and correct assembly of its specific and remarkable lipid and protein constituents.

1.1 Myelin Composition: Major lipids and Proteins

Myelin membrane is unique among other plasma membrane equivalents due to its higher than normal lipid content - 70% to 80% of the dry weight – where cholesterol is the major constituent, essential for myelin sheath production and assembly (Rasband and Macklin, 2012;Mathews and Appel, 2016). Besides cholesterol, cerebroside is also strongly associated with CNS myelin, and although not essential for myelin formation, its presence is crucial for myelin's primary function as a highly stable axonal insulator (Faria, 2017). Moreover, this specialized membrane is also composed of high levels of sphingolipids, saturated long-chain fatty acids together with a considerable amount of plasmalogen and sulfatide, among others (Eckhardt, 2008;Chrast et al., 2011).

Regarding myelin-associated proteins, they significantly differ between the CNS and Peripheral Nervous System myelin. For this study, we will focus on CNS myelin where Myelin Basic Protein (MBP) and Proteolipid Protein (PLP) make up 60% to 80% of the total protein content (Rasband and Macklin, 2012)

MBP is the only myelin-associated protein known to be vital and crucial for myelin biogenesis (Boggs, 2006). Among the different MBP isoforms, the most abundant one within human myelin has only five – 1B, 3, 4, 5 and 7 – out of seven exons, with approximately 18,5kDa. Importantly, MBP is an arginine-rich protein and due to that**,** has an extreme net positive charge which allows it to bring closer together the two opposing negatively charged cytoplasmic surfaces of myelin membrane. Indeed it is mostly known for its involvement in myelin intracellular compaction (see section 2.3)**,** though other additional roles have also been observed such as mediating cytoskeleton interactions, signaling and plasma membrane reorganization (Harauz et al., 2009;Boggs, 2006;Rasband and Macklin, 2012;Harauz et al., 2004;Fitzner et al., 2006). PLP, on the other hand, is the most abundant protein within the CNS myelin and, unlike MPB, is not essential for myelination to occur (Luders et al., 2019). However is necessary to ensure the correct apposition of the membranes at the myelin's exoplasmic side, thus stabilizing myelin extracellular compaction (see section 2.3) (Klugmann et al., 1997;Duncan et al., 1989;Boison and Stoffel, 1994).

Another myelin-specific protein is Myelin-oligodendrocyte glycoprotein (MOG), a small protein localized on the outside surface of myelin sheaths and together with MBP has been a protein of interest**,** once they are both strongly associated with immune responses and myelin degradation (Genain et al., 1999;Lalive et al., 2006;Linington et al., 1988). Finally, Myelin-associated glycoprotein (MAG)**,** as well as 2',3'-cyclic nucleotide phosphodiesterase (CNP) protein are also important constituents of CNS myelin, yet present in minor concentrations and both excluded from compacted regions. Particularly, CNP protein not only prevents MBP-mediated compaction but also favors the maintenance of intact noncompact cytoplasmic areas described in detail in section 2.3 (Snaidero et al., 2017). Furthermore, both MAG and CNP are also believed to play important roles as mediators of neuronal-glia signals providing greater axonal stability and ensuring efficient myelination (Lappe-Siefke et al., 2003;Gravel et al., 2009;Lee et al., 2005).

1.2 Biological functions of myelin

1.2.1 Myelin-mediated saltatory conduction

Neurons - the basic units in the Nervous System - receive millions of either chemical or physical stimuli, transforming them rapidly into electric chain that flows through their axonal membrane to be further transmitted to another neuron(s) (Faria, 2017). Any increase in the speed of nerve conduction is an obvious priority once it represents an evolutionary advantage allowing organisms to respond faster to the information they receive from the outside environment. Therefore, the Nervous System has evolved two mechanisms for increasing the conduction speed of the electrical impulse; either by increasing axonal diameter - as nerve impulse speed augments in proportion to the square root of the interior diameter - or through myelin ensheathment (Hartline and Colman, 2007). Remarkably, by both decreasing the transverse capacitance between the

inside and the outside of the axonal membrane and preventing electric current from leaking out, myelin ensheathment enables faster action potential propagation along the axons - via saltatory conduction - without the need of increasing axonal diameter, thus conserving space and energy. Ultimately becoming not only a critical evolutionary acquisition and an adaptative advantage but also making possible the development of highly complex and large body-sized vertebrates (Hartline and Colman, 2007;Salzer and Zalc, 2016;Nave, 2010;Sherman and Brophy, 2005;Funfschilling et al., 2012)

Besides being a requirement for proper and faster transmission of nerve impulses, additional myelin functions are also of great matter and importance being those of providing metabolic support and axonal integrity, as discussed below (Lee et al., 2012b;Bando et al., 2008).

1.2.2. Axonal metabolic support

Compact myelin sheaths while covering almost the entire axons, insulate them electrically, which is crucial for speed up the conduction of action potentials. However, as a result, it also isolates them from the extracellular milieu limiting the access of nutrients and metabolites (Simons and Nave, 2015). Even though myelination tends to reduce the energy requirements for action potential (AP) propagation, axons still need adenosine triphosphate (ATP) for axonal transport, AP regeneration, and axonal metabolism. Thereby, to overcome this problem, axons naturally became metabolically dependent on myelinating glia. Indeed, specific transporters, monocarboxylate 1 (MCT1) and 2 (MCT2) - localized in the innermost myelin layer and in the axonal membrane, respectively - ensure the delivery of metabolites such as pyruvate and lactate from the OL cell body into neuronal axons to be utilized for mitochondrial ATP production (Funfschilling et al., 2012;Lee et al., 2012b). After all, myelin surrounding axons provide an appropriate environment for axonal survival, supply the necessary nutrients thus maintaining axonal homeostasis (Bando et al., 2008).

1.2.3. Axonal integrity and function

Myelin is long known to be involved in the maintenance of axonal integrity and support. Particularly loss of myelin-specific proteins PLP, MAG and CNP, rather than myelin membrane itself, are enough to cause axon degeneration (Griffiths et al., 1998;Lappe-Siefke et al., 2003;Nave and Trapp, 2008;Yin et al., 1997). Evidence for this fact came from studies using the respective null mutants. For instance**,** PLP-absence from myelin has been associated with axonal swellings, abnormal axonal microtubules arrangement and impaired retrograde axonal transport (Yin et al., 2006;Yin et al., 2016;Garbern et al., 2002;Griffiths et al., 1998). MAG-null mice on the other hand, have a decrease in neurofilament (NF) phosphorylation which consequently reduces NF spacing and axonal caliber causing inevitably degeneration of myelinated fibers, suggesting a possible role for MAG protein in the modulation the axon cytoskeleton by regulating NF phosphorylation (Yin et al., 1997;Hirokawa et al., 1984;de Waegh, 1992;Nave, 2010). Finally, CNP-null mice also develop axonal swellings early in life. Once associated with non-compact regions where most of axonal-glia interactions take place, CNP is thought to provide axonal

support by coordinating communications between OLs and its related axons (Rasband et al., 2005;Trapp et al., 1988;Lappe-Siefke et al., 2003).

This being said, it is important to highlight the necessity for the correct assembly of these proteins through the myelinating process in order to maintain such stringent interplay with axonal segments, critical for preventing severe axonopathies and neuronal loss.

2. Myelination in the Central Nervous System (CNS)

Myelination, the process where OL-extended plasma membranes surround the axonal segments, is an ongoing event from birth until human adulthood (Miller et al., 2012). It is probably the best example of intimate cellular interaction and a process of enormous importance for the nervous system function and repair, as the absence or deregulation of myelination directly results in neurodegenerative disorders. As expected, it is a multistep process rather complex and tightly regulated, which include the following sequence of events: 1) OL proliferation, migration, and differentiation; 2) membrane outgrowth and axonal wrapping; 3) trafficking of membrane components and 4) myelin compaction and axonal polarization (Watkins et al., 2008;Lee et al., 2012a).

2.1. Oligodendrocytes: proliferation, migration and differentiation

OLs were first discovered by Robertson in 1899 but only named and characterized later, by Rio Hortega, as the myelinating cells of the vertebrate CNS (as reviewed by (Jakovcevski and Zecevic, 2005). OLs derive from Oligodendrocyte progenitor cells (OPC), highly proliferative and motile cells which are characterized by the expression of Platelet-derived growth factor receptor-α (PDGFRα) and proteoglycan neuro-glia antigen (NG)-2, both markers of the early stages in developmental maturation of myelinating cells (Barateiro and Fernandes, 2014;Back et al., 2005;Rakic and Zecevic, 2003;Rivkin et al., 1995;Stallcup and Beasley, 1987). Strikingly, until reaching the mature/myelinating state, OPC suffer a multi-step transition identified by differences in their morphology, expression of specific markers and migratory capacities. However, not all OPCs differentiate into myelin-forming cells. Some actually persist in the adult brain in their precursor and proliferative state having a fundamental role in myelin turnover, regeneration, and remyelination (Dawson et al., 2003;Hughes et al., 2013).

OPC arrive from two different sources before establishing themselves within the white and gray matter. The first wave generates in the subventricular zone (SVZ) and is then joined and overtaken by a second dorsally-derived OPC population (Vallstedt et al., 2005;Kessaris et al., 2006). The way migration is regulated precisely is still unknown. It is only known to be mediated by some cell surface molecules and growth factors – semaphorins, chemokine CXCL1, fibroblast growth factor (FGF) and PDGF – and also by contact-mediated mechanisms with extracellular matrix proteins (Jarjour, 2003;Frost et al., 1996;de Castro and Bribian, 2005;Wang et al., 1994;Redwine et al., 1997). Importantly, all these populations although they can be intense space

competitors, they are functionally redundant, essential for restoring normal distribution of OPCs any time one source is destroyed (Kessaris et al., 2006).

Later on, OPCs differentiate into preoligodendrocytes and further into immature OLs highly ramified and with broader processes (Barateiro and Fernandes, 2014). Immature OLs extend and retract their branches towards an axonal membrane and the moment OL processes stabilizes at the right axonal surface it triggers the developing switch to mature/myelinating OL followed by initiation of myelin assembly (Czopka et al., 2013). The exact mechanism behind OPC development in the CNS is still unknown. Only that is negatively regulated by jagged1, the axonal ligand of OL receptor NOTCH1 (Barres, 2008;Watkins et al., 2008;Zhang et al., 2009;Wang et al., 1998).

Finally, immature OL maturation into myelinating glia is marked by the enlargement of membrane processes, dramatic molecular rearrangements – of interest the local enrichment of phosphatidylinositol-4,5-bisphosphate (PIP_2) and phosphatidylinositol-3,4,5-trisphosphate (PIP_3) in the glial membrane - and by the expression of myelin-specific proteins, such as CNP, MBP, MAG and PLP (Goebbels et al., 2010;Barateiro and Fernandes, 2014). Mature/myelinating OLs, will further extend their plasma membranes and initiate myelin assembly forming multiple segment of myelin either along a single or multiple axonal fibers (Paz Soldan and Pirko, 2012).

2.2. Membrane outgrowth, axonal wrapping and cell polarization

The onset of myelination is tightly regulated. Czopka and colleagues even observed that all different axons are myelinated within the same brief window of time - within five hours after having formed the first myelin layer, suggesting the existence of some neuronal and axonal signals regulating and controlling the beginning of myelin ensheathment (Czopka et al., 2013;Barres, 2008;Watkins et al., 2008). Indeed, electrically active neurons ready to be involved by the multilamellar myelin sheath release adenosine and ATP known to mediate neuron-glia communications thus promoting myelination. At the same time, polysialylated-neural cell adhesion molecule (PSA-NCAM) expressed at the surface of the axonal segment, which prevent premature myelination, is also down-regulated in this same window of time, all contributing to such coordinated event (Watkins et al., 2008;Stevens et al., 2002;Barres, 2008;Charles et al., 2000).

Various models have been proposed trying to explain myelin assembly during development (Sobottka et al., 2011;Snaidero et al., 2014). Using a combination of several techniques - mainly *in vivo* live-imaging and high-pressure freezing - Snaidero and colleagues proposed the one model accounting both temporal and three-dimensional resolution. According to their findings, OLs wrap their processes around the axonal surface through two coordinated movements (**Figure I.1**). First, in a continuous movement - in a triangle-like shape - the inner tongue wraps around the axon underneath the previously deposited membrane layers. At the same time, myelin layers suffer lateral extension, with the inner or periaxonal layers having shorter lateral extensions when compared with the outermost or abaxonal ones. Notably, such non-

random way of membrane outgrowth allows each tip of myelin sheaths to be always in contact with the axonal surface, forming the noncompacted paranodal loops (see in detail in section 1.2.4) (Snaidero et al., 2014). PI3K/AKT/mTOR is one of the key signaling pathways involved in the regulation of myelin growth, whereas the number of myelin sheaths is regulated by the tyrosine-kinase Fyn (Flores et al., 2008;Tyler et al., 2009;Goebbels et al., 2010;Czopka et al., 2013).

Strikingly, concomitant with myelin assembly there is an intense cell polarization giving rise to this myelin membrane with markedly different protein composition when compared to the one of OL's plasma membrane (Baron and Hoekstra, 2010). As an example of OL's tight intracellular trafficking regulation, MBP mRNA itself is transported and targeted directly to the innermost myelin membrane being only synthesized locally to prevent aberrant effects and avoid unwanted adhesive interactions with other membranes (Muller et al., 2013;Ainger et al., 1993;Snaidero et al., 2014). PLP is also tightly regulated in time and space, being this protein accumulated in late endosomes in pre-myelinating OLs until myelination starts, only then transported and released to the myelin membrane (Simons and Trotter, 2007;Baron and Hoekstra, 2010).

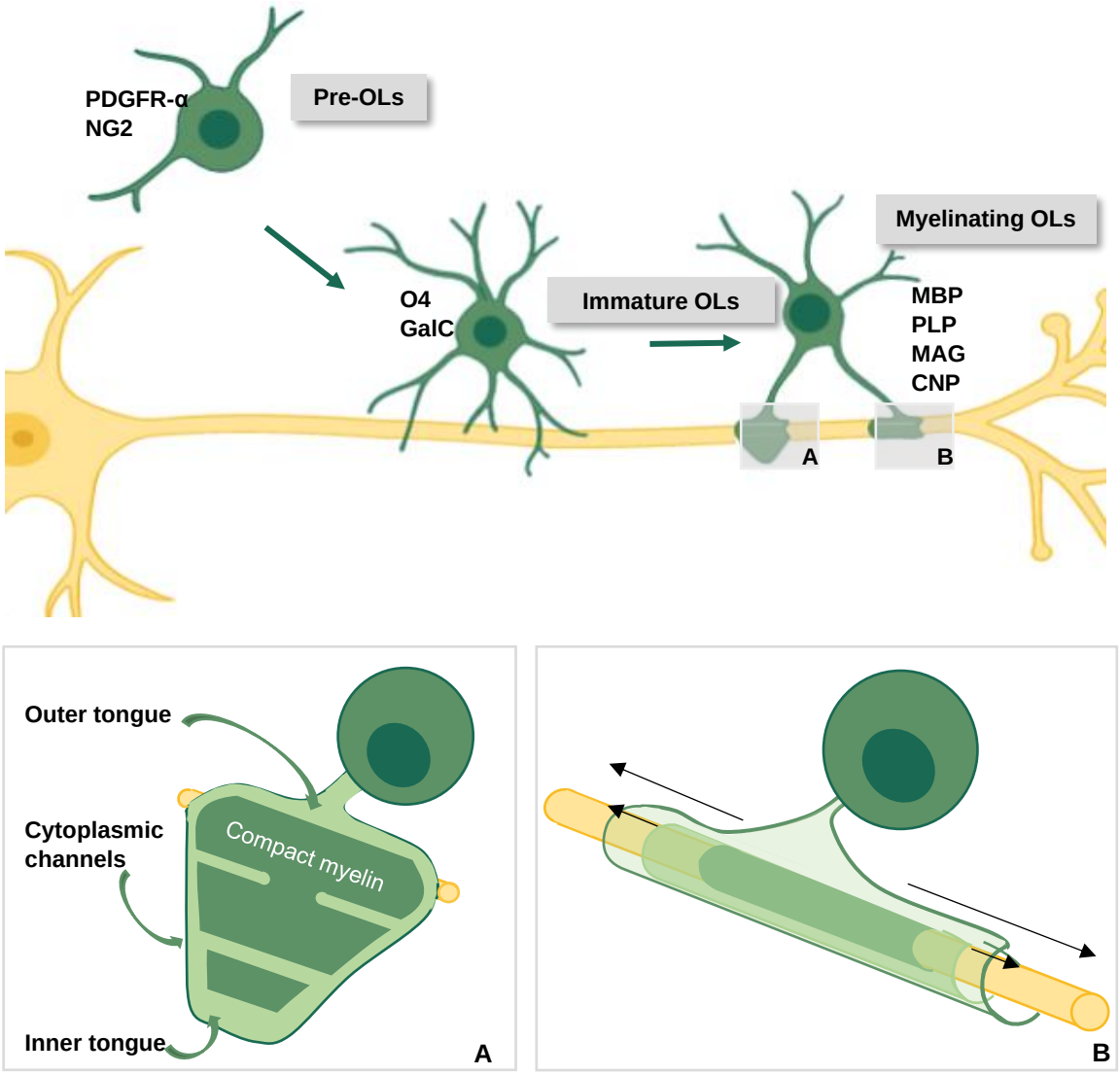


Figure I. 1. Schematic representation of oligodendrocyte differentiation, myelin outgrowth and wrapping. Differentiation into mature/myelinating oligodendrocytes (OLs) is regulated by the interaction of OPCs with axonal signals and other extrinsic factors, and marked by the expression of myelin-specific proteins – myelin basic protein (MBP), proteolipid protein (PLP), 2',3'-cyclic nucleotide phosphodiesterase (CNP) protein and myelin-associated glycoprotein (MAG) - together with the loss of other markers characteristic of early stages of differentiation, platelet-derived growth factor (PDGFR- α), neuron-glia antigen 2 (NG2), a sulfatide recognized by O4 antibody and galactocerebroside (GalC) and Myelinating OLs extend and flat their processes in a triangle-like shape and wrap them around the axonal surface. Unwrapped myelin scheme shows the compact (dark green) and non-compact regions (light green) with cytoplasmic spaces connecting the inner tongue with the OL soma (A). Upon myelination, the inner tongue grows underneath the previous stablished myelin membrane with concomitant lateral extension of myelin layers towards the nodes (B).

2.3. Myelin compaction

Myelin is an almost fully compacted membrane with compact regions alternating between electron-dense layers or major dense lines (MDL) and electron-light layers or intraperiod lines (IPL) (**Figure I.2**). To form these specific structures, the already formed myelin layers undergo a two-phase process of compaction. While MDL consist in a dense apposition of the cytoplasmic

leaflets, IPL result from the compaction of the extracellular layers of myelin membrane (Simons and Nave, 2015;Garcia-Mateo et al., 2018;Bakhti et al., 2014).

MBP plays a key role mediating MDL formation or myelin intracellular compaction corroborated by its specific distribution, barely found in uncompacted regions but increasingly distributed throughout compacted ones (Chernoff, 1981;Weil et al., 2016). As a result of the electrostatic forces formed between PIP_2 and MBP's positively charged residues, this protein brings together the two adjacent cytoplasmic leaflets (Snaidero et al., 2014). Moreover, based on MBP physicochemical properties, it is self-repulsive in solution but when bound to membranes MPB neutralizes its positive charge switching to a self-associating state and forming a polymeric network. Therefore, acting as a molecular zipper, MBP drives protein segregation and extrusion and closes the two adjacent membrane bilayers into a compacted myelin membrane (Aggarwal et al., 2011a;Aggarwal et al., 2011b;Bakhti et al., 2013;Harauz et al., 2004;Smith, 1992;Kattnig et al., 2012;Aggarwal et al., 2013).

Both intracellular and extracellular correct compaction is essential to achieve the final goal of producing a stable and fully compact myelin sheath surrounding the axons. Although less studied, recent findings observed compaction of the extracellular sides of myelin to be dependent on an efficient lysosomal-dependent myelin recycling system. Two major proteins, Apolipoprotein D (ApoD) and PLP, are crucial in the process. Indeed, ApoD by ensuring the functional integrity of the lysosomes indirectly assures the removal of the extracellular glycocalyx – limiting step for PLP function. Once removed the glycocalyx from the outer myelin surface, PLP is then able to bring the extracellular layers together, resulting in the formation of a zipped IPL (Rosenbluth et al., 2006;Garcia-Mateo et al., 2018;Bakhti et al., 2014).

Besides compact regions, there are few areas or cytoplasmic spaces that remain uncompacted throughout life. Noncompact myelin is mostly restricted to the outer- an innermost edges of the myelin sheath - as well as to the lateral or paranodal regions. Occasionally, cytoplasmic-rich longitudinal incisures can also be observed within the compact membrane and together, these noncompact areas assure cytoplasmic continuity from the OL soma to the innermost myelin layer. Furthermore, initially neglected, non-compact regions are now characterized as highly dynamic and metabolically active regions, facilitating the trafficking of myelin-specific proteins - PLP-rich vesicles and MBP mRNA – and the delivery of essential components necessary to ensure all the metabolic, transport and stability functions (Snaidero et al., 2017;Snaidero et al., 2014;Velumian et al., 2011). How these structures are preserved - despite all MBP-dependent compaction forces - depends on the presence of CNP protein. Noncompact myelin wraps are enriched in CNP which specifically delays and prevents myelin compaction. Indeed, while the overexpression of CNP results in areas lacking myelin compaction, in the absence of CNP, not only myelin compaction is fastened but there is also a decrease in the number of cytoplasmic spaces accompanied by axonal swellings and spheroid formation (Edgar et al., 2009;Lappe-Siefke et al., 2003;Yin et al., 1997). Consistent with these findings, Snaidero and colleagues proposed that, by interacting with F-actin, CNP was able to prevent myelin

compaction, working as an MBP antagonist, both competing to regulate cytoplasmic compartments formation within myelin sheaths (Snaidero et al., 2017).

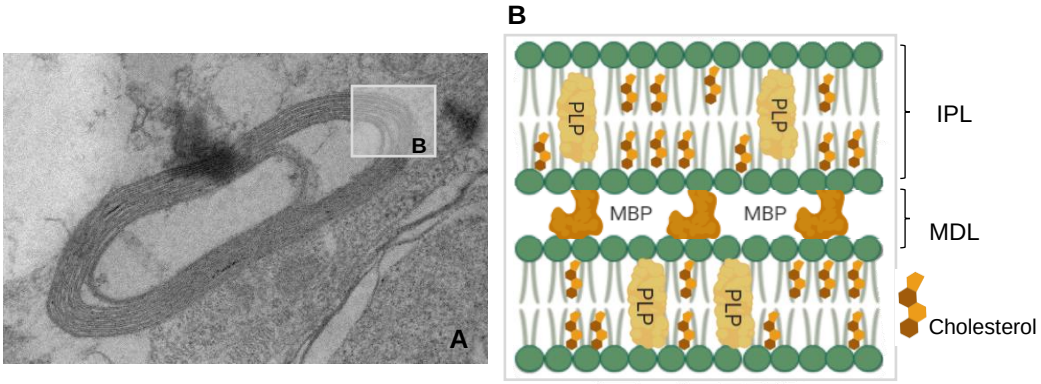


FIGURE I. 2. Schematic representation of myelin membrane structure and composition. (A) Myelin ultrastructure in the mouse cerebellar organotypic slice culture with the axon in the center surrounded by compact myelin layers showing dense lines, indicative of the major dense line (MDL), separated by less dense lines, indicative of intraperiod line (IPL). (B) Schematic representation of myelin hypothetical distribution of lipids (80%) and proteins (20%) with cholesterol, the main lipid of myelin membranes, being highly distributed throughout the lipid membrane. Myelin compact regions – IPL and MDL – together with two major proteins - proteolipid protein (PLP) and myelin basic protein (MBP) - are illustrated. PLP brings the two cytoplasmic leaflets together resulting in the formation of the IPL whereas MBP is essential for the fusion of the adjacent membrane bilayers to form the IPL.

2.4. Axonal domains

Unlike unmyelinated fibers, myelin allows axonal ion channels, proteins, and cytoskeleton molecules to acquire a specific organization giving rise to different polarized axonal domains – Internode, the node of Ranvier, paranode and juxtaparanode regions (**Figure I.3**) (Inouye et al., 2014;Salzer, 2003). The internode is the entire axonal region covered by compact myelin sheath - nearly 99% of the total axonal length (Salzer, 2003). Here, MAG by interacting with sialylated glycoconjugates within the axon regulates the spacing and adhesion between myelin and the axon (Trapp, 1990;Kelm et al., 1994;Sadoul et al., 1990).

Between each myelinated segment or internode, axons are exposed to the extracellular environment through periodic gaps called nodes of Ranvier where voltage-gated sodium (Na+) channels cluster (Boiko et al., 2001;Waxman and Ritchie, 1993). Na^+ channels, firstly dispersed along the axon, tend to cluster around the edges of OL processes while myelination is taking place. Later on, the lateral extension of those processes ultimately relocates these Na^+ channels clusters so they can reach their final destination within the internodal gaps. Otherwise, in the absence of OLs or myelination, Na^+ channels are dispersed along the axon (Rasband et al., 1999;Dugandzija-Novakovic et al., 1995). Importantly, such ion channels are subsequently stabilized by nodal-specific adhesion molecules and cytoskeletal proteins such as neurofascin-186, neuronal cell-adhesion molecules (Nr-CAMs), AnkyrinG, spectrin and tenascin R or C (Srinivasan et al., 1998;Davis et al., 1996;Weber et al., 1999;Bennett and Lambert, 1999;Komada

and Soriano, 2002). Sodium channels rearrangements restrict action potentials to these nodes, imperative for faster and efficient saltatory nerve conduction.

Right before the nodes, internode layers extend as non-compacted cytoplasmic-filled regions – the paranodal loops. Here, through septate junctions, the contact between OLs and axons is stabilized by the adhesion proteins neurofascin-155, on the glial surface, and axonal Caspr and Contactin (Salzer et al., 2008). Therefore, paranodes work as a lateral membrane diffusion barrier limiting the diffusion of axolemmal proteins and maintaining Na^+ clusters, separating them from the voltage-gated potassium (K^+) ones – restricted to the juxtaparanodal regions (Rosenbluth, 1976;Boyle et al., 2001;Rios et al., 2003;Rosenbluth et al., 2003). These latter are small regions localized beyond the paranodal junction and flanked by the internode. Potassium clustering, due to the presence of the juxtaparanodal complex containing Caspr2 and contactin-2 proteins, is vital to maintain internodal resting potential and essential to prevent re-entrant excitation (Traka et al., 2003;Vabnick et al., 1999;Chiu and Ritchie, 1984).

Axonal differentiation and specific organization into different regions/domains throughout myelination are a requisite for these fibers to conduct impulses via saltatory conduction.

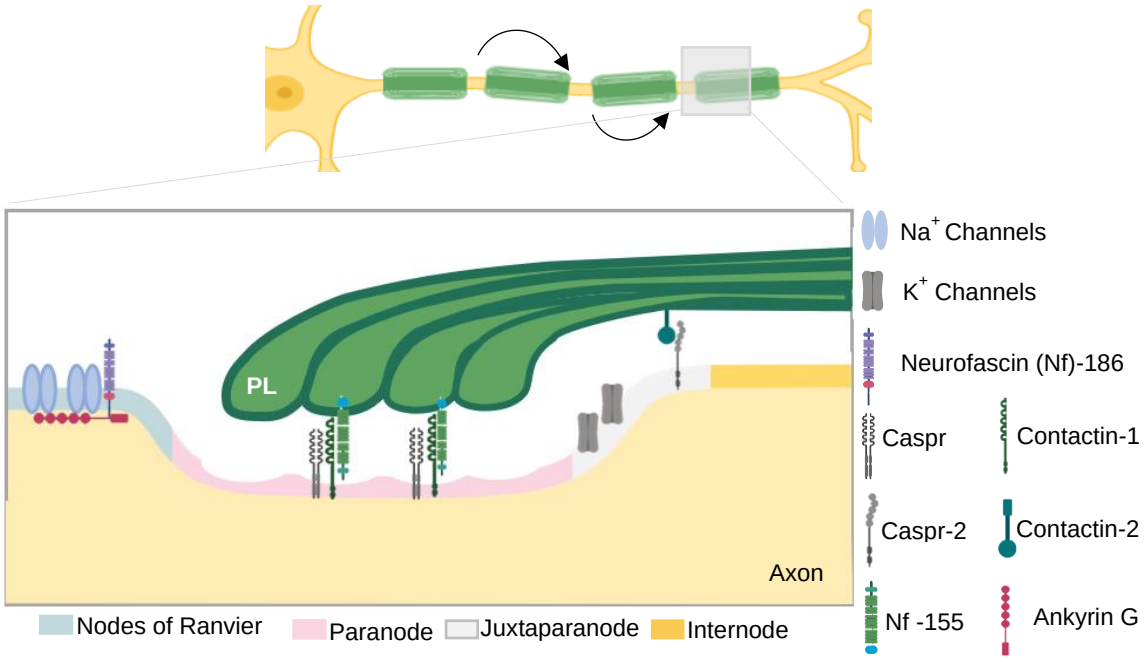


FIGURE I. 3. Schematic representation of the different domains of a myelinated axon. The different axonal domains: node, paranode, juxtaparanode and internode are depicted alongside with the paranodal loops (PL) of myelin layers (in green) All axonal segments have characteristic proteins. The nodes of Ranvier are characterized by the high amount of Sodium (Na) channels stabilized by axonal ankyrin and neurofascin (Nf)-186 proteins. In the paranode region, myelin is tightly attached to the axon as a result of the interactions between Nf-155 and axonal Caspr and Contactin-1 thus preventing ion channels diffusion maintaining the Na^+ clusters. Right before the internode (axonal region covered by compact myelin) Potassium (K^+) channels accumulate in the juxtaparanode region, separated from the other regions thanks to interactions between specific proteins such as Caspr-2 and contactin-2.

3. Myelin degeneration

Myelin formation and preservation is crucial for the maintenance of a healthy and functional nervous system. Otherwise, all the above-described functions will be impaired originating myelin-associated disorders with clear neuronal deficits, mainly affecting speech, cognition and motor function (as reviewed in(Love, 2006)). Myelin deficiencies from birth - manifested in the CNS as Leukodystrophies - are long known to result from an inappropriate production of myelin (dysmyelination) on the basis of genetic abnormalities in genes coding for critical myelin-associated enzymes (Faria, 2017). Rather challenging is the process of myelin deterioration after myelin's assembly is complete (demyelination).

Upon demyelination, there is a destruction of the previously correctly-established myelin membrane as a result of a direct attack against myelin constituents or myelinating glia itself. Multiple events can trigger demyelination thereby originating different categories of demyelinating diseases. The most common demyelinating disorder in the CNS is Multiple Sclerosis (MS), where the loss of myelin results from an autoimmune attack against myelin-specific constituents – inflammatory-mediated demyelination (Love, 2006). Strikingly, following demyelination, OLs can reverse myelin loss by promoting remyelination. Indeed, NG2/PDGFαR-expressing adult progenitors – already known to be distributed throughout the brain as a potential reservoir for remyelination – become activated in such demyelinating conditions reverting their phenotype into a more neonatal OPC. With higher motile and proliferative capacities, OPCs are spontaneously recruited to the lesioned region where they will differentiate into myelinating OLs restoring myelin sheaths and protecting them from further degeneration (Zawadzka et al., 2010;Moyon et al., 2015;Boyd et al., 2013;Franklin and Goldman, 2015;McMurran et al., 2016).

Unfortunately, remyelination is often ineffective or even inhibited in the vast majority of myelin diseases thanks to the misguided inflammatory response and activated milieu, frequently associated with demyelination which limits OLs remyelination capacity extensively (Boyd et al., 2013;Franklin and Goldman, 2015;McMurran et al., 2016). Being so, besides the immediate loss of AP conduction, denuded neurons of demyelinating lesions also become vulnerable to irreversible damage which ultimately leads to neurotoxicity and axonal degeneration - the primary cause of permanent neurological disability in myelin diseases (Griffiths et al., 1998;Trapp and Nave, 2008).

For this reason, it is of great interest to better understand the pathophysiology of these diseases as well as the demyelinating-associated inflammatory response to further potentiate remyelination as an effort to repair myelin-associated damaged, restore axonal integrity and the majority of neuronal conduction.

3.1. The process of demyelination: pattern and molecular insights

Early analysis of demyelinating lesions of MS patients - using both light and electron microscopy - characterized myelin as a vesiculated membranous network either loosely associated with the parent axon - "honeycomb arrays" - or as vesicular debris in the extracellular

space (Raine et al., 1999). In agreement to this, recent studies from Weil and colleagues - using high-resolution EM analyses - observed the same pattern of myelin fragmentation among both toxin- and inflammatory-induced models of demyelination, and further established a common sequence of events culminating in such vesiculated profile (**Figure I.4**). Thereby, upon a demyelinating event, the earliest evidence of demyelination is the enlargement of the inner tongue. Being connected with OL soma via cytoplasmic channels, the inner tongue is the earliest to detect and respond to alterations and metabolic disturbances. Later on, such initial event is followed by membrane decompaction concomitant with mild vesiculation within the innermost layers. Finally, vesiculation of the layers progresses outward culminating in complete myelin degeneration in the form of lipid-rich vesicles dispersed around the axons (Weil et al., 2016).

Much work was put into characterizing and understanding the molecular basis of demyelination. Experiments using shiverer mice – lacking MBP – showed consistent patterns of myelin degeneration as the ones described earlier. Again, the same degeneration was achieved when inducing the release of MBP from its membrane-bound state, inhibiting MBP/Pip2 interaction (Weil et al., 2016). Furthermore, posttranslational modifications in MBP such as citrullination and phosphorylation - altering MBP charge distribution, although they do not inhibit MBP interaction with myelin membranes completely, are enough to affect the compactness of myelin layers, suggesting that myelin disassembly is tight regulated and strongly MBP-dependent (Musse et al., 2006;Harauz et al., 2009;Kim et al., 2003;Moscarello et al., 2007;Jang et al., 2018).

Accumulation of fragmented myelin in the extracellular space is a crucial step in the progression of demyelinating diseases. Indeed, myelin-containing inhibitory molecules such as Nogo A, Oligodendrocyte-myelin glycoprotein and MAG have shown to inhibit neurite growth and axonal regeneration but most importantly, their accumulation within the lesion prevents OPC differentiation into mature OLs thus sharply limiting effective remyelination (Chen et al., 2000;Plemel et al., 2013;Lampron et al., 2015;McKerracher and R. j. Dunn, 1994). Therefore for efficient remyelination to be achieved, myelin vesiculated debris must be efficiently internalized and cleared away by glial cells and perivascular macrophages (Goodrum et al., 1994;Weil et al., 2016;Kotter et al., 2006). Intracellularly, myelin debris remain in the endocytic pathway to be hydrolyzed in the lysosomal compartment where myelin-derived lipids – mainly free cholesterol – accumulate before exportation to endoplasmic reticulum for *de novo* lipid biosynthesis (Thelen and Zoncu, 2017;A. M. Petrov, 2016).

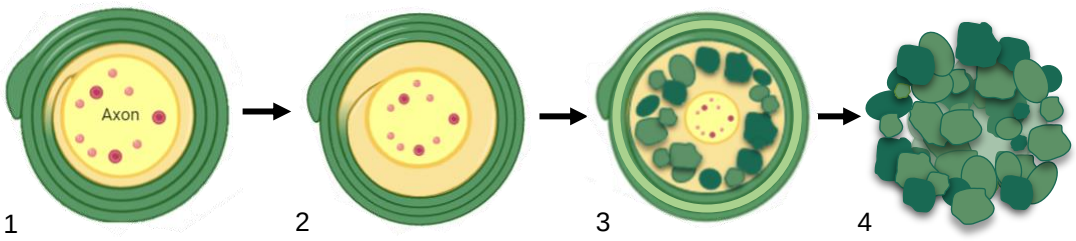


FIGURE I. 4. Schematic representation of myelin degeneration stages. (1) Compact myelin layers (in green) wrapped around the axon (in yellow) are depicted. (2) After a demyelinating stimuli, myelin´s first evidence of degradation is the enlargement of the cytoplasmic rich inner tongue, (3) which further collapses into myelin vesicles. (4) Afterwards, myelin loses compaction and membrane fragmentation progresses from the inner layers to the outermost ones until myelin is completely degraded into myelin debris.

4. Glial role in myelination & remyelination

OLs, together with astrocytes and microglia, form the glial cell population from the CNS, and collectively they are the most abundant cells of the nervous system. Glia – from the Greek word meaning glue – reflects the ancient view of neuroglia as connective tissue with a unique function, the one of holding the nervous system together. However, today's research highlight the importance of neuroglia as a master regulator of the nervous system development, plasticity, and disease, where they can be implicated in many diverse aspects such as mediating synaptic functions, CNS homeostasis, nerve signal propagation and myelination/remyelination (Fields et al., 2015;Zuchero and Barres, 2015).

OLs have a preponderant role as myelinating cells, providing neuronal metabolic support, integrity, and protection through myelination (detailed in section 1.2) being ultimately the main targets in demyelinating diseases. Rather complex is the role of astrocytes and microglia. Although their function and crosstalk is essential for myelination, demyelinating-associated gliosis is also responsible for the inflammatory outcome through the release of pro-inflammatory cytokines and other inflammatory modulators, impairing remyelination and exacerbating lesion formation and neuronal injury (Xanthos and Sandkuhler, 2014;DiSabato et al., 2016;McMurran et al., 2016). It is well accepted that glial contribution can have either beneficial or detrimental outcomes when it comes to CNS repair and demyelination in particular, so that understanding cells' complex behaviors and dynamics markedly increase the knowledge of their function in health and disease, allowing future modulation and targeting approaches in the context of demyelination and neurodegenerative diseases.

4.1. Astrocytes

Astrocytes, deriving from embryonic neuronal cells, are quite abundant among glial cells - approximately 30% (von Bartheld et al., 2016). Based on their morphology and location within the brain, these cells have been subdivided into protoplasmic and fibrous (reviewed by(Sofroniew and Vinters, 2010). Astrocytic role in CNS homeostasis is well studied and goes beyond the maintenance of physical and metabolic neuronal support, ranging from maintaining Blood Brain

Barrier (BBB) integrity (Sofroniew and Vinters, 2010;Sofroniew, 2015;Bush TG, 1999) regulating synaptic transmission (Ullian et al., 2001); promoting OPC maintenance and proliferation (Liu et al., 2017); to ensuring efficient myelination (Moore et al., 2011;Sorensen et al., 2008;Watkins et al., 2008).

When exposed to pathologic insult astrocytes range from inactive or quiescent into an active state where this astroglial reaction - astrogliosis - is accompanied by an increase in proliferation, cellular hypertrophy, process extension and enhanced expression of structural molecules such as glial fibrillary acidic protein (GFAP), vimentin and nestin (Eng and Ghirnikar, 1994;Norenberg, 1994;Leadbeater et al., 2006). Although initially associated only with diseased tissue and pathological conditions, today, reactive astrocytes are known to be highly plastic changing their phenotype along reactive astrocytic response, thus playing a wide variety of functions in CNS homeostasis. Indeed, based on their activation and response to neuroinflammation, reactive astrocytes were recently differentiated into A1 (neurotoxic) and A2 (neurotrophic) phenotypes, yet such descriptions can be somewhat simplistic according to the phenotypes found *in vivo* (Liddelow et al., 2017).

Astrocytic-mediated regulation of myelination was early hypothesized by Müller in 1904 when realizing that astrocytic dysfunction boosted the demyelination process in MS disease (reviewed in (Kiray et al., 2016;Williams et al., 2007)). Remarkably, astrocytes participate in almost every step of myelination either through the secretion of potent inducers of OPC differentiation, such as leukemia inhibitory factor-like protein (LIF), neuregulin-1, and other soluble molecules and growth factors or directly ensuring myelin correct assembly and maintenance via physical contact with OLs – through OL α6β1 integrin and astrocytic laminin or Cx47/Cx30 gap junctions in OLs and astrocytes, respectively (Gard et al., 1995;Stankoff et al., 2002;Taveggia et al., 2008;Ishibashi et al., 2006;Tress et al., 2012;Sakurai et al., 1998;Watkins et al., 2008;Ye et al., 2004;Liu et al., 2017).

Upon demyelination, astrocytes initiate the first line of response to myelin injury accumulating among areas of acute demyelination where they phagocyte myelin debris, thus contributing to efficient myelin clearance (Brosnan and Raine, 2013;Ponath et al., 2017). However, astrogliosis has long been associated with the formation of the glial scar surrounding demyelinated areas. Although crucial for helping restore the BBB and prevent the spread of immune cells into healthy tissues, glial scar also function as a barrier to tissue regeneration by preventing OPC entry and interaction with the denude axons and as a source of neurodegeneration-inducing molecules - mainly S100B - along with direct inhibitors of remyelination such as tenascin C, hyaluronan, PDGF-α and FGF-2 (Bianchi et al., 2011;Wang et al., 2011;Back et al., 2005;Messersmith et al., 2000;Brosnan and Raine, 2013;Sofroniew and Vinters, 2010;Silver and Miller, 2004). Nevertheless, once again, it has become increasingly clear that the role of activated astrocytes is not entirely negative. Recent studies have indeed shown that astrocytes activated with ciliary neurotrophic factor (CNTF) increase myelination, again emphasizing the multiple roles of these glial cells which together with microglia participate either in lesion progression and repair (Nash et al., 2011a;Nash et al., 2011b;Liberto et al., 2004).

4.2. Microglia

Microglia are the most abundant of the resident macrophage population in the CNS yet only representing around 10% of the total glial cells (Perry et al., 2010;Soulet and Rivest, 2008). Microglial myeloid precursors - fetal macrophages, monocytes, mesodermic and hematopoietic stem cells - settle in the brain during embryogenesis, before the BBB is wholly established, where they differentiate under the influence of CNS microenvironment (Ling, 1980). This resident myeloid population resides in the healthy nervous system as a highly stable population, ubiquitously distributed throughout the neural parenchyma in a dynamic “surveillant state”, with their characteristic morphology of small cell body, and long and ramified processes (von Bernhardi et al., 2016). By extending and retracting their branches, “surveillant” microglia interact with blood vessels, neurons, and other glial cells acting as sensors of pathologic events, recognizing and scavenging dead cells, as an effort to maintain CNS homeostasis (von Bernhardi et al., 2016;Hanisch and Kettenmann, 2007). More than maintaining homeostasis, surveillant microglia also regulate critical biological events. Indeed, co-cultures of OPCs with conditioned media from non-activated microglia showed enhanced OPC survival and differentiation, due to microglial release of the posteriorly identified insulin growth factor-2 (IGF-2) and galectin-3, respectively, which indirectly promotes myelination (Nicholas et al., 2001;Pasquini et al., 2011;Hoyos et al., 2014;Hamilton and Rome, 1994).

Being extremely sensitive to pathology, microglia becomes activated upon any insult or alteration in brain homeostasis, which like in astrocytes is accompanied by profound morphological and molecular changes. Microglia suffer partial retraction and hypertrophy of cell processes thus evolving from a ramified state to an intermedium one or “Bushy” and further to a complete rod and amoeboid shape (Stence et al., 2001). Parallel to their morphological transformation, activated microglia are molecularly characterized by the upregulation of ionized calcium binding adaptor molecule-1 (Iba1), CD11b and myeloid CD45 markers as well as by the elevated expression of major histocompatibility complex (MHC) antigen class II (Ito et al., 1998;Perry et al., 1985;Finsen et al., 1993).

Microglial activation has been described extensively under several pathological conditions, where they play critical roles as regulators of CNS immunity and mediators of inflammation (Cherry et al., 2014). Analogous to the classification of peripheral macrophages, microglial cells exist as functionally distinct states, those being “classical” activation, “alternative” activation and “acquired” deactivation, depending on the differential inflammatory outcomes as well as the stimulating milieu and specific time of activation (Mikita et al., 2011;Colton and Wilcock, 2010;Colton, 2009;Cherry et al., 2014;Tang and Le, 2016).

Indeed, upon acute infection or stimulation with lipopolysaccharide (LPS) and interferon-γ (IFN-γ), microglia develop into classical activated phenotype or pro-inflammatory state, classically classified as M1, mainly characterized by the production and release of pro-inflammatory cytokines - tumor necrosis factor-α (TNF-α), interleukins (IL)-1β, IL-12, IL-6 - and expression of other innate inflammatory mediators, like inducible nitric oxide synthase (iNOS) (Block et al., 2007;Li et al., 2004). Pro-inflammatory microglia are the first responders when

sensing tissue injury or infection, playing critical roles as antigen presenting cells and promoting the inflammatory response necessary for lesion repair (Soehnlein and Lindbom, 2010;Orihuela R, 2016).

Both “alternative” activation and “acquired” deactivation belong to the immunosuppressive category, classically classified as M2, characterized by the expression of specific markers, such as arginase 1 (Arg1), the heparin-binding lectin Ym1 and Found in inflammatory zone 1 (FIZZ1) (Gordon, 2003;Cherry et al., 2014). When stimulated with IL-4 and IL-13 cytokines, these cells assume the “alternative activated” state producing anti-inflammatory cytokines and other growth factors, like IL-10 and transforming growth factor-β (TGF-β), respectively. These latter cells are mainly involved in the phagocytosis of cellular debris, apoptotic bodies and pathogens, as well as in the suppression of both production and release of inflammatory mediators (Butovsky et al., 2005;Colton, 2009;Ponomarev et al., 2007;Ledeboer et al., 2000;Zhao et al., 2006;Park et al., 2005;Sawada, 2001;Satoh, 2018). Alternatively, through the exposure to anti-inflammatory cytokines or glucocorticoids, microglial cells evolve to an “acquired” deactivation state playing an essential role for tissue repair and extracellular matrix reconstruction (Colton and Wilcock, 2010;Colton, 2009;Satoh, 2018;Cherry et al., 2014).

Strikingly, phenotypic switch towards an anti-inflammatory/neuroprotective state usually initiates in response to “classical” activation in order to rapidly recover brain homeostasis. Otherwise, any imbalance of pro-/anti-inflammatory dynamics in which there is late or absent microglial polarization into M2, ultimately lead to the unregulated M1 polarization that further potentiates neurotoxicity as a result of the uncontrolled and continuous release of the former pro-inflammatory cytokines, thereby contributing to an extremely inflammatory/cell-damaging environment, strongly associated with many CNS disorders (Kigerl et al., 2009;Colton and Wilcock, 2010). Notwithstanding, similarly to A1/A2 astrocytic phenotypes, the M1/M2 paradigm is only illustrative of the two opposing sides of an inflammatory response, incapable of accurately modeling the environmental complexity and microglial plasticity found *in vivo* where they are known to coexist or even be transient into both extremes over disease progression (Grajchen et al., 2018;Ransohoff and Perry, 2009).

4.2.1 Microglia plasticity over demyelination

Among demyelinating lesions, although astrocytes slightly participate in myelin clearance, the bulk of this degenerated fat layer is cleared away by microglia, the professional phagocytes of the CNS. As so, microglia arrives at the lesion site and phagocytes myelin in a receptor-dependent way of endocytosis (reviewed in (Grajchen et al., 2018). Notably, myelin clearance is the most important event following demyelination not only because it is a limiting step for efficient remyelination but also because myelin is a potent inducer of microglial activation.

Both inflammatory and regenerative microglial states have been described upon internalization of myelin strongly indicating that myelin impact microglia differently, being capable of changing their properties and phenotypes along the process (Miron et al., 2013). In line with this, Grajchen and colleagues recently proposed a triphasic pattern of myelin-dependent

microglial polarization (**Figure I.5**) describing: (i) an early microglia activation in **inflammatory/M1** which later evolves to (ii) a more **M2/regenerative-like** phenotype that further re-shifts again, as degeneration progresses,(iii) towards an **M1 and neurotoxic state** (Grajchen et al., 2018). Indeed, myelin as a strong inflammatory stimulus is known to directly activate microglial complement receptor 3 (CR3) which further initiates a subsequent inflammatory signaling cascade with the expression of neurotoxic cytokines and inflammatory modulators via activation of the NF-kB through FAK/PI3K/AKT signaling pathway (Zani et al., 2015;Sun et al., 2010). Therefore, myelin entrance polarizes microglia towards an inflammatory/M1 state already known to be associated with axonal degeneration and impaired remyelination. Particularly, TNF-α and IL-1β are potent inducers of inflammation and axonal damage, whether IL-6 release mediates neurotoxicity and neuronal loss (Campbell et al., 1998). Furthermore, the oxidative burst from M1 cells is particularly damaging for OPC – cells with low expression of antioxidant enzymes and enhanced production of proapoptotic proteins – thus directly limiting remyelination (Blomgren and Hagberg, 2006).

Notwithstanding, myelin-rich M2 microglia have also been observed among demyelinated lesions releasing anti-inflammatory molecules while lacking, at the same time, the expression of pro-inflammatory ones (Boven et al., 2006;Zhang et al., 2011). The presence of such microglia is critical to repress the inflammatory response and potentiate further remyelination by inducing OPC differentiation, through the release of TGF-β and activin A (Diemel et al., 2003;McKinnon et al., 1993).

Such inflammatory to regenerative phenotypic switch is most likely concomitant with signaling pathways activated during intracellular myelin processing. Supporting this hypothesis is the activation of Liver X receptor (LXR) and peroxisome proliferator-activated receptor β and δ (PPARβ/δ) by cholesterol and phosphatidylserine, respectively – both major constituents of myelin membrane (Mailleux et al., 2018;Bogie et al., 2012). LXR- and PPARβ/δ-dependent signaling pathways are known to repress inflammatory responses mediated by NF-kB and inhibit the release of inflammatory mediators (Bogie et al., 2012).

Finally, characteristic of demyelinating and some neurodegenerative diseases - like MS - is this enormous amount of degraded myelin, accumulated extracellularly as a result of the ongoing demyelination. In response, it occurs an increased expression of many receptors responsible for microglia phagocytosis of myelin, as described in MS chronic demyelinating lesions, together with the down-regulation of myelin protein CD47 - inhibitor of myelin entrance through the activation of microglial receptor SIRPα - contributing to an excessive and continuous myelin uptake culminating in the formation of lipid-engorged microglia (Hendrickx et al., 2013;Gitik et al., 2011;Gorska et al., 2004). At this point, excessive amounts of free cholesterol accumulate in the lysosomes forming cholesterol crystals, which are not only associated with lysosomal dysfunction but also with activation of the NLRP3 inflammasome. NLRP3 activation ultimately leads to the production of IL-1β and IL-18 inflammatory cytokines, finally shifting microglial phenotype again into disease-promoting M1-like, potentiating brain neuroinflammation and

disease progression (Kaushik et al., 2012;Cantuti-Castelvetri et al., 2018;Hornung et al., 2008;Duewell et al., 2010;Cox et al., 2007;Li et al., 1998).

Interestingly, it was recently described that upon demyelination, microglial shift was in fact dependent on cell death by necroptosis. Indeed, Lloyd and colleagues observed that inhibition of necroptosis both *in vivo* and *ex vivo* was enough to prevent pro-regenerative microglia repopulation, contributing at the same time to an abnormal prevalence of a more pro-inflammatory microglia over time and to the inhibition of remyelination thus identifying microglial necroptotic cell death as an essential feature for reinstating axon health and CNS function (Lloyd et al., 2019). Although accumulation of lipids, particularly long chain fatty acids, have been proposed to be involved in membrane disruptions associated with necroptosis, more in-depth studies are needed in order to evaluate whether excessive accumulation of myelin-derived lipids is indeed associated with the activation of microglial necroptotic pathway (Parisi et al., 2017).

Altogether, the above described dynamics of microglia activation emphasizes both cells plasticity and functional duality in maintaining CNS homeostasis. As the main targets of the excessive myelin debris dispersed within demyelinated lesions, microglial cells are essential players in the course of demyelination where their involvement is a prerequisite for CNS repair either by simply promoting myelin clearance or through its remyelinating-related properties. However, when overactivated, and unable to differentiate into the M2 state - either due to the unbalance of inflammatory cytokines, intracellular lipid processing or necroptosis - microglia is no longer capable of repairing the CNS damage thereby potentiating excessive neuroinflammation throughout the brain which will interfere with glia crosstalk resulting in a complete change in the nervous system microenvironment prevailing axonal degeneration and neurotoxicity with little or no chance for remyelination to take place.

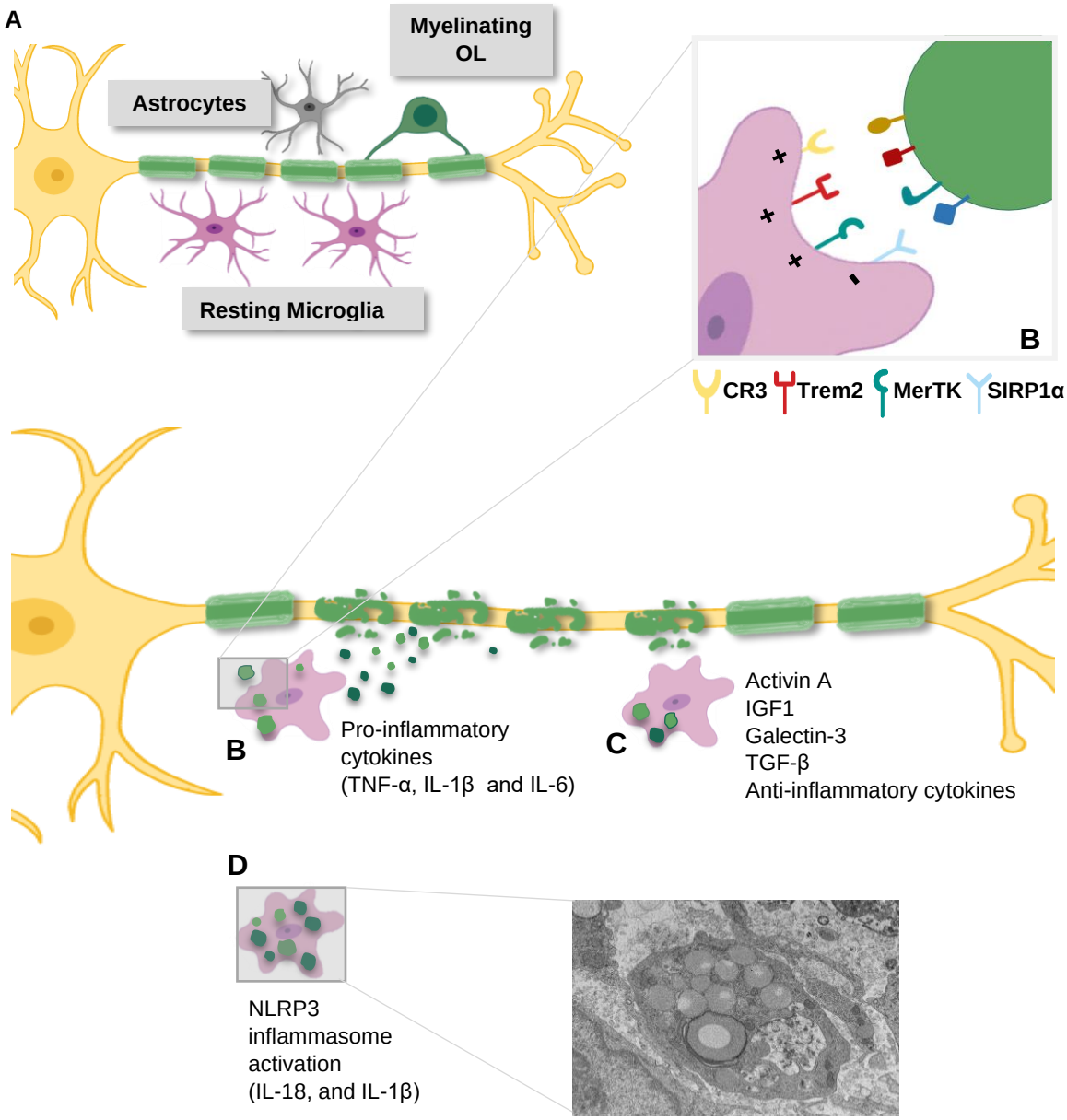


Figure I. 5. Schematic representation of microglial dynamics over demyelination. (A) in homeostatic conditions, resting microglia survey the microenvironment for molecular indicators of damage or infection and regulate processes like neuronal survival, OLs survival and differentiation. (B) Upon demyelination, microglia become activated, changing to an amoeboid/rod morphology, migrate to the lesion site and phagocytes the bulk of myelin debris. Myelin internalization by microglia is dependent on the expression of many receptors that positively or negatively regulate myelin uptake. While complement receptor 3 (CR3), triggering receptor expressed on myeloid cells 2 (Trem2) and mer tyrosine kinase (MerTK) enhance myelin uptake, activation of signal regulatory protein α (SIRP1α) by myelin-associated protein CD47 inhibits myelin entrance as a feedback mechanism to suppress excessive myelin intracellular accumulation. Myelin entrance polarizes microglia towards and inflammatory state releasing inflammatory cytokines, TNF-α, IL-1β and IL-6, favoring axonal damage and oligodendrogenesis. (C) This pro-inflammatory population shift, due to myelin intracellular processing, giving rise to a more anti-inflammatory one, suppressing the inflammatory status and releasing pro-remyelinating factors like activin A, TGF-β and insulin growth factor 1 (IGF1). (D) Finally, dysregulated phagocytosis or excessive demyelination give rise to foamy microglia with accumulation of myelin-derived cholesterol crystals, causing lysosomal dysfunction which further activates NLRP3 inflammasome, shifting again to a pro-inflammatory neurodegenerative microglia.

5. Methods to detect demyelination

5.1 Specific markers of myelin and degraded myelin

Once identified the different sequential events upon ongoing demyelination, is rather interesting to develop mechanisms to fairly distinguish demyelinated regions from healthy myelin as well as to identify the different phases of myelin degeneration – compact, non-compact and vesiculated – to further understand myelin processing pathway and address potential mechanisms favoring lesion repair.

Regarding the identification of compact myelin, commonly used histological markers include Luxol fast blue dye and, for myelin staining of peripheral nerves, the Sudan black dye. Moreover, as described earlier, MBP is the one needed for myelin compaction. Due to that, for immunohistochemistry analysis, the anti-MBP antibody has long been used to detect intact myelin sheaths, whereas degenerated myelin can be detected using antibodies against MBP specific residues. As described above, MBP's unattachment to the opposing membrane layers is concomitant with the loss of myelin compaction. Strikingly, because it is not bound to any membrane, MBP becomes self-repulsing exposing the phenylalanine residues, the ones once required to mediate its molecular assembly, at the same time, unmasking a small epitope QDENPVV (QD9) – 82-88 MBP residues – the one that will be recognized by anti-QD9 antibody then used to recognize specifically these myelin-degenerated areas (Matsuo et al., 1997;Matsuo et al., 1998;Weil et al., 2016).

Apart from anti- MBP and QD9, myelin debris at the final stage of myelin degradation have been commonly stained using Oil red O (ORO) dye which stains lipid in general or Nile Red (NR) Dye, specific for lipid droplets (Greenspan et al., 1985;Mehlem et al., 2013;Boven et al., 2006).

5.3 BASHY dyes: new molecules for myelin characterization

Boronic acid salicylidenehydrazone (BASHY) dyes offer a promising alternative for the detection of degenerating myelin, particularly for the detection of myelin debris. Through the association of boronic acids with Schiff-base ligands, it was possible to synthesize these highly stable fluorescent complexes with structural and photophysical properties suitable for live cell bioimaging applications (Santos et al., 2016). With their hydrophobic asymmetric structure and fluorescence in nonpolar environments, these BASHY complexes were envisioned to stain lipid reservoirs, thus meeting all the requirements to be considered promising tools for the detection of cholesterol-rich myelin debris. Indeed, our collaborators Gois P and Santos F showed that when labeling HeLa cells with BASHY dyes, there was a rapid accumulation within lipid reservoirs without staining plasma membranes confirming their specificity and ability to distinguish between different lipid structures with variable packing properties (**Figure I.6**) (Santos et al., 2016).

Remarkably, another striking feature of BASHY platforms is the possibility of bioconjugation with specific relevant small ligands so that they can be used as powerful tools for

selectively target particular cells or biological processes, then easily followed by simple fluorescence techniques (Santos et al., 2016).

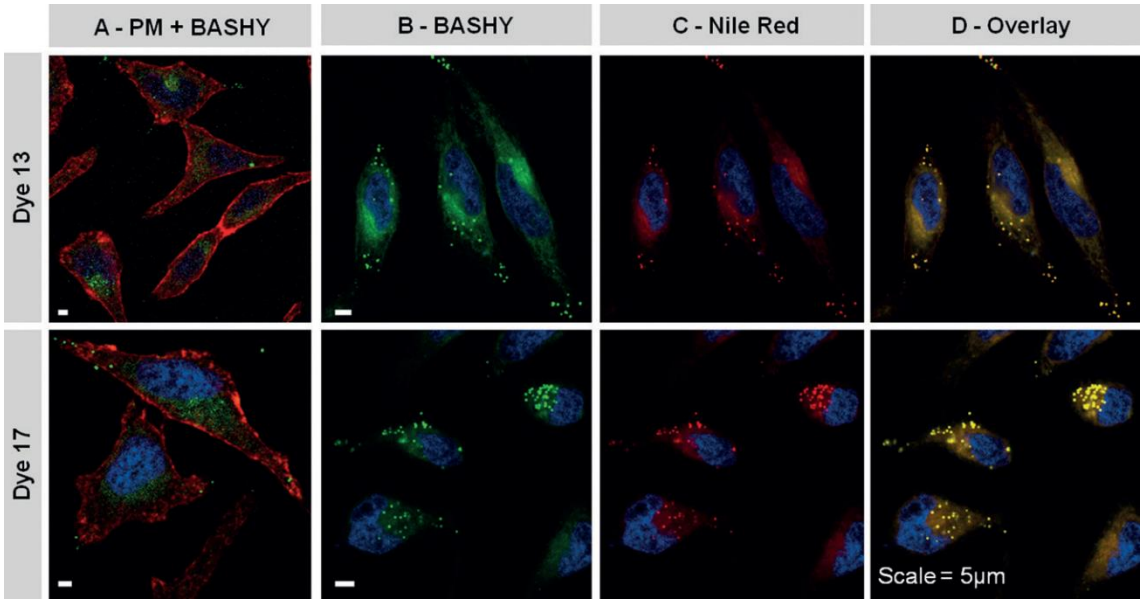


Figure I. 6. BASHY dye specifically label lipid aggregates. Representative images of BASHY labeling lipid aggregates in HeLa cells incubated with BASHY, observed by the co-localization of BASHY (green) and Nile Red (Red) with no labeling of lipid plasma membranes. Data from (Santos et al., 2016).

Overall and emphasizing once again the importance of myelin in the normal function of the brain it is urgent the need for more specific tools to better address myelin changes along demyelination, that may not only allow a better understanding of the phenomenon but also may potentially permit the targeting of specific cells to ultimately improve remyelination and CNS recovery.

5. Aims

The major aim of our study is to understand if BASHY dyes may be used for myelin characterization during demyelination and be a potential vector for a targeted therapeutic strategy to improve remyelination/recovery. With this in mind, our specific aims are:

1) To test whether BASHY dyes can function as a better alternative to address myelin processing during demyelination. Being so, we will use organotypic cerebellar slice cultures (OCSC) from wild type (WT) 10 postnatal rats that will be demyelinated by using lysophosphatidylcholine (LPC). 18 and 48h after insult, control and LPC-treated OCSC will be incubated with BASHY dyes and characterized in terms of myelin/cholesterol staining and immunostained for specific cell markers to address which myelin and specific cell types co-localize with BASHY dyes.

2) To characterize targeted microglia according to their phenotype and inflammatory profile. For that we will use again control and LPC-treated OCSC incubated with BASHY and further immunostain for specific markers of inflammatory status at both 18 and 48h. We will use a general microglia marker together with pro-inflammatory and anti-inflammatory ones – Iba1, iNOS and Arg-1, respectively.

3) To assess whether BASHY dyes-associated small molecule may be used to modulate microglial phenotype. To achieve our final aim, control and LPC-treated OCSC will be incubated with BASHY conjugated with a specific small molecule. In this phase, microglia reactivity, demyelination and inflammatory response will be evaluated.

II- Material and Methods

1. Animals

For the *ex vivo* studies, we used Wistar rats with 10 days, acquired from Instituto de Higiene e Medicina Tropical (IHMT).

Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional Animal Care and Use Committee. The best efforts were made to minimize the number of animals used and their suffering.

2. Ex vivo model - Organotypic Cerebellar Slice Cultures (OCSC)

To evaluate BASHY specificity for demyelinating states, experiments were conducted using an *ex vivo* demyelinating model. First we used Organotypic Cerebellar Slice Cultures (OCSC), which better reproduces the myelin tract complexity and functionality of cell relationships and tissue microenvironments found *in vivo*. In short, cerebella from postnatal day 10 (P10) rats were isolated from brains in phosphate buffered saline (PBS) and sagittal slices of 400 µm were obtained using a McIlwain tissue chopper. Four slices from different animals were placed into each membrane culture insert, with 0.4 µm pores (BD Falcon, #353493, Lincoln Park, NJ, USA), and inserts placed in 6-well cell culture plates kept at 37 °C, in 5% CO_2 conditioned atmosphere. For 7 days slices were maintained *in vitro* to allow slice recovery and significant myelination to occur (Birgbauer, 2004). For the first 3 days *in vitro* (DIV) slices were cultured with 1 mL per well of culture medium consisting of 50% minimal essential media (MEM), (Gibco, Life Technologies, Inc., Grand Islands, USA), 25% of both heat-inactivated horse serum (Gibco) and Earle's balanced salt solution (EBSS, Gibco), 6.5 mg/mL glucose, 36 mM HEPES (Biochrom AG, Berlin, Germany), and 1% of both L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and antibiotic-antimycotic (Sigma-Aldrich). Half the culture media was renewed every day. At 4 DIV, to improve neuronal viability, culture media was totally replaced by 1 mL of a serum-free media, containing 98% Neurobasal-A (NB) (Gibco), 2% B-27 (Gibco), supplemented with 1% L-glutamine, 36 mM glucose, 1% of antibiotic-antimycotic and 25 mM HEPES. Again, half the media was daily replaced by new NB medium.

After 7 DIV, slices were treated with lysophosphatidylcholine (LPC) (0.5mg/mL, in NB) for 18h to induce demyelination after which, media was completely replaced by NB fresh medium for a recovering period of 30h. At both 18h- and 48h-post LPC-treatment, treated and control slices were fixed with paraformaldehyde (PFA) for future immunohistochemistry assays and BASHY, Lysotracker and Filipin staining analysis.

Regarding the assay of new microglia targeting compounds, OCSC were kept in the same culture and NB media for 7DIV however, slices were treated with LPC in parallel with the

administration of the following molecules - BASHY solubilized in acetonitrile and diluted in NB media to a final concentration of 5µM; BASHY + Galantamine or BASHY linked to Galantamine (FFS2B) both solubilized in dimethyl sulfoxide (DMSO), reaching a DMSO concentration of 5mM, and diluted in NB media, to a final concentration of 5µM. 18h-post LPC treatment the medium was replaced with fresh one LPC-free but supplemented with BASHY; BASHY + Galantamine or FFS2B, for 30h. Being so, we had 6 treatment groups for each time point (18h- and 48h-post LPC incubation): (1) BASHY (Control); (2) BASHY + Galantamine; (3) FFS2B; (4) LPC with BASHY; (5) LPC with BASHY + Galantamine and (6) LPC with FFS2B (Figure II.1). Slices from the mentioned groups were (i) fixed with PFA for immunohistochemistry analysis and (ii) stored in RiboZol™ reagent, at -80°C for further RNA extraction.

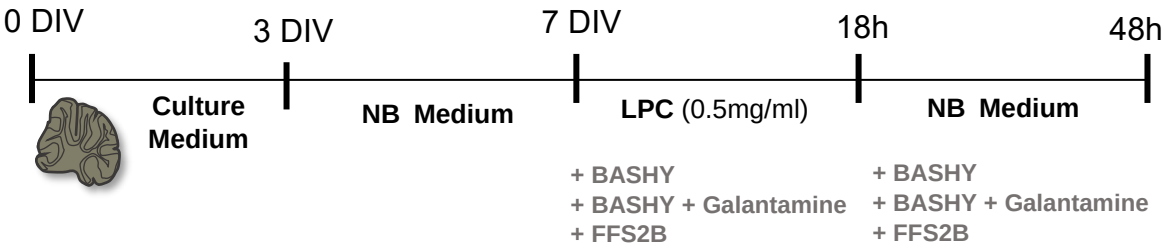


Figure II. 1. Schematic illustration of culture treatment. Cerebellar slices from 10 postnatal rats were kept in culture medium for 7 days *in vitro* and posteriorly incubated with neurobasal (NB) medium supplemented with LPC for 18h. After the incubation period, LPC-containing medium was discarded and replaced by fresh NB for 30h. For the galantamine assay, 7 DIV slices were treated with LPC and in parallel incubated with BASHY, BASHY + galantamine or FFS2B molecules maintained for the remaining 48h.

3. Staining Procedure

Regarding BASHY staining procedure, control and LPC-treated slices were fixed in each insert with 4% PFA for 1 hour and posteriorly washed three times for 10 minutes with PBS. Fixed slices were then cut out form the insert, placed into glass slides and washed one time for 30 seconds with sterile water. Afterwards, slices were incubated for 20 min at room temperature (RT) with 100 µl of BASHY molecule (5µM), solubilized in acetonitrile and diluted in PBS.

For Lysotracker and Filipin staining, slices were incubated with 100 µl of lysotracker (1:20000, TermoFisher), for 30 minutes at 37°C and posteriorly fixed with 4% PFA for 1 hour after which they were washed one time with PBS for 1 minute. Then, these slices were incubated with filipin (50ng/µl in PBS) for 1h at 37°C. In both cases slices were carefully washed three times with PBS for 1 minute.

Finally, in all staining procedures slices were mounted using Fluoromount-G (Southern Biotech, Birmingham, AL) for fluorescence/confocal microscopy. Fluorescent images were acquired using Leica DMi8-CS inverted microscope with Leica LAS X software and the different z-stacks were merged and analyzed with ImageJ (Fiji Is Just) software.

4. Immunohistochemistry procedure

For immunostaining procedure, slices were fixed in each insert as described above, cut out form the insert, placed into glass slides and blocked for three hours at RT with blocking solution containing 2% heat-inactivated horse serum (Gibco), 10% fetal bovine serum (Biochrom), 1% bovine serum albumin (BSA, Sigma-Aldrich), 0.25% Triton X-100 (Roche Diagnostics, Indianapolis, USA) and 1nM HEPES in Hank's balanced salt solution (HBSS, Gibco). Afterwards, slices were incubated with primary antibody (**Table II.1**) diluted in blocking solution, for approximately 48 hours, at 4°C. Following this, slices were washed three times for 20 minutes with 0.01% Triton X-100 in PBS solution, under shaking at RT and posteriorly probed with the secondary fluorescent antibodies (Table II.2) also diluted in blocking solution, for approximately 24h at 4°C. Slices were again washed three times in the same conditions and incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:1000 in PBS) for 5 minutes. Slices were washed one time with PBS for 1 minute before staining with fluorescent BASHY dye and finally mounted with Fluoromount-G (Southern Biotech, Birmingham, AL) for fluorescence/confocal microscopy. Images were acquired and analyzed in the same conditions as above.

With ImageJ software, it was possible to observe BASHY labeling, and in parallel evaluate the demyelinating process and microglia activation. Three cerebellar regions containing myelin tracks were taken from each slice and approximately 20-30 z-stacks were taken per region per condition. The area of co-localization with GFAP/QD9/Iba-1/MBP, was calculated with ImageJ software, given by the ROI number resulting from the co-localization of the total area of BASHY and the total area occupied by GFAP/QD9/Iba-1/MBP, respectively. Furthermore, for microglia phenotype analysis, BASHY, Iba-1, Arg-1 and iNOS positive cells were counted from merged z-stacks and the results were given by the mean cell number from each region.

Table II. 1. List of Primary antibodies used for immunohistochemistry assays.

Antibody	Target	Host	Dilution	Brand
MBP Myelin binding protein	Mature Oligodendrocytes	Rat	1:200	BioRad MCA409S
QD9 QD9 epitope of MBP	MBP residues (82-88)	Mouse	1:100	Abnova MaB8817
GFAP Glial fibrillary acidic protein	Astrocytes	Mouse	1:100	NovoCastra 6035278
Iba-1 Ionized calcium-binding adapter protein-1	Microglia	Rabbit	1:250	Wako 019-19741
Arg-1 Arginase	Anti-inflammatory Microglia	Goat	1:50	Santa Cruz Biotechnology Sc- 18355
iNOS Inducible nitric oxide synthase	Pro-inflammatory Microglia	Mouse	1:100	BD Biosciences 610329

Table II. 2. List of secondary antibodies used for immunohistochemistry assays.

Secondary Antibody	Host	Dilution
Alexa 594, red Anti-rabbit	Goat	1:500
Alexa 594, red Anti-rat	Donkey	
Alexa 488, green Anti-mouse	Goat	
Alexa 594, red Anti-goat	Chicken	
Alexa 647, far red Anti-mouse	Goat	
Alexa 405, blue Anti-rabbit	Goat	

5. Gene Expression - Semi-quantitative Real-Time PCR

In order to evaluate the expression levels of the genes of interest, total cytoplasmic RNA of OCSC slices was extracted at both timepoints (18h- and 48h-post LPC and galantamine incubation), using RiboZol™ reagent method following the manufacture's guidelines (VWR Life Science, USA). RNA concentration and purity were quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). After extraction and quantification, RNA samples were reversely transcribed into complementary DNA (cDNA) using Xpert cDNA synthesis Mastermix kit (GRiSP), according to manufacturer's guidelines. Quantitative Real-Time PCR (qRT-PCR) for cDNA amplification was performed on a 7300 Real-Time PCR detection system (Applied Biosystem, Madrid, Spain) using an Xpert Fast SYBR Mastermix (GRiSP) Kit under optimized conditions of 50ºC for 2 min, 95ºC for 10 min followed by 40 cycles at 95ºC for 5 s and 62ºC for 30 s. The PCR was performed in 384-well plates with each sample being performed in duplicate. The genes and respective sequences used as primers are listed in **Table II.3** with β-actin gene being used as an endogenous control to normalize the expression levels.

Table II. 3. List of genes analyzed for each sample with respective primers

Gene	Primers Forward	Primers Reverse
PSD95	5' cgaggatgccgtggcagcc3'	5' catggctgtggggtagtcagtgcc 3'
SYP	5' tcaggactcaacacctcagtgg 3'	5' aacacgaaccataagttgccaa 3'
MBP	5' agtcgcagaggacccaagat 3'	5' cacaggcctctccccttc 3'
NG2	5' gggctgtgctgtctgttga 3'	5' tgattcccttcaggtaaggca 3'
IL-1β	5' caggctccgagatgaacaac 3'	5' ggtggagagctttcagctcata 3'
TNF-α	5' tactgaacttcggggtgattggtcc 3'	5' cagccttgtcccttgaagagaacc 3'
IL-10	5' atgctgcctgctcttactga 3'	5' gcagctctaggagcatgtgg 3'
Arg-1	5' ccacggtctgtgggaaaagccaat 3'	5' ttgccatactgtggtctccaccca 3'
iNOS	5' acccacatctggcagaatgag 3'	5' agccatgacctttcgcattag 3'
NLRP3	5' tggtcaaggagcatccaagca 3'	5' aagtgttcatcctcaggctcaaa 3'
IL-18	5' tggagacttggaatcagacc 3'	5' ggcaagctagaaagtgtcct 3'
TREM2	5' tgtggccagtgggctagact 3'	5' ctctgggtccagtgagga 3'
CR3	5' tgctgagactggaggcaac 3'	5' ctccccagcatccttgttt 3'
β-actin	5' tatggagaagatttggcacc 3'	5' ccaccaatccacacagagta 3'

IL- Interleukin; PSD95- Postsynaptic protein density; SYP- Synaptophysin; MBP- Myelin basic protein; NG2- Neuron-glia antigen 2; TNF- Tumor Necrosis Factor; Arg-1- Arginase 1; iNOS- Inducible nitric oxide synthase; NLRP3- NLR family pyrin domain containing 3; Trem2-Triggering Receptor Expressed On Myeloid Cells 2; CR3- Complement receptor type 3

For semi-quantitative analysis of the transcription levels of our genes of interest we used the ΔΔCT comparative method. The results were normalized to the housekeeping gene, β-actin and were obtained by the $2^{-\Delta\Delta CT}$ formula where the CT value is the cycle number at which the amplification curve intersects the threshold line. The ΔCT value results from the difference between the CT values from our gene of interest and the mean CT value of the endogenous gene, β-actin. For each sample, ΔΔCT results from the difference between its ΔCT value and the ΔCT value of the sample chosen as reference, in this case the group non-demyelinated and only incubated with BASHY.

6. Statistical analysis

All results are presented as mean ± SEM. Differences between two groups were determined by the two-tailed t-test performed on the basis of equal and unequal variance or by one-way ANOVA with Tukey post-test for multiple comparisons, using GraphPad PRISM 5.0 (GraphPad Software, San Diego, CA, USA), as appropriate. The P-values of $P<0.05$, $P<0.01$ and $P<0.001$ were considered as being statistically significant.

III. Results

1. BASHY fluorescence increases in cerebellar slices following LPC-induced demyelination

Demyelination is the main cause of neurological symptoms and cognitive dysfunction in MS, as well as in other demyelinating diseases. It starts with myelin decompaction followed by mild vesiculation that culminates in total membrane destruction that detaches from the internode and accumulates as cholesterol-rich myelin debris within the extracellular space (Weil et al., 2016;Raine et al., 1999).

As it was mentioned before, *in vitro* studies showed that BASHY fluorescent molecules were highly specific for lipid structures, particularly for the nonpolar lipid aggregates, therefore envisioned to be a good chemical tool for the identification of later stages of myelin degradation (Santos et al., 2016). In line with this, we firstly decided to clarify the specificity of these BASHY dyes in the context of demyelination using an *ex vivo* demyelinating model of OCSC. For that, demyelination was induced by the administration of LPC, a demyelinating agent, and fixed slices were stained with BASHY, 48h-post LPC treatment.

As it is clearly illustrated in **Figure III. 1A**, upon demyelination we observed a marked increase in BASHY fluorescence, when compared to the controls, which is corroborated by (2.88-fold) increase in the area stained with BASHY ($P<0.05$) shown following quantitative analysis as shown in **Figure III. 1B.** These results suggest that BASHY identify degraded myelin which accumulates among demyelinating process.

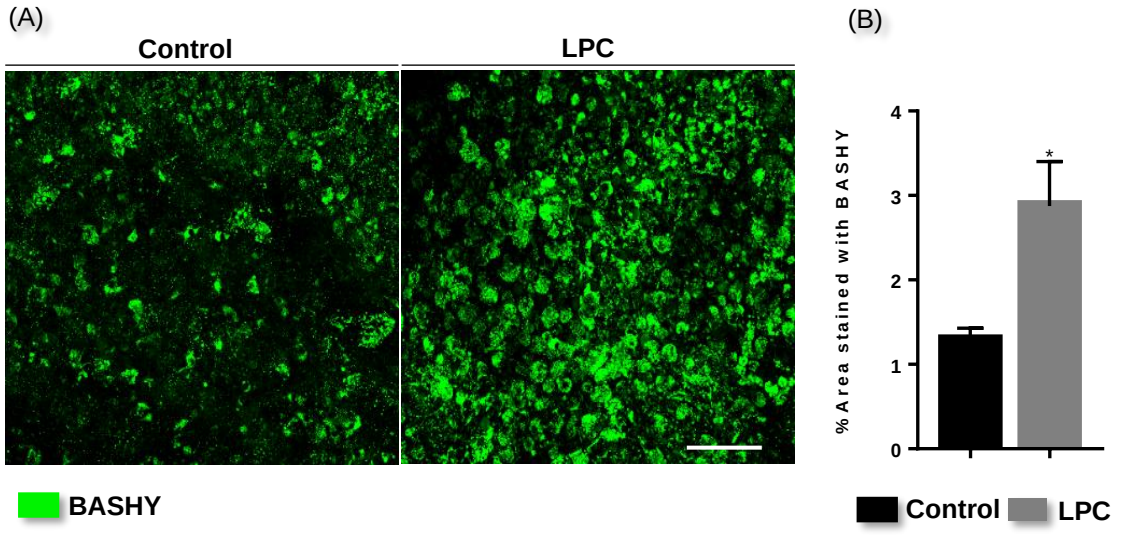


FIGURE III. 1. BASHY fluorescence increases in demyelinated slices following treatment with LPC. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) at 7 day *in vitro* (DIV) for 18 hours, to induce demyelination. After LPC exposure, slices were incubated with new neurobasal (NB) fresh medium for a recovering period of 30h. 48 hours post-incubation with LPC, cerebellar slices were fixed and stained with BASHY dye (BASHY, green) (A) Representative images of BASHY enhanced labeling after LPC insult (B) Graph bars represent the quantitative analysis of the percentage of area stained with BASHY. Scale bar equals 100 microns. Results are expressed as mean ± SEM. *P<0.05 vs Control

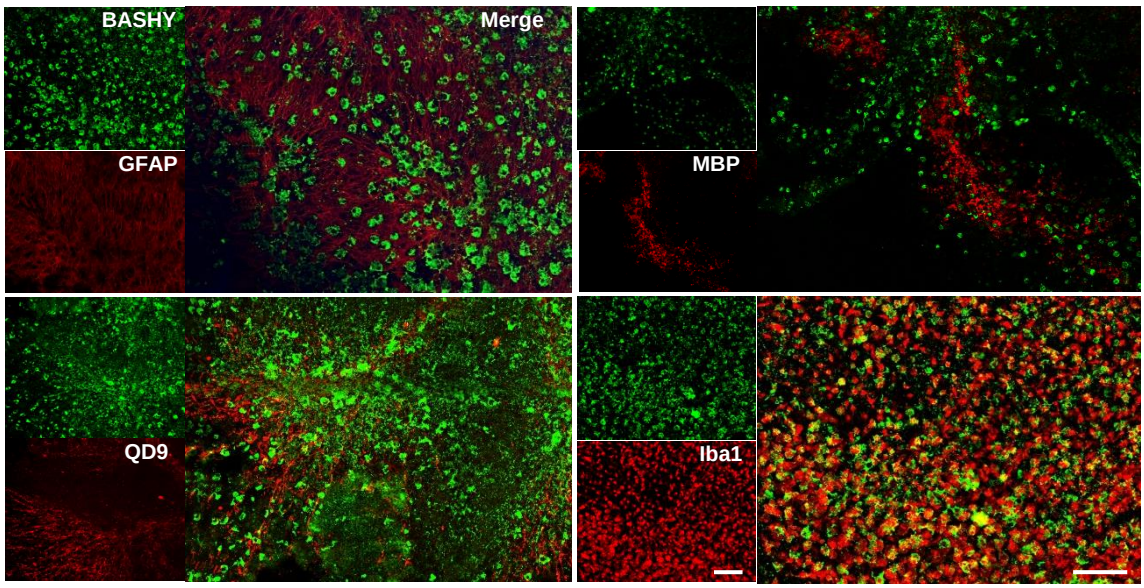
2. BASHY dye co-localize with myelin debris phagocyted by microglia

Degraded myelin comprises both non-compact myelin and lipid debris. As described earlier, non-compact myelin can be identified by specific MBP residues using anti-QD9 antibody (Weil et al., 2016). On the other hand, myelin debris, once they are highly toxic and contribute to a more inflammatory environment, are rapidly cleared from the extracellular space by glial cells as astrocytes and microglia. Indeed, this uptake is mostly driven by microglia, and such myelin aggregates are further destroyed intracellularly through the lysosomal pathway, which is responsible for cholesterol and other lipids degradation (Brosnan and Raine, 2013;Ponath et al., 2017;Lampron et al., 2015;Thelen and Zoncu, 2017;A. M. Petrov, 2016)

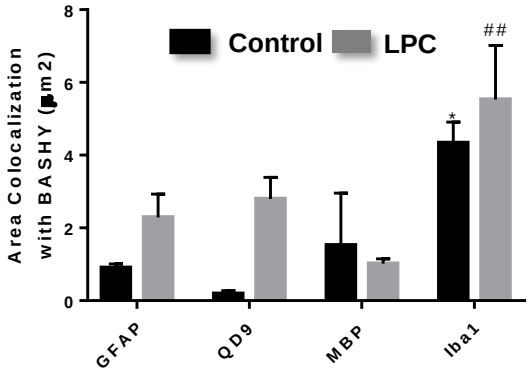
Knowing this and given the previous results, we next decided to assess BASHY cellular location, to understand which cell type mostly stain for BASHY at the cerebellum. For that, we performed immunohistochemistry assays to detect both myelin degradation patterns and glial cells and evaluated their co-localization with BASHY. Indeed, 48h-post LPC incubation, fixed slices were immuno-stained for mature OLs/compact myelin (MBP), degraded myelin (QD9), astrocytes (GFAP), and for microglia (Iba1), and posteriorly stained with BASHY molecules. As depicted in **Figure III.2A**, upon demyelination, although we had some co-localization with astrocytes, we observed that BASHY staining was mostly confined to lipid vesicles inside Iba1-positive microglial cells, as it can be observed by the enhanced area of co-localization of microglia with BASHY both in non-treated slices ($P<0.05$ vs QD9) and in LPC-induced ones (27%**,** $P<0.01$ vs LPC) **(Figure III.2B)**. Curiously, BASHY labeling accompanies the formation of some early vesiculated structures concomitant with the loss of compaction, evidenced by the increased, yet not significant, area of co-localization with QD9 in slices incubated with LPC.

Furthermore, in order to confirm BASHY specificity for myelin aggregates, we performed triple staining with BASHY in addition with filipin and lysotracker, which are commonly used markers to stain cholesterol and lysosomes, respectively. Indeed, as shown in **Figure III.2C**, BASHY molecules co-localize with cholesterol-rich vesicles accumulated in lysosomes. Overall, the above results strongly indicate that BASHY dyes are indeed specific for myelin aggregates and lipid debris. Interestingly, BASHY dyes also preferentially stain microglial cells, the professional phagocytes of myelin debris and the main inflammatory mediators in demyelinating conditions, allowing a possible new way of targeting for these cells.

(A)



(B)



(C)

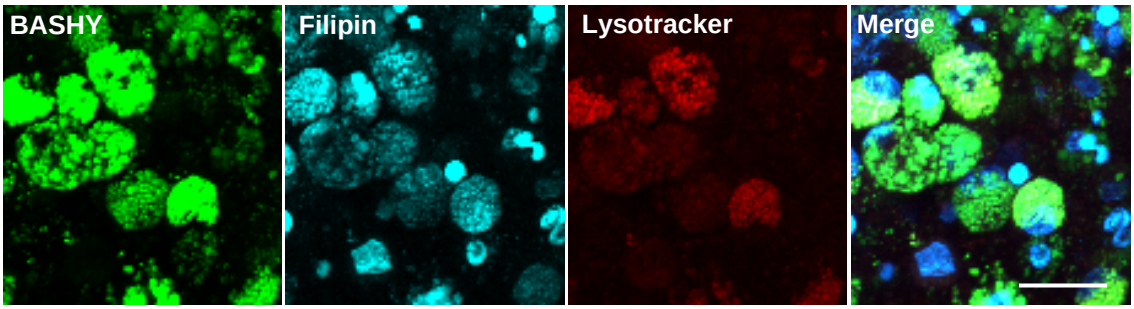


Figure III. 2 BASHY dyes co-localize mostly with microglial cells. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) at 7 day *in vitro* (DIV) for 18 hours, to induce demyelination. Slices were posteriorly maintained in NB medium for a recovery period of 30h. 48 hours post-incubation with LPC slices were fixed and double stained to observe astrocytes (GFAP, red), mature oligodendrocytes/compact myelin (MBP, red), degenerated myelin (QD9, red) and microglia (Iba1, red) and then stained with BASHY (A). 48-post LPC and BASHY incubation, OCSC were incubated with lysotracker for 30 minutes at 37ºC, fixed and stained with filipin (50 ng/µl) for 1h at 37ºC (C). (A) Representative images of BASHY labelling being mostly co-localized with microglial cells (magnification 20X) and (B) respective quantitative analysis of the area of co-localization with BASHY. Scale bar equals 120 microns. (C) Representative images of triple staining to observe BASHY molecules, cholesterol (filipin, light blue), and lysosomes (lysotracker, red). Magnification 40X. Scale bar equals 20 microns. Results are mean ± SEM. *P<0.05 for Iba1 control vs QD9 Control. $^{\#\#}$P<0.01 for Iba1 LPC vs MBP LPC. GFAP, glial fibrillar acidic protein; MBP, myelin basic protein; Iba1, ionized calcium binding adaptor molecule 1.

3. BASHY molecule preferentially stains amoeboid lipid-rich microglia

Microglial cells are the main vigilant guards of the CNS. Notwithstanding, they also fulfill a crucial role as in the development and progression of neurological disorders. Very sensitive to the microenvironment, microglia respond to environmental cues, CNS injuries and diseases with complex reactions that require cell activation. Characteristic of microglial activation is its increase proliferation, motility and phagocytic capacity. These latter changes are accompanied by an evident morphological transformation, where resting and ramified microglia suffer hypertrophy of their processes, evolving to a bushy cell shape until finally acquiring a rod and amoeboid morphology (Lively and Schlichter, 2018;Bogie et al., 2014). So, to characterize our microglial population in terms of activation and morphological differences, 48h after LPC incubation slices were fixed and immuno-stained for microglia (Iba1).

As illustrated in **Figure III.3A,** although we observed around 60% of amoeboid/activated microglia in non-treated slices, resulting from the mechanical injury and cell death associated with the cerebellum slicing procedure, LPC-treated slices, not only showed a decrease in microglial cells with ramified and bushy morphologies ($P<0.05$), but we also observed a significant increase of amoeboid cell ($P<0.01$). Indeed, after demyelination, the great majority of microglia population (around 80%) exhibited a completely round shape with few or non-existent processes, characteristic of an activated state. The quantitative analysis also reflected what was observed. This findings are in line with previous studies that highlight microglial shift in cell shape after demyelinating stimuli as LPC-treatment (Sheikh et al., 2009).

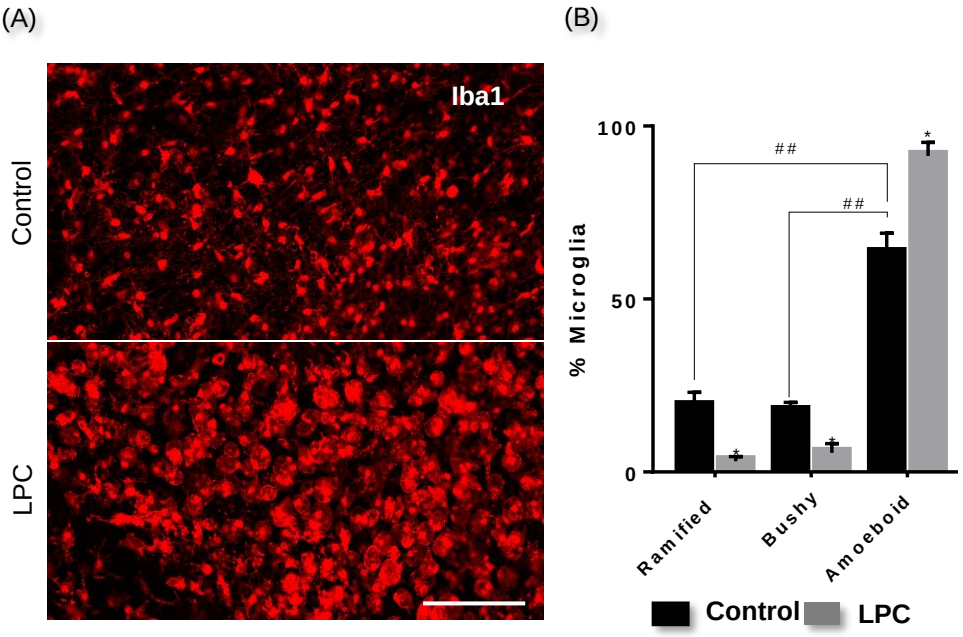


Figure III. 3. LPC induces microglial switch in morphology from a more ramified, to a bushy and amoeboid/activated microglia. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) at 7 day *in vitro* (DIV) for 18 hours, to induce demyelination. Slices were posteriorly maintained in NB medium for a recovery period of 30h. 48h-post LPC treatment, fixed slices were immunostained for microglia (Iba1, red). (A) Representative images of microglial morphology shift in control and LPC-treated slices. Scale bar equals120 microns. (Magnification 20x). (B) Quantitative analysis of the different microglial phenotypes, including ramified, bushy and amoeboid. Results are mean ± SEM. $^{\#\#}P<0.01$ vs. Amoeboid Control. $^{*}P<0.05$ vs. respective Control.

Given microglia activation-associated properties in demyelinating conditions such as MS disease, activated/amoeboid microglia rapidly arrive to demyelinated lesions and clears the extracellular bulk of damaged myelin vesicles. Knowing this and taking in consideration the BASHY specificity for myelin debris, we next evaluated BASHY labelling of particular microglial cell morphologies. For this, fixed slices immune-stained with anti-Iba1 antibody were posteriorly incubated with BASHY dyes.

As depicted in **Figure III. 4A** and **B**, BASHY molecules were internalized by microglial cells with an increased presence in demyelinating conditions and following microglia morphological changes: from a more ramified microglia, to a bushy microglia but being particularly internalized by amoeboid microglia (~70%, P<0.05), resembling the characteristic “foamy phagocytes” found in MS cases (Kuhlmann et al., 2017).

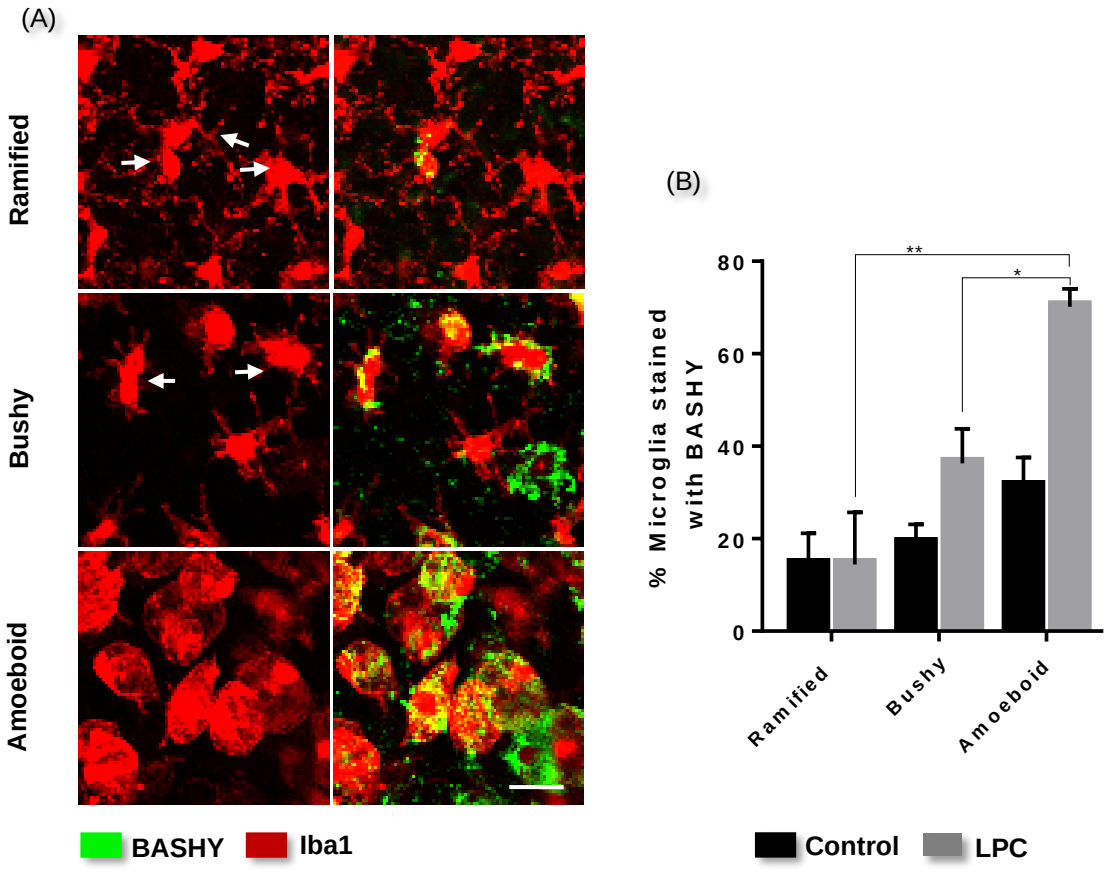


Figure III. 4. **BASHY molecules are increasingly present along microglial changes, barely seen in ramified but markedly staining amoeboid lipid-rich (foamy) microglia**. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) at 7 day *in vitro* (DIV) for 18 hours, to induce demyelination. Slices were posteriorly maintained in NB medium for a recovery period of 30h. 48h-post LPC treatment, fixed slices were immune-stained for microglia (Iba1, red) and further stained with BASHY dyes (BASHY, green). (A) Representative images of the various microglial phenotypes (white arrows) and respective co-localization with BASHY. Scale bar equals 20 microns. (Magnification 20x) (B) Quantitative analysis of the percentage of co-localization of microglia with BASHY. Results are mean ± SEM. *P<0.05 and **P<0.01 vs Amoeboid LPC.

4. BASHY preference for activated microglia

Activated microglia also undergo functional changes accompanied by differences in gene expression, transitioning from an homeostatic and surveillant stage to various stages of activation and reactivity with opposing roles as diseases progress (Lively and Schlichter, 2018). The first and most-known microglial response to CNS damage is the release of pro-inflammatory and other cell-damaging mediators. Particularly, myelin engulfment by microglia was seen to promote the release of substantial amounts of NO, TNF-α and IL-1β (K. Williams, 1994). Although such pro-inflammatory phenotype - usually categorized into an over simplistic M1 phenotype - is necessary for CNS protection, excessive neuroinflammatory response may also exacerbate damage leading to demyelination and neurodegeneration.

As previously described, after myelin internalization, microglia were observed to evolve into a less inflammatory or even reparative phenotype – commonly characterized as M2 state. Through the release of anti-inflammatory cytokines and growth factors, microglia is an active player participating in neuroprotective processes as well as in CNS repair (Miron et al., 2013).

Taking into account this differential microglial activation, we decided to evaluate microglial phenotype in our *ex vivo* demyelinating model. For that, we assessed microglia from two different time points – 18h- and 48h-post LPC incubation – and, finally, evaluated BASHY preference for either pro- or anti-inflammatory microglial phenotypes.

In order to fulfill this task, 18h- and 48h-post LPC incubation, control and treated slices were fixed and immuno-stained for microglia (Iba1), Arginase 1 (Arg1) – common marker for anti-inflammatory microglia – and for inducible nitric oxide synthase (iNOS) – characteristic of pro-inflammatory microglial cells. Afterwards, slices were incubated with BASHY molecule.

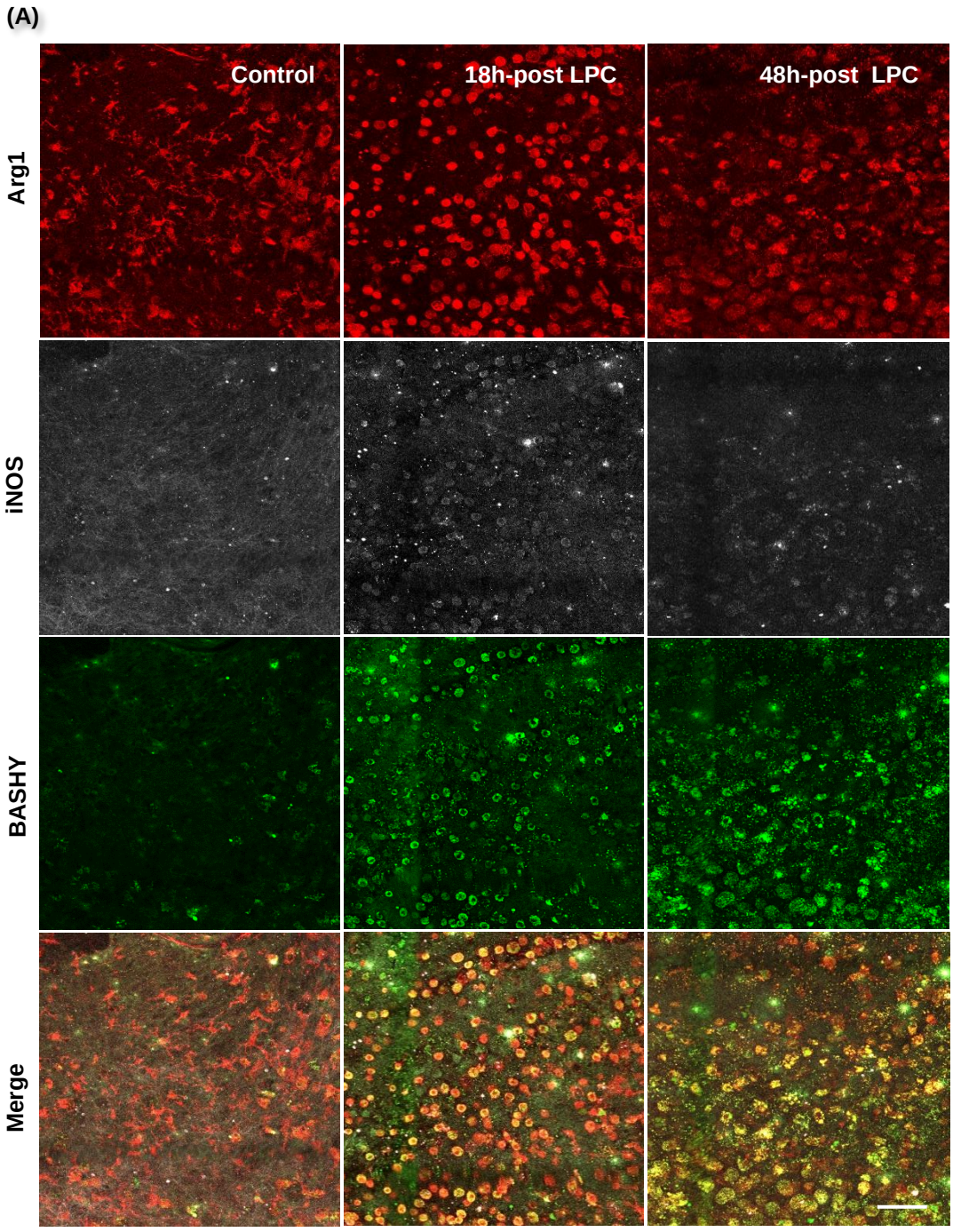
As depicted in **Figure III.5A and B,** demyelinating insult induced differences in cell phenotype, when compared to the controls and also along both time points after LPC-treatment. Although the majority of the cells were $Arg1^+$ in all conditions, iNOS expression was specially elevated in 18h-post LPC treated slices ($P<0.001$). From 18h- to 48h-post LPC treatment, we observed a marked reduction in $iNOS^+$ cells which may suggest a shift in phenotype from a pro-inflammatory to a more anti-inflammatory one over time.

Curiously, at both time points we did not find microglial cells expressing iNOS marker alone. Instead, we observed only two populations, $Arg1^+/iNOS^-$ and $Arg1^+/iNOS^+$, which latter decreased from 18h to 48h after LPC incubation, as a result of the loss of the pro-inflammatory marker.

Regarding BASHY co-localization, we observed internalization of this fluorescent molecule in both $Arg1^+/iNOS^+$ and $Arg1^+/iNOS^-$ microglia at each time points. So, focusing on the phenotype of $BASHY^+$ cells (**Figure III.5C**), we observed that 18h after LPC-treatment, ~45% of the $BASHY^+$ cells are in a more pro-inflammatory state ($P<0.001$), whereas at 48h ~90% of $BASHY^+$ microglia are in a more anti-inflammatory phenotype. This transition is accompanied with the loss of iNOS marker, although maintaining the increased number of cells expressing Arg1.

Overall, in line with recently published results from Lloyd and colleagues, under demyelination we observed a shift in microglial phenotype over time. In our model, at 18h cells must be in a transient time point from an earlier homogeneous pro-inflammatory population that eventually

evolves to a mixed population co-expressing both anti- and pro-inflammatory markers until it forms a late anti-inflammatory microglial population. Importantly, such phenotypic changes were accompanied by BASHY labelling (**Figure III.5C**).



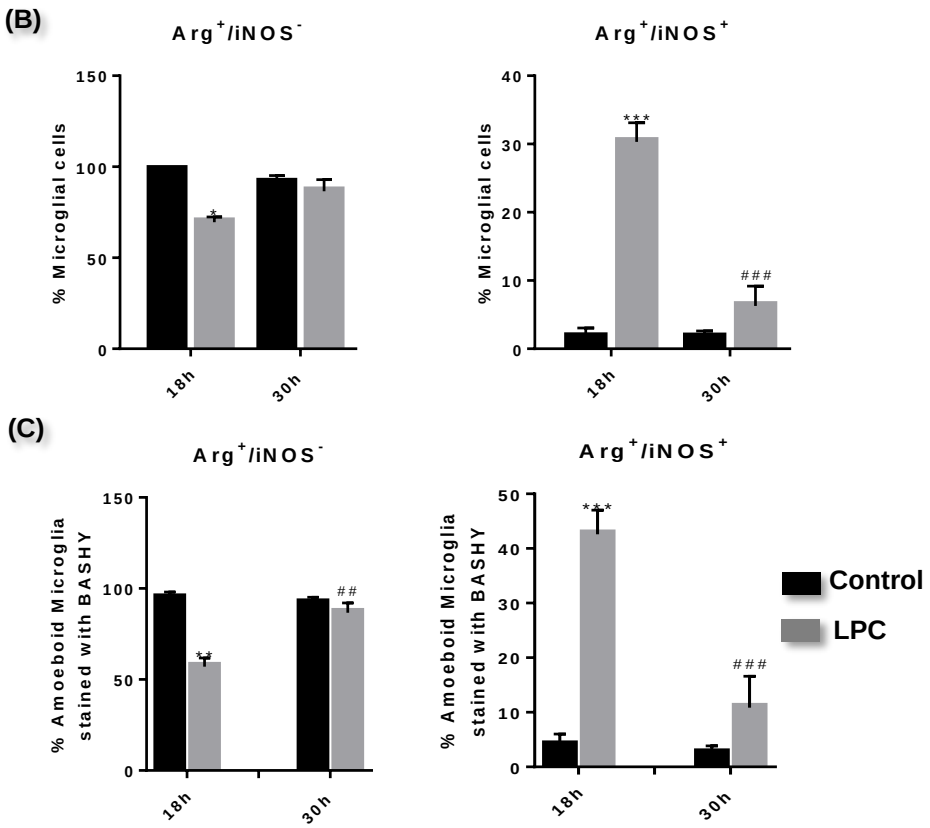


Figure III. 5. LPC induces an early pro-inflammatory/M1-like microglial state with both arginase (Arg1) and inducible nitric oxide synthase (iNOS) expression that switches to an M2-like Arg1-expressing state over the course of demyelination. Moreover, microglial phenotypic changes are accompanied with BASHY labelling. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) at 7 day *in vitro* (DIV) for 18 hours, to induce demyelination. After that slices were fixed (1) or maintained in NB medium for a recovery period of 30h (2) and then fixed for immunohistochemistry analysis. After fixation slices were triple stained to observe microglial anti-inflammatory marker Arginase, (Arg1, red), inducible nitric oxide synthase, a common marker of inflammatory microglia (iNOS, white), and further co-localization with BASHY molecules, (BASHY, green); (A) Representative images of microglial changes in phenotype over time with BASHY co-localization with activated microglia, both $Arg1^+$ and $iNOS^+$. (B) and (C) Quantitative analysis of the percentage of total microglia and $BASHY^+$ microglia, respectively, with both phenotypes at each time point. Scale bar equals 120 microns. Results are mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. 18h Control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. 18h LPC

5. Assay of a new BASHY-associated compound to target activated and lipid-rich microglia

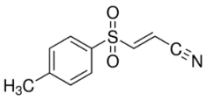
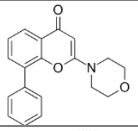
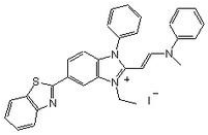
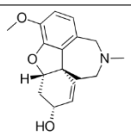
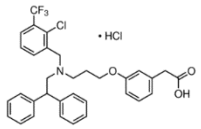
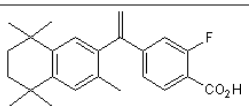
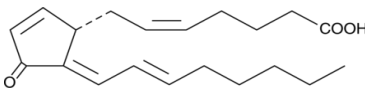
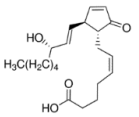
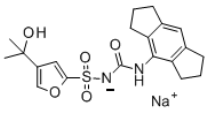
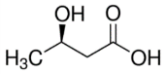
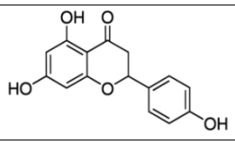
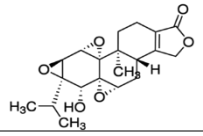
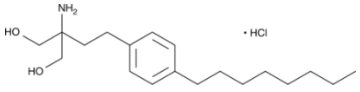
Understanding the different timings of phenotypic changes throughout demyelination is vital for the identification of new therapeutic strategies to either reduce secondary damage or enhance regeneration. Indeed, potentiating cell rearrangement towards a regenerative CNS protective or M2 microglial state proved to be fundamental for remyelination to occur and could be a promising therapeutic intervention in the context of demyelinating diseases (Miron et al., 2013). Taking into account the latest results and knowing that BASHY molecule can be used as a drug delivery vehicle, we decided to conjugate BASHY molecule with a specific microglial inflammatory modulator in order to decrease their inflammatory response, facilitating OPC survival and consequently *de novo* synthesis of myelin sheaths.

For that, after extensive research in the literature we identified several possible modulating molecules for the different timings of microglial activation (**Table III.1**). From a long list of molecules we decided to proceed the work using galantamine, not only because it was a well described allosteric activator of the cholinergic inhibitory pathway thus having powerful anti-inflammatory properties *in vivo* and *in vitro* (Liu et al., 2018;Wang et al., 2019) , but also because it was chemically suitable to be linked to BASHY molecule. With this in mind, galantamine was linked to our BASHY vehicle, giving rise to FFS2B molecule (**Figure III.6A**).

In order to evaluate if BASHY maintained its specificity to phagocytic activated microglia in spite of its conjugation with galantamine, Control and LPC-treated slices were incubated with FFS2B and analyzed 18h-post LPC incubation. Slices were posteriorly immune-stained with Arg1 marker for activated microglia in order to confirm its internalization by microglial cells (**Figure II.1**).

As Illustrated in **Figure III.6B,** FFS2B maintained the same specificity for lipid debris as previously seen for BASHY, also being internalized by amoeboid microglial cells, corroborated by the co-localization of FFS2B with Arg1 marker.

Table III.1. List of potential molecules for the modulation of microglia along demyelination.
The table highlights the name function and respective chemical representation of the different molecules chosen for each step of microglial polarization

TIMEPOINT	DESIGNATION	FUNCTION	CHEMICAL REPRESENTATION	REFERENCE
M1 POLARIZATION (INITIAL MYELIN PHAGOCYTOSIS)	Bay 11-7082	NF-kB inhibitor		Ref (Sun et al., 2010)
	LY-294002	NF-kB inhibitor		Ref (Sun et al., 2010)
	AKt inhibitor IV	NF-kB inhibitor		Ref (Sun et al., 2010)
	Galantamine	Indirect inhibitor of NF-kB via allosteric activation of α7-nACh receptors		Ref (Liu et al., 2018)
SHIFT M1/M2 (MYELIN PROCESSING)	GW3965 hydrochloride	LXR agonist		Ref (Secor McVoy et al., 2015)
	Fluorobexaroteno	RXR agonist		Ref (Secor McVoy et al., 2015)
	15 pgj2 15-deoxy-Δ12,14-Prostaglandin J2	PPAR γ agonist		Ref (Diab et al., 2002)
	PGA2 prostaglandin A2	PPAR γ agonist		Ref (Storer et al., 2005)
RE-SHIFT TO M1 STATE (FOAMY PHASE)	MCC950	NLRP3 inhibitor		Ref (Coll et al., 2015)
	BHB	NLRP3 inhibitor		Ref (Youm et al., 2015)
OTHER ANTI-INFLAMMATORY MOLECULES	Narigenin	Inhibitor of LPS-dependent JNK activation		Ref (Zhang et al., 2018)
	Triptolide (pg490)	Nf-Kb inhibitor		Ref (Zhou et al., 2003)
	FTY720 fingolimod	Increases expression of NPC1		Ref (Blom et al., 2010)

NF-kB- factor nuclear kappa B; α7-nACh- alpha-7 nicotinic acetylcholine receptor; LXR-liver X receptor; RXR- retinoid X receptor; PPAR- peroxisome proliferator-activated receptor; NLRP3- NLR family pyrin domain containing 3; NPC1-Niemann-Pick type C disease 1.

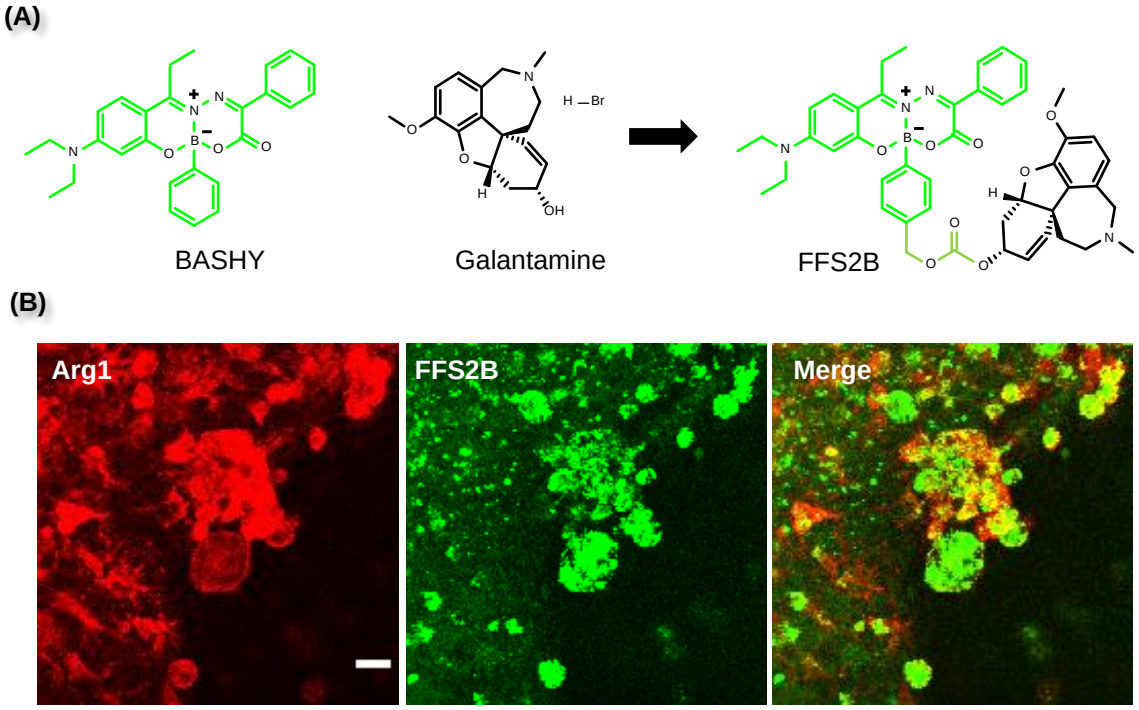


Figure III. 6. Schematic representation of FFS2B compound synthesis and representative images of its internalization by microglia. (A) Schematic representation of FFS2B molecule that results from the binding of BASHY with galantamine. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) and FFS2B at 7 day *in vitro* (DIV) for 18 hours, to induce demyelination. After slices were fixed and immune-stained for anti-inflammatory microglia marker, arginase-1 (Arg1). (B) Representative images of co-localization of FFS2B (FFS2B, green) with microglia (Arg1, red). Scale bar equals 10 microns.

6. LPC-induced synaptic impairment is transiently attenuated by FFS2B and galantamine

Loss of healthy myelin sheaths around the axon is strongly associated with neuronal damage and synaptic dysfunction, both exacerbated by the inflammatory environment resulting from glial reactivity in these conditions (Dutta et al., 2011).

Recently, chronic administration of galantamine in *in vivo* models of neuroinflammation had beneficial effects on synaptic function, preventing neurodegeneration and the associated reduction of both synaptophysin and PSD-95 proteins (Liu et al., 2018). In line with this, we decided to evaluate neuronal dysfunction in our *ex vivo* demyelinating model and detect if it could be prevented with FFS2B treatment. To do so, control and demyelinated slices were incubated with BASHY (non-treated slices), BASHY + galantamine or FFS2B for 18h or 48h (**Figure II.1**). mRNA expression levels of post- and pre-synaptic marker proteins, e.g. postsynaptic density protein 95 (PSD-95) and synaptophysin, respectively, were analyzed by qRT-PCR at 18- and 48h-post LPC incubation.

Consistent with previous studies (unpublished data, Santos, G. 2018. PhD thesis), as depicted in **Figure III.7A and B**, LPC-mediated demyelination had a negative role in synaptic plasticity. Indeed, we observed a continuous decrease in mRNA expression of synaptophysin

over time. Specifically, 48h-post LPC induction we observed a significantly reduction of this pre-synaptic marker by almost 70% (P<0.01), when compared to non-treated controls (**Figure III.7A**). Demyelinating effect in PSD-95 expression, on the other hand, was delayed. Although we observe slight increase in PSD-95 gene expression in early demyelinated slices (1.96-fold) this compensatory effect is lost at 48h where we observe a decrease by around 50% (0.51-fold) when compared to non-treated controls (**Figure III.7B**).

Curiously, at early demyelination (18h) both treatments with BASHY + galantamine or FFS2B prevented the loss of synaptophysin gene expression, maintaining the expression levels near to non-treated controls. Similarly, treatment with FFS2B and BASHY + galantamine equally increased PSD-95 gene expression either in control and demyelinated slices, when compared to respective non-treated controls.

However, 48h-post LPC induction, the effect of both treatments was lost and therefore could not prevent the loss of both synaptophysin and PSD-95 expression, suggesting that both treatments may only temporarily prevent LPC-induced synaptic and neuronal injury.

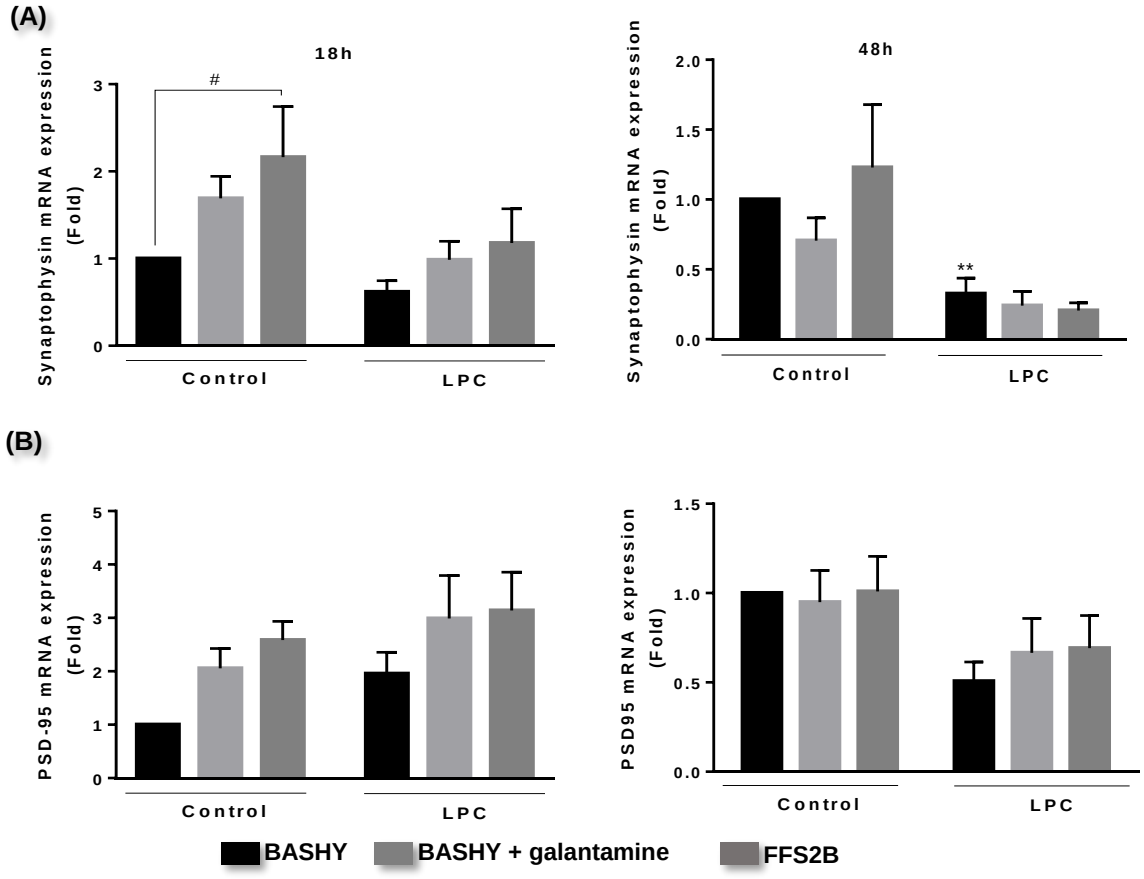


Figure III. 7. LPC-induced synaptic and neuronal dysfunction is ameliorated with FFS2B and galantamine only for short periods after treatment. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) and FFS2B at 7 day in vitro (DIV) for 18 hours to induce demyelination Relative (A) synaptophysin and (B) postsynaptic protein density 95 (PSD-95) mRNA levels were determined by qRT-PCR with the ΔΔCT method at 18h- and 48h-post LPC treatment. The results are normalized to β-actin. Results are expressed as mean ± SEM of n=9 per group. **P<0.01 vs. respective Control; #P<0.05 vs. Control BASHY

7. LPC-induced myelin loss and oligodendrogenesis impairment is prevented by galantamine

Another feature of myelin disorders is the loss of mature OLs, the myelinating cells of the CNS, and the recruitment of progenitors NG2 cells (Moyon et al., 2015). Indeed, OPCs tend to migrate and accumulate in lesioned areas in order to differentiate into new OLs allowing neuronal functional recovery through remyelination. However, accumulation of degenerated myelin and glial-mediated inflammatory imbalance resulting from extensive demyelination are known to inhibit OPCs migration, proliferation and differentiation, strongly promoting oligodendrogenesis impairment (Plemel et al., 2013;Lampron et al., 2015;Blomgren and Hagberg, 2006). Knowing this, we wanted to evaluate whether treatment with galantamine or FFS2B could prevent oligodendrogenesis impairment. For that, expression of MBP and NG2, markers of mature oligodendrocyte and OPCs, respectively, was analyzed by qRT-PCR.

In line with previous results from our group (unpublished data, Santos, G. 2018. PhD thesis)**,** LPC insult reduced by around 50% the MBP mRNA expression levels in non-treated slices at 48h, which was not evident in the earliest time point (**Figure III.8A)**. Moreover, as a result of myelin insult and OL's loss, it was observed a significant increase of NG2 expression levels (9.14-fold, $P<0.01$) only at 18h, in non-treated demyelinated slices when compared to non-treated controls. This is indicative of OPC proliferation and accumulation, as an attempt to promote oligodendrogenesis and restore myelin sheaths.

Interestingly, targeting of microglia with FFS2B had no effects on myelin loss and oligodendrogenesis as no differences were observed in gene expression between FFS2B and non-treated slices either in Control or LPC conditions. Notwithstanding, galantamine treatment seem to prevent the loss of mature OLs 48h-post LPC incubation, observed by the slight increase of MBP mRNA expression (0.93-fold) when compared to non-treated demyelinated slices (0.49-fold). Curiously, although preventing OL's loss, NG2 expression levels were markedly increased after treatment with galantamine, at both 18h (19.22-fold, $P<0.01$) and 48h (2.38-fold, $P<0.01$), when compared to non-treated demyelinated slices.

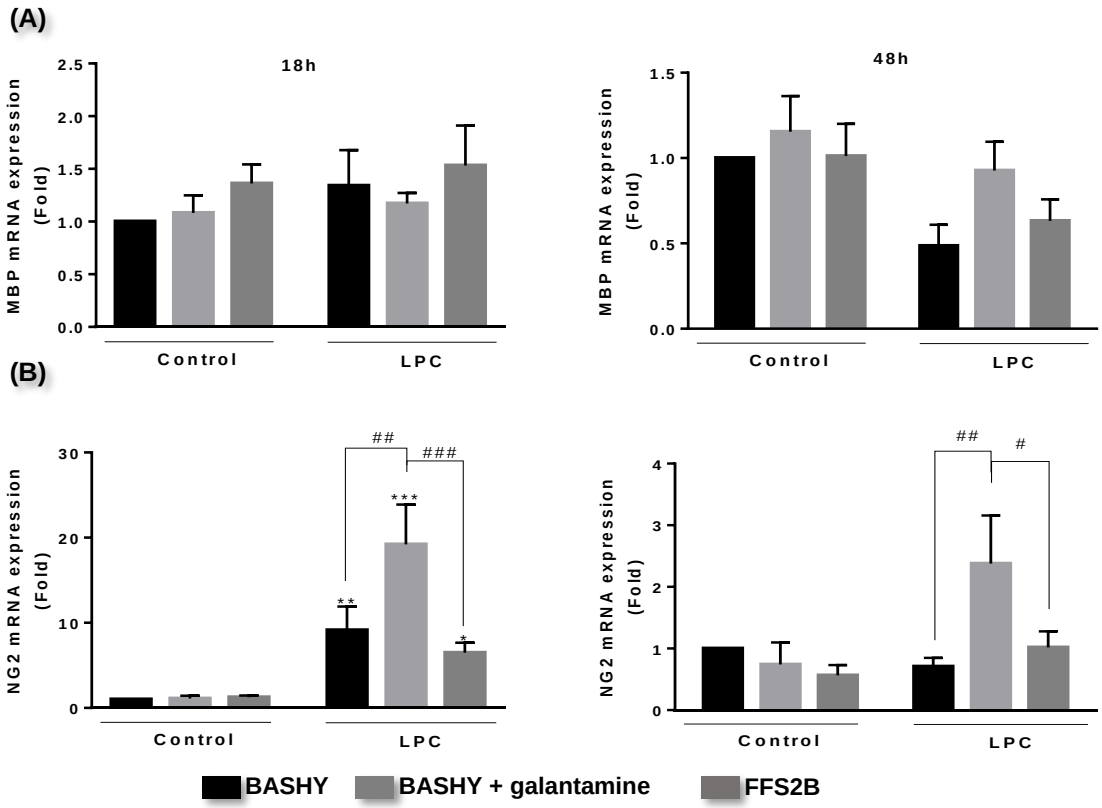


Figure III. 8. Galantamine treatment prevents LPC-induced myelin and oligodendrogenesis impairment. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) and FFS2B at 7 day in vitro (DIV) for 18 hours to induce demyelination Relative (A) myelin basic proteins (MBP) and (B) neuron-glia antigen 2 (NG2) mRNA levels were determined by qRT-PCR with the ΔΔCT method at 18h- and 48h-post LPC treatment. The results are normalized to β-actin. Results are expressed as mean ± SEM of n=9 per group *P<0.05, **P<0.01, ***P<0.001 vs. Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. LPC BASHY + galantamine

Indeed, these data suggests a possible role of galantamine in preventing demyelination as well as in exacerbating OPC differentiation thus promoting subsequent remyelination.

8. FFS2B exacerbates early LPC-induced inflammatory response whereas galantamine activates late NLRP3-mediated response

As already discussed, together with neuronal injury and oligodendrogenesis impairment, myelin degeneration is associated with a strong inflammatory response, inducing the release of pro-inflammatory mediators and cytokines as a result of glial activation and reactivity (Ponath et al., 2017;Moore et al., 2011;Sun et al., 2010). With this in mind and taking into account the anti-inflammatory effect of *in vivo* administration of galantamine, through inhibition of inflammatory molecules – NF-kB – and its downstream production of TNF-α and IL-1β, we further decided to evaluate the effect of FFS2B on demyelination-associated inflammatory response. Again, total RNA was extracted from both control and demyelinated slices in all conditions at both time points and gene expression pro-inflammatory cytokines TNF-α and IL-1β, and anti-inflammatory cytokine IL-10 was analyzed by qRT-PCR.

Expectably, in non-treated demyelinated slices we observed an early significant increase of TNF-α (1.85-fold, P<0.05) and IL-1β (6.12-fold, P<0.05) mRNA expression levels when compared to non-treated controls (**Figure III.9A**). Parallel to the increase of such inflammatory

cytokines, 18h-post LPC induction also induced a marked increase of IL-10 mRNA expression levels in non-treated demyelinated slices (10.85-fold, $P<0.01$), when compared to non-treated controls, to counteract the observed inflammatory response (**FigureIII.9B**).

Interestingly, treatment with FFS2B only seemed to have an effect on the early release of the first line proinflammatory cytokines. Indeed, after 18h not only we observe a marked increase in TNF-α expression levels (4.77-fold, $P<0.01$), on demyelinated slices, but also IL-1β mRNA levels showed a tendency to be augmented upon LPC induction, when compared to respective non-treated demyelinated slices **(Figure III.9A and B)**.

Unexpectedly, after galantamine treatment, TNF-α mRNA expression at 18h was also significantly augmented in demyelinated slices (4.73-fold, $P<0.01$) when compared to non-treated demyelinated ones (**Figure III.9A**). Moreover, IL-1β mRNA expression levels showed a tendency to be increased 48h after galantamine incubation (3.2-fold), when compared to non-treated demyelinated slices (**Figure III.9B**), suggesting a possible activation of a delayed inflammatory response which was accompanied by anti-inflammatory IL-10 cytokine expression. Although not significant galantamine enhanced IL-10 expression (4.53-fold) when compared to non-treated demyelinated slices 48h-post LPC induction (**Figure III.9C**).

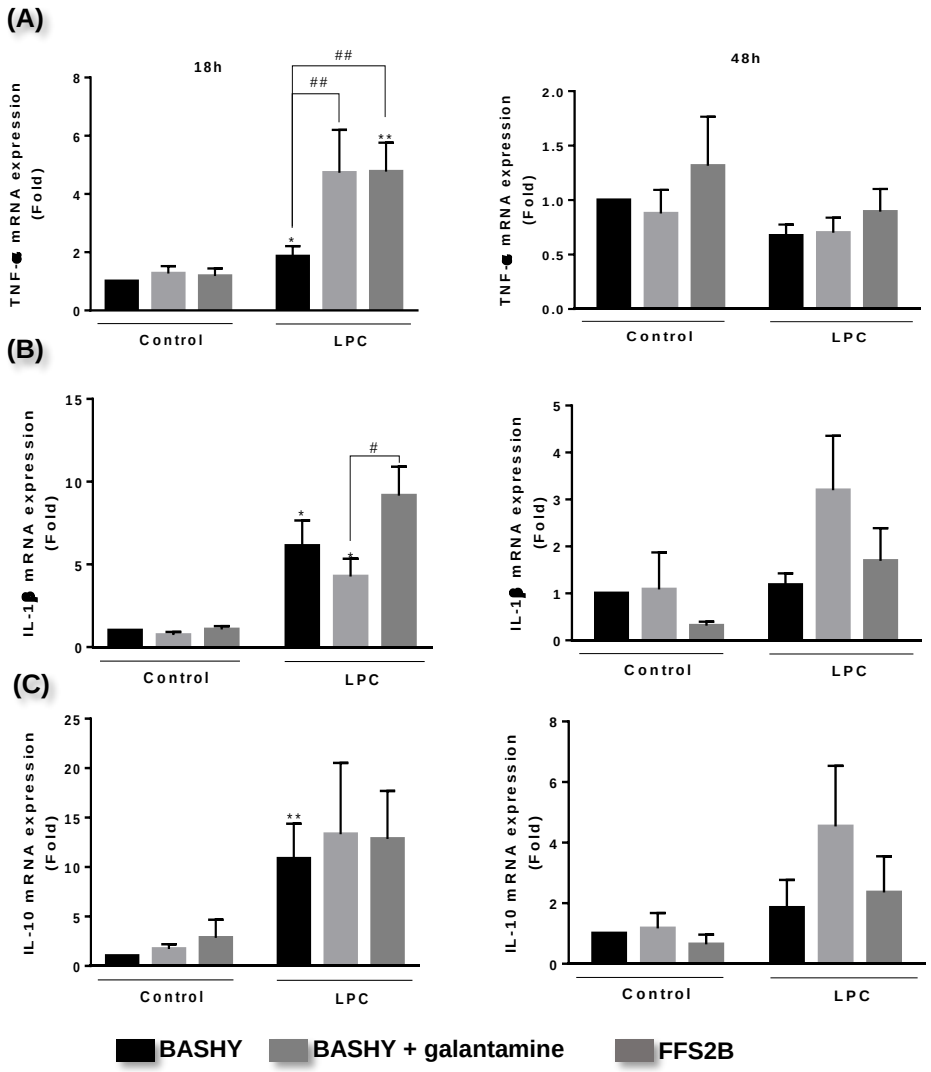


Figure III. 9. FFS2B potentiates early pro-inflammatory response induced by LPC, while galantamine exacerbates a later inflammatory response. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) and FFS2B at 7 day in vitro (DIV) for 18 hours to induce demyelination. Relative (A) TNF-α and (B) IL-1β and (C) IL-10 mRNA levels were determined by qRT-PCR with the ΔΔCT method at 18h- and 48h-post LPC treatment. The results are normalized to β-actin. Results are expressed as mean ± SEM of n=9 per group. *P<0.05, **P<0.01 vs. respective Control; $^{\#}$P<0.05 vs. LPC BASHY + galantamine, $^{\#\#}$P<0.01 vs. LPC BASHY

Next, as already described, myelin-derived cholesterol excessive accumulation in foamy phagocytic cells after demyelination induce the activation of NLRP3 inflammasome complex promoting the release of its downstream IL-1β and IL-18 cytokines (Cantuti-Castelvetri et al., 2018;Duewell et al., 2010). Furthermore, NLRP3 activation is, in turn, associated with neurodegenerative diseases contributing to the exacerbation of neuroinflammation, demyelination and neurodegeneration (Gris et al., 2010). With this in mind, in order to evaluate the effect of FFS2B treatment in NLRP3 activation and in the subsequent release of associated pro-inflammatory cytokines, total mRNA of both control and demyelinated slices in all conditions and time points was isolated, and gene expression of NLRP3 inflammasome and pro-inflammatory cytokine IL-18 was analyzed by qRT-PCR.

As illustrated in **Figure III.10A.** LPC induced a 90% augment of NLRP3 gene expression at 48h (1.90-fold), when compared to control slices which was not accompanied by the expression of its downstream cytokines (**Figure III.10B** and **Figure III.9B).**

Curiously, FFS2B-treated slices did not alter NLRP3 expression levels and subsequent cytokine expression (**Figure III.10**). However, galantamine treatment seemed to strongly induce NLRP3 inflammasome along the course of demyelination, corroborated by the significant increase in NLRP3 mRNA expression levels (11.87-fold, P<0.05) at 18h and by the slight increase, yet not significant, at 48h when compared to non-treated demyelinated slices (3.83-fold) (**Figure III.10A**). Consequently, we observed a slight augment of IL-18 mRNA expression (1.80-fold) at 48h in demyelinated slices treated with galantamine when compared to the non-treated demyelinated ones (**Figure III.10B**). Furthermore, NLRP3 augmented expression could also explain IL-1β increased tendency observed at 48h in demyelinated slices treated with galantamine (**Figure III.9B**).

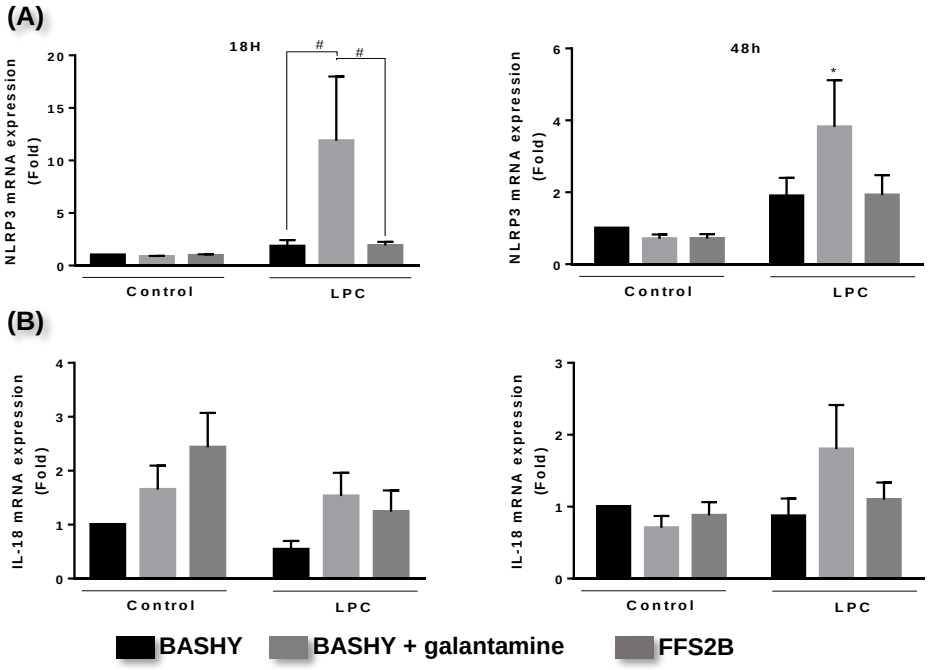


Figure III. 10. Galantamine is a strong inducer of NLRP3 inflammatory pathway. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) and FFS2B at 7 day in vitro (DIV) for 18 hours to induce demyelination. Relative (A) NLRP3, (B) IL-1β and (C) IL-18 mRNA levels were determined by qRT-PCR with the ΔΔCT method at 18h- and 48h-post LPC treatment. The results are normalized to β-actin. Results are expressed as mean ± SEM of n=9 per group. *P<0.05 vs.respective Control; #P<0.05 vs. LPC BASHY + galantamine

9. Galantamine exacerbates LPC-induced microglial activation and CR3-mediated myelin phagocytosis

As discussed and analyzed in the previous section, myelin uptake after demyelination induce phenotypic alterations in activated microglia over time. Knowing this, we wanted to assess FFS2B-mediated role in microglial inflammatory response. To do so, gene expression of anti- and pro-inflammatory microglial markers, arginase-1 (Arg1) and inducible nitric oxide synthase (iNOS) respectively, was analyzed by qRT-PCR.

Consistent with the above results concerning microglial phenotypic changes, LPC induced an early and significant increase in the expression of anti-inflammatory marker, Arg1, (3.44-fold, P<0.05) which was maintained for the remaining 30h (3.17-fold, P<0.05), when compared to non-treated controls **(Figure III.11A)**. Regarding iNOS expression, on the other hand, we observed a vast augment of expression levels 18h-post LPC induction when compared to non-treated control slices (157.2-fold, P<0.01), that decreased over the following 30h, which is also in line with the above described immunohistochemistry results **(Figure III.11B)**.

Surprisingly, following FFS2B treatment no changes in gene expression were observed for each of the analyzed genes as it can be seen by the lack of differences between treated and non-treated slices in all conditions. Treatment with galantamine, however, significantly increased mRNA levels of Arg1 in demyelinated slices at 18h when compared to non-treated demyelinated slices (16.11-fold, P<0.05), also showing a tendency to be augmented in demyelinated slices when compared to non-treated demyelinated ones at 48h **(Figure III.11A)**. Furthermore, after galantamine treatment, although not significant, iNOS expression levels were also slight increased (326-fold) in earlier time points, when compared with non-treated demyelinated slices (157-fold), while no changes were observed at 48h.

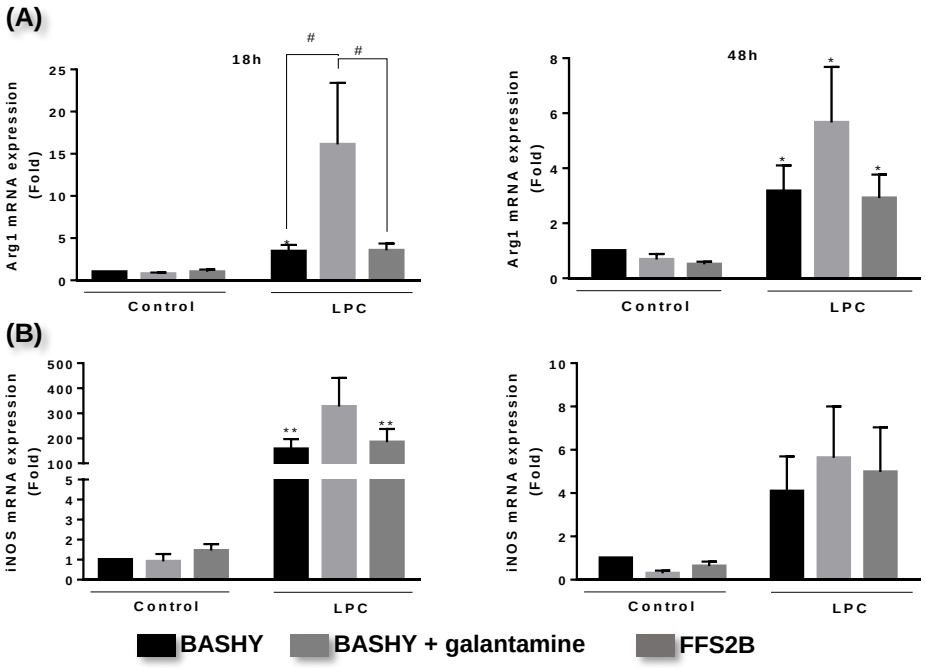


Figure III. 11. Galantamine but no FFS2B enhanced microglial activation induced by demyelination at both timepoints. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) and FFS2B at 7 day in vitro (DIV) for 18 hours to induce demyelination Relative (A) Arg1 and (B) iNOS mRNA levels were determined by qRT-PCR with the ΔΔCT method at 18h- and 48h-post LPC treatment. The results are normalized to β-actin. Results are expressed as mean ± SEM of n=9 per group. *P<0.05, **P<0.01, vs. Control; #P<0.05 vs. LPC BASHY + galantamine

Finally, a hallmark of activated microglia in demyelinating lesions is the overexpression of myelin-specific receptors thus favoring oligodendrogenesis and remyelination through myelin phagocytosis. In line with this we decided to assess FFS2B role in microglial expression of myelin-specific receptors. For that we finally evaluated gene expression of two receptors involved in the uptake of myelin debris, Trem2 and CR3, by qRT-PCR.

As depicted in **Figure III.12**, LPC induced a sustained downregulation of Trem2 expression levels from 18h (0.23-fold, P<0.001) until 48h (0.36-fold, P<0.01) after demyelination, when compared to non-treated respective controls. On the other hand, LPC induced a significant increase of CR3 mRNA expressing levels at 18h (2.82-fold, P<0.001), when compared to non-treated controls, that was reduced at 48h (0.77-fold, P<0.05)

Following FFS2B treatment no changes in gene expression of myelin receptors were observed for each of the analyzed genes as can be observed by the lack of differences between treated and non-treated slices in all conditions.

Surprisingly, galantamine treatment although did not affect Trem2 expression, induced a slight increase in CR3 gene expression at 18h (5.12-fold). CR3 increased expression was maintained in the following 30h corroborated by the observed significant augment in gene expression (1.99-fold, P<0.01) 48h-post LPC induction, when compared to non-treated demyelinated slices.

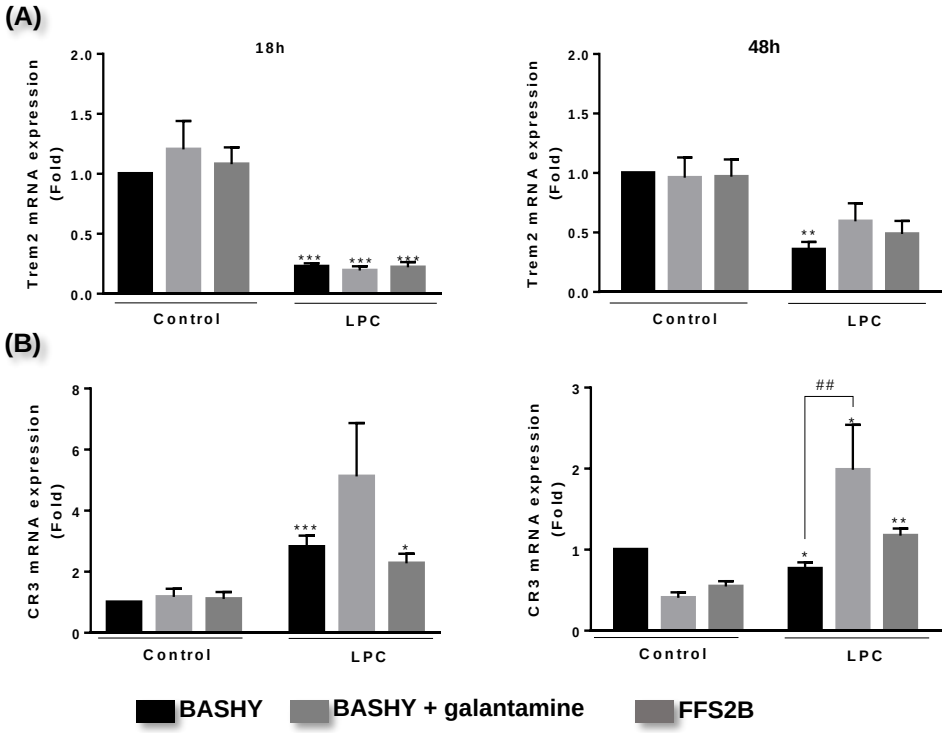


Figure III. 12. Galantamine increases LPC-induced CR3 mediated myelin phagocytosis. Organotypic cerebellar slice cultures (OCSC) were incubated with lysophosphatidylcholine (LPC) and FFS2B at 7 day in vitro (DIV) for 18 hours to induce demyelination. Relative (A) Trem2 and (B) CR3 mRNA levels were determined by qRT-PCR with the ΔΔCT method at 18h- and 48h-post LPC treatment. The results are normalized to β-actin. Results are expressed as mean ± SEM of n=9 per group. *P<0.05, **P<0.01, ***P<0.001 vs.respective Control; ##P<0.05 vs. LPC BASHY

Overall our results suggest that galantamine alone has a higher beneficial effect on LPC-induced demyelination than if bound to BASHY dye, which may indicate that this later combined compound is masking galantamine ability to interact with its specific receptors at cell surface and therefore losing its reported anti-inflammatory effect.

IV. Discussion

Myelin is a specialized and lipid-rich membrane that plays essential functions in the brain, namely in promoting a faster neuronal conduction, axonal function and stability. Its loss – or demyelination – is indeed a critical phenomenon and the known cause of neurodegeneration and associated motor, sensor and cognitive dysfunctions in demyelinating diseases like MS (Lee et al., 2012b;Bando et al., 2008;Trapp and Nave, 2008). Demyelination results from a succession of myelin structural alterations that are identical among demyelinating models. As mentioned before, it is characterized by an initial loss of myelin compaction – from the inside to the outside - followed by a subsequent full destruction of myelin layers into a vesiculated network of lipid-rich myelin debris surrounding the axons (Weil et al., 2016;Cedric S. Raine and and Claude P. Genain, 1999). Following demyelination, there is usually a re-ensheathment of denude axons – remyelination – which restores axonal function while preventing at the same time further neuronal injury (Franklin and Ffrench-Constant, 2008). However, particularly in progressive types of MS, uncontrolled myelin loss and upcoming excessive inflammatory environment are known to impair this restorative event (Jacques Penderis, 2003). Therefore, studying myelin alterations and understanding the demyelinating process is of great interest to better comprehend myelin-related diseases and promote remyelinating-based therapies.

In line with this, in an *in vivo* system magnetic resonance imaging (MRI) is extensively used for the identification of myelin alterations. Indeed, MRI-mediated detection of myelin disturbances is based on differences in diffusion and relaxation time of water molecules among different lipidic arrangements. Although sensitive to myelin degeneration, there is also lack of specificity for lesions given that water disturbances can also be due to inflammation or edema (Brugarolas et al., 2018;Vavasour et al., 2011). Besides MRI, positron emission tomography (PET) is another neuroimaging system which also exploits myelin high lipid content to locate demyelinating lesions. Notwithstanding, there is also associated background resulting from nonspecific lipid binding (Brugarolas et al., 2018).

Furthermore, for post-mortem studies there are also current histological and immunohistochemical markers used for the identification of the different stages in the demyelinating process. Indeed, compact myelin sheaths are commonly stained with Luxol Fast Blue dye (Carriel et al., 2017) or identified using an antibody against MBP, the main protein involved in myelin compaction. On the other hand, earlier stages of myelin decompaction are identified by an antibody against MBP residues 82–88, the QD9 epitope (Weil et al., 2016;Matsuo et al., 1997) while final stages of myelin degeneration with the formation of myelin-derived lipid aggregates are commonly stained with Oil Red O and Nile Red dyes, both non-specific markers that stain for general lipids and lipid droplets, respectively (Mehlem et al., 2013;Greenspan et al., 1985).

Overall, there is an urgent need for more specific biomarkers and as well as more specific indicators of the different stages/structures along the demyelinating processes, that may be used either in patients, live animal models and in post-mortem samples. As previously mentioned, a recently synthesized BASHY molecule, showed to be specific for non-polar lipid aggregates when

tested *in vitro* (Santos et al., 2016). So, in collaboration with P Gois and F Santos, we intended to test BASHY labeling, in an *ex vivo* demyelinating model of OCSC (Birgbauer, 2004), as a promising alternative for characterization of demyelination, particularly for the identification of the later stages of this process and possibly address the mechanisms of myelin processing during demyelinating and remyelinating events. Indeed, we began by analyzing BASHY fluorescent labeling after a demyelinating insult with LPC in the OCSC. Our first results showed a basal BASHY labeling in control slices that was significantly increased after inducing demyelination, suggesting a BASHY labeling of lipid structures that could possibly be degenerated myelin – non-compact or vesiculated - accumulated during ongoing demyelination (Weil et al., 2016).

Previous reports showed that vesiculated myelin are extensively phagocyted by microglial cells, which are the main glial cells responsible for myelin clearance. Interestingly, although not contributing significantly to myelin clearance, it has also been reported the presence of myelin-positive astrocytes in areas of active myelin breakdown (Lampron et al., 2015;Skripuletz et al., 2013;Ponath et al., 2017). Concordantly, we observed that BASHY labeling was slightly co-localizing with astrocytes but preferentially confined to microglial cells, further suggesting that BASHY was being specific for phagocyted myelin debris. As pointed out already, we know that myelin is mostly composed by cholesterol and once phagocyted, is degraded intracellularly via endosomal-lysosomal pathway (Rasband and Macklin, 2012;Mathews and Appel, 2016;Thelen and Zoncu, 2017;Ponath et al., 2017). In accordance we observed co-localization of our fluorescent molecule with cholesterol marker – filipin – and lysosome marker – lysotracker, corroborating BASHY specificity for myelin debris. These results also validate previous *in vitro* results from Santos and colleagues demonstrating BASHY discrimination between different lipid structures (Santos et al., 2016).

Furthermore, since we previously observed that BASHY colocalized preferentially with microglial cells, we next aimed to characterize BASHY specificity for the different stages of microglia activation. In fact, microglia response to CNS injuries or environment alterations requires cell reorganization and activation. Activation of microglia – characteristic of MS demyelinated lesions - is accompanied by intense morphological changes and alterations in proliferation, motility and enhanced phagocytic capacity (Stence et al., 2001). Similar to previous reports inducing demyelination with LPC in *in vivo* (Sheikh et al., 2009) and *ex vivo* models (unpublished data, Santos, G. 2018. PhD thesis), after LPC insult we detected a complete shift in microglial cell morphology from a ramified one to a fully amoeboid one, which is characteristic of activated microglia. Moreover, we also observed BASHY enhanced prevalence along microglial morphological changes, therefore being mostly internalized by phagocytic amoeboid/activated microglia.

Activated microglia can alternate between two opposing phenotypes either pro-inflammatory or anti-inflammatory and regenerative one. Currently, PET imaging is also being use for imaging of activated microglia *in vivo* using radioisotope labeled ligands to bind to the translocator protein-18 (TSPO), which is a mitochondrial molecule upregulated in microglia upon their activation and highly expressed in lesion-associated microglia (Tronel et al., 2017). Although, already used to

assess microglial activation in MS Patients (Airas et al., 2018), some limitations like nonspecific binding, TSPO human polymorphisms or low brain uptake are still needed to overcome. Given BASHY preference for activated microglia, we decided to evaluate the potential of this molecule as an alternative biomarker for a specific microglial phenotype. According to recent studies in an *ex vivo* model of demyelination, 12h-post LPC induction, pro-inflammatory microglia reached a maximum peak whereas at 24h-post LPC induction it was again observed a shift to a more anti-inflammatory microglia populations with regenerative and remyelinating properties (Lloyd et al., 2019). In line with this, we assessed microglial phenotypes in two time points, 18h and 48h after demyelination and further evaluated BASHY specificity for either one of the phenotypes. Consistent with the above findings, we found our results indicative of a sequential changing in phenotype over demyelination. Although we did not find microglial cells expressing iNOS marker alone, at earlier time points almost half the microglial population was $Arg1^{+}/iNOS^{+}$ while 48h after LPC insult this population was replaced by a more anti-inflammatory one only expressing the anti-inflammatory marker Arg1, as a result of the loss of the pro-inflammatory marker. In accordance with studies from Lloyd and colleagues, at 18h cells must be in a transient time point from an earlier homogeneous pro-inflammatory population that eventually evolves to a mixed population co-expressing both anti- and pro-inflammatory markers until it forms a late anti-inflammatory microglial population (Lloyd et al., 2019). Further, by analyzing BASHY co-localization with microglial cells at both timepoints we observed that BASHY labeling was accompanying these phenotypic changes, therefore was being internalized by both $Arg1^{+}/iNOS^{-}$ and $Arg1^{+}/iNOS^{+}$ microglial populations. Altogether, these latter results suggest that BASHY could not specify microglial phenotypes though had the potential for being used as a fluorescent label for phagocytic microglia, thus a biomarker for active demyelinating lesions that are under repair.

The balance of anti- versus pro-inflammatory microglial factors are determinants in the role of microglia over demyelination. Whereas polarization into pro-inflammatory microglia has been strongly associated with axonal degeneration, impaired oligodendrogenesis and remyelination failure (Campbell et al., 1998;Blomgren and Hagberg, 2006), anti-inflammatory microglia are implicated in regenerative events through the clearance of myelin debris and secretion of growth and neurotrophic factors. In fact *in vivo* analysis from Miron and colleagues showed that microglial shift into a more repairing and regenerative role was concomitant and necessary for remyelination to occur (Miron et al., 2013). Taking this into account and knowing that BASHY molecule offer the possibility to be linked to specific modulator molecules, we further decided to use BASHY as a delivery vehicle for the modulation of the initial inflammatory burst of microglia in favor of a more reparative one.

Following a thorough literature review, from all the potential molecules, galantamine stood up given its anti-inflammatory properties, ability to cross the blood brain barrier, know safety as being in use in Alzheimer´s patients to improve cognitive function and, possibly a major characteristic, its suitable structure for binding to BASHY molecule. Once again, in collaboration with P Gois and F Santos, a new BASHY-associated galantamine compound was synthetized – FFS2B – and we

assessed its role on our demyelinating *ex vivo* model, focusing on synaptogenesis, myelination and oligodendrogenesis, inflammatory outcome and microglial activation and phagocytosis.

Demyelination is characterized by severe synaptic loss. Indeed, according to our previous studies (unpublished data, Santos, G. 2018. PhD thesis), *ex vivo* LPC administration induced an apparent synaptic impairment revealed by down regulation of synaptophysin expression levels and by a late post-synaptic reduction. Interestingly, both treatments with either co-incubation with separated BASHY + galantamine and bound FFS2B transiently prevented synaptic dysfunction which goes in line with a recent study showing that galantamine administration *in vivo* was able to ameliorated the LPS-induced decrease of synaptic proteins (Liu et al., 2018). Additionally, as previously observed (unpublished data, Santos, G. 2018. PhD thesis) our results also showed a decrease on the number of mature OL after 48h of LPC injury indicative of loss of mature OLs. For remyelination, there is a recruitment of NG2 expressing OPC to the injured area that will differentiate into mature OL allowing functional recovery through remyelination (Franklin and Ffrench-Constant, 2008). Concordantly, after LPC injury we also observed a significant increase of NG2 expression levels only at 18h, when compared to controls, suggesting a rapid OPC proliferation and accumulation as an attempt to restore myelin sheaths. Curiously, treatment with BASHY + galantamine seemed to promote a continuous recruitment and accumulation of OPCs which therefore prevented loss of mature OL 48h-post LPC induction. With FFS2B treatment, on the other hand, we had no effects on myelin loss or oligodendrogenesis. These results suggest a possible role of galantamine in the regulation of myelination and oligodendrogenesis which therefore results in enhanced myelin recovery.

As pointed out already, myelin uptake after demyelination induce phenotypic alterations in activated microglia over time. In accordance with the above immunohistochemistry analysis, after LPC insult we observed an early activated microglia into a more pro-inflammatory state, expressing the anti-inflammatory marker, Arg1, but with substantially augmented iNOS expression levels when compared to respective controls. 48h-post LPC insult this pro-inflammatory microglia is further replaced by a more anti-inflammatory one, with reduced expression of iNOS, necessary to recover brain homeostasis. In recent studies using a cultured microglia model of HIV-associated dementia, galantamine showed inhibitory effects on the release of pro-inflammatory TNF-α and microglial cell activation (B. Giunta and D. Shytle, 2004). In comparison with the non-treated group, treatment with BASY + galantamine seemed to mediate an early reparative response by microglia, revealed by the significant augment of cells expressing Arg1. Surprisingly, FFS2B-mediated modulation of microglia showed no effects on the inflammatory outcome. This could be due to the fact that galantamine-mediated anti-inflammatory effect occurs through the allosteric activation of the extracellular α7 nicotinic acetylcholine receptors (Wang et al., 2007) and possibly galantamine as part of the FFS2B compound that is located in lipid droplets may have no function intracellularly. Furthermore, it must be taking into account that BASHY conjugation with galantamine could be masking the active site of galantamine, thus preventing its action on the specific receptors.

The marked inflammation following gliosis is a characteristic feature of demyelinating lesions. We previously showed that LPC-induced demyelination increased NF-kB activation with subsequent enhanced production of the first line pro-inflammatory cytokines, TNF-α and IL-1β (unpublished data, Santos, G. 2018. PhD thesis). Moreover, sustained demyelination with accumulation of excessive cholesterol in lysosomes showed to induce NLRP3 activation thus contributing to a late inflammatory response (Gris et al., 2010;Duewell et al., 2010). Here we observed enhanced expression of both pro-inflammatory cytokines at 18h and, parallel to that, a marked increase of IL-10 mRNA expression levels, possibly to suppress the observed inflammatory response. LPC-mediated demyelination had no effects on NLRP3 activation, but elicited a fast increase of IL-1β mRNA expression. Regarding inflammation, galantamine administration *in vivo* also showed to decrease NF-kB as well as IL-1β, TNF- α and IL-6 expression (Liu et al., 2018). Unexpectedly, FFS2B treatment seemed to potentiate the early pro-inflammatory response by enhancing the expression of TNF-α and IL-1β, 18h after LPC insult, while treatment with BASHY + galantamine had marked effects on late inflammatory response, mediated by the activation of NLRP3 inflammasome showed by an apparent increase of IL-18 and IL-1β expression, 48h-post LPC insult, suggesting an enhanced shift to the final stage of foamy microglia upon a demyelinating insult.

Another hallmark of microgliosis in demyelinating lesion is the over expression of myelin-specific receptors (e.g. Trem2 and CR3) (Cantoni et al., 2015;Friede, 1990) to enhance myelin clearance and favor remyelination. Indeed, after LPC insult, although we observed a sustained decrease of Trem2 expression, there was a vast increase in CR3 expression levels at 18h but significantly reduced 48h-post LPC insult, suggesting a transient augment of myelin phagocytosis via CR3 while there is intense demyelination, in order to clear the inhibitory myelin debris from the extracellular space. Interestingly, previous reports found that treatment with galantamine could enhance amyloid-β phagocytosis in primary cultures of rat microglia (Takata et al., 2010). Again, we did not observe any changes following FFS2B treatment, however when incubating slices with BASHY + galantamine we surprisingly observed an apparent increase in the expression of CR3 in demyelinated slices which was significantly augmented 48h-post LPC insult.

Overall our results suggest that the early augment of CR3 expression by galantamine could be favoring the establishment of a permissive environment for the accumulation and proliferation of OPCs, through promoting effective clearance of myelin debris, thus favoring oligodendrogenesis and preventing OL's loss. However, CR3/myelin axis is known for its pro-inflammatory outcome through the activation of NF-kB (Sun et al., 2010), so that enhanced expression of CR3 could also be on the basis of the observed augment of TNF-α, which expression is induced by the transcription factor NF-kB, at 18h-post LPC insult. Alternatively, by promoting a vast increase in CR3 expression long after demyelinating insult (48h), galantamine is possibly promoting excessive intracellular accumulation of myelin debris leading to the formation of cholesterol crystals in foamy phagocytes and therefore activating the NLRP3 inflammasome pathway.

Altogether, these results suggest that BASHY labeling could be a good biomarker of later stages of demyelination and even for activated phagocytic microglia. Taking advantage of this specificity, BASHY offered the possibility to be used as drug delivery vehicle. However, in our *ex vivo* model of demyelination, when testing BASHY targeting effects using galantamine, we could see a specific targeting of myelin-containing microglia, but galantamine did not exert its known anti-inflammatory properties as expected. On the other hand, galantamine alone prevented both synaptogenesis and oligodendrogenesis impairment in early stages of damage and showed an additional role as a possible activator of CR3-mediated myelin phagocytosis, favoring the subsequent remyelination.

Concluding Remarks

The study of the mechanisms behind demyelination and remyelination is of great interest when studying demyelinating diseases like MS. Recently, newly synthesized fluorescent molecules – BASHY dyes – when tested *in vitro* could differentiate between lipid membranes with different packing properties, particularly labeling lipid droplets. Therefore, being demyelination a process of continuous alterations of myelin lipid membranes, with this thesis we aimed to assess BASHY labeling in an *ex vivo* demyelinating model and hopefully characterize/distinguish the different stages of demyelination and assess myelin processing pathways

We started by staining with BASHY, slices treated with LPC and our first results suggested BASHY specificity for degenerated myelin, that we later confirmed to be cholesterol-rich myelin debris phagocyted by microglial cells. Given BASHY specificity for myelin debris we further observed that BASHY molecule was preferentially labelling activated/amoeboid microglia, which are the main phagocytes involved in the clearance of myelin debris and the main inflammatory mediators in demyelinating conditions. Based on this latter results, further interest emerged on the possible bioconjugation of BASHY dye with an anti-inflammatory molecule – galantamine – (FFS2B) in order to modulate early pro-inflammatory microglia into a more anti-inflammatory one to reduce the inflammatory outcome and promote remyelination. However, galantamine when bound to BASHY lost its known properties and only treatment with galantamine alone prevented both synaptogenesis and oligodendrogenesis impairment. More attractively, we also observed an additional role of galantamine as a possible activator of myelin-specific receptor CR3 in microglia.

However, further studies with galantamine should be performed to understand this new role on microglia modulation during demyelination. In line with this it should be interesting to address possible relations between CR3 activation and binding of galantamine to its known targets - the α7 nicotinic acetylcholine receptors. Furthermore, it could also be interesting to use BASHY for bioconjugation with other molecules from the presented **Table III.1**., namely other molecules with well described microglial intracellular effects. Or, concerning demyelinating diseases like MS, with fingolimod, which is already used in the clinic for the treatment of MS, and also shows an interesting role on cholesterol metabolism via an increase of NPC1.

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