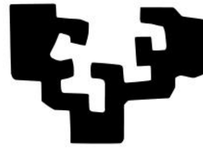

Universidad del País Vasco / Euskal Herriko Unibertsitatea

Facultad de Medicina y Enfermería
Departamento de Neurociencias

Role of GABAergic signaling in oligodendroglial differentiation, myelination and remyelination after demyelinating lesions

DOCTORAL THESIS

María Paz Serrano Regal

2019

Thesis Supervisors:

Mª Victoria Sánchez Gómez PhD.
Carlos Matute Almau PhD.

(c)2019 MARIA PAZ SERRANO REGAL

Part of the results presented in this doctoral thesis has already been published in the article:

Serrano-Regal MP, Luengas-Escuza I, Bayón-Cordero L, Ibarra-Aizpurúa N, Alberdi E, Pérez-Samartín A, Matute C, Sánchez-Gómez MV (2019) Oligodendrocyte differentiation and myelination is potentiated via $GABA_B$ receptor activation. *Neuroscience*, Jul 23. pii: S0306-4522(19)30488-9. doi: 10.1016/j.neuroscience.2019.07.014.

This work was supported by:

- Ministry of Economy and Competitiveness, Government of Spain – Ayudas para contratos predoctorales para la formación de doctores 2014 (FPI 2014).
- Ministry of Economy and Competitiveness, Government of Spain (SAF2013-45084-R and SAF2016-75292-R).
- Department of Economic Development and Competitiveness, Basque Government (IT702-13).
- Centro de Investigación en Red de Enfermedades Neurodegenerativas (CIBERNED).

ABBREVIATIONS I

INTRODUCTION 1

1. THE GLIAL CELLS 3

2. OLIGODENDROCYTES 3

2.1. Oligodendrocyte proliferation 4

2.2. Oligodendrocyte differentiation 5

2.3. Oligodendrocyte functions 7

2.4. Myelination by oligodendrocytes 9

3. MULTIPLE SCLEROSIS (MS) 11

3.1. Etiology of MS 13

3.2. MS subtypes 14

3.3. Patophysiology of MS 16

3.4. Experimental animal models of MS 19

3.4.1. Experimental autoimmune encephalomyelitis (EAE) 19

3.4.2. Virus-induced inflammatory demyelination 20

3.4.3. Toxin-induced demyelination 20

3.5. Treatments for MS 22

4. THE GABAERGIC SYSTEM 24

4.1. GABA synthesis and metabolism 24

4.2. GABA receptors 25

4.3. GABA transporters 29

4.4. GABA receptors and transporters in oligodendrocytes 30

HYPOTHESIS AND OBJECTIVES 33

MATERIAL AND METHODS 37

1. ANIMALS 39

2. *IN VITRO* MODELS 39

2.1. Rat cortical oligodendrocyte primary culture 39

2.2. Rat optic nerve-derived oligodendrocyte primary culture 41

2.3. Dorsal root ganglion neurons-oligodendrocyte precursor cells coculture 41

2.4. Cerebellar organotypic culture 42

2.4.1. EdU labeling and detection 42

2.4.2. Lysophosphatidylcholine (LPC)-induced demyelination in cerebellar slices 43
2.5. Optic nerve-derived organotypic culture 43
3. *IN VIVO* MODELS 43
3.1. Developing Sprague-Dawley rats 43
3.2. LPC-induced demyelination in C57BL/6 mice 44
4. SUBSTANCES 44
5. IMMUNOFLUORESCENCE 44
5.1. Cultured oligodendrocytes 44
5.2. DRG neurons-OPCs coculture 46
5.3. Cerebellar organotypic slices and brain sections 47
5.4. Mice spinal cord tissue samples 47
5.5. Image acquisition and analysis 49
6. PROTEIN EXTRACTS PREPARATION AND DETECTION BY WESTERN BLOTTING 51
6.1. Oligodendrocyte protein preparation 51
6.2. Cerebellar slice protein preparation 51
6.3. Western blotting 51
7. RNA EXTRACTION AND QUANTIFICATION 53
7.1. RNA isolation 53
7.2. Retrotranscription and Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) 53
8. CELL VIABILITY ASSAY 54
9. ELECTROPHYSIOLOGY 54
10. STATISTICAL ANALYSIS 55
RESULTS **57**
1. Cultured oligodendrocytes express $GABA_B$ receptors ($GABA_BRs$) 59
2. Expression of GABA transporters in oligodendroglial cells *in vitro* 61
3. Oligodendrocytes express GABA synthesizing enzymes 62
4. Cultured oligodendrocytes contain endogenous GABA 63
5. GABA regulates myelination in DRG-OPC coculture system 65
6. GABA upregulates MBP expression in isolated cortical oligodendrocytes 67

7. $GABA_BR$ stimulation by baclofen enhances myelin protein levels in OL cultures 68
8. $GABA_BRs$ stimulation increases process branching and MBP levels in peripheral areas of oligodendrocytes 70
9. Src kinase family mediates baclofen-induced MBP upregulation in isolated OPCs 71
10. Baclofen-induced upregulation of MBP requires CREB activation 74
11. Expression of $GABA_BRs$ in cerebellar organotypic slices 75
12. $GABA_BR$ activation upregulates myelin-related protein levels in organotypic cultures 76
13. Modulation of the GABAergic system does not affect oligodendrocyte proliferation in cerebellar slices 80
14. $GABA_BR$ activation upregulates MAG protein levels after LPC insult in cerebellar organotypic slices 83
15. GABAergic system manipulation modulates LPC-induced myelin damage in cerebellar organotypic cultures 85
16. Expression of $GABA_BRs$ and GABA transporters in oligodendrocytes *in vivo* 87
17. Baclofen accelerates oligodendrocyte differentiation in the *corpus callosum* at early stages of development 90
18. Baclofen enhances MBP expression in the *corpus callosum* of developing rats 93
19. Baclofen increases myelinated fiber conduction velocity in the *corpus callosum* of developing rats 95
20. $GABA_BR$ activation by baclofen promotes oligodendrocyte differentiation after LPC-induced demyelination in the mouse spinal cord 97
21. $GABA_BR$ activation by baclofen does not modify OPC population nor microglial cell number in LPC-demyelinating lesions in the mouse spinal cord 99

DISCUSSION **101**

1. Oligodendrocytes express the major components of the GABAergic system and synthesize GABA 104
2. GABA via $GABA_BRs$ regulates oligodendrocyte differentiation and myelination *in vitro* 106

3. Baclofen-induced MBP upregulation *in vitro* requires the participation of Src-family kinases and the phosphorylation of CREB 107

4. GABAergic signaling modulates oligodendrocyte differentiation in organotypic cultures 107

5. GABAergic signaling modulates LPC-induced myelin damage and restores MAG levels in cerebellar organotypic cultures 109

5. Baclofen promotes *in vivo* OL differentiation and accelerates postnatal myelination in rats 110

6. $GABA_B$R activation promotes OPC differentiation without modifying proliferation *in vivo* after LPC-induced demyelination 112

CONCLUSIONS **114**

REFERENCES **117**

ABBREVIATIONS

aCSF	Artificial CSF
A-KG	Alpha-ketoglutaric acid
ANOVA	Analysis of variance
APC (CC1)	Adenomatous poliposis coli clone CC1
ATP	Adenosine triphosphate
A.U.	Arbitrary units
BAC	Baclofen
BBB	Blood brain barrier
BGT-1	Betaine-GABA transporter 1
BME	Basal medium with Earle´s salt
BSA	Bovine serum albumin
BZ	Benzodiacepine
Calcein-AM	Calcein-acetoxymethyl
CaMKIIβ	Calcium/calmodulin-dependent protein kinase II subunit beta
cAMP	Cyclic adenosine monophosphate
CAP	Compound action potential
CCP	Complement control protein
CD	Cluster of differentiation
cDNA	Complementary DNA
CEEA	Comité de ética en experimentación animal
CEID	Comité de ética en la investigación y la docencia
CicA	Cyclophilin A
CIS	Clinically isolated syndrome
CNPase	2’, 3’-Cyclic nucleotide-3’-phosphodiesterase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CREB	Cyclic-AMP response element binding protein
CSF	Cerebrospinal fluid

Dcc	Netrin 1 receptor (deleted in colorectal carcinoma)
DIV	Days *in vitro*
DMEM	Dulbecco´s modified Eagle´s medium
DMT	Disease modifying treatment
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DPL	Days post-lesion
DRG	Dorsal root ganglion
DTT	Dithiothreitol
E	Embryonic day
EAE	Experimental autoimmune encephalomyelitis
EdU	5-ethynyl-2´-deoxyuridine
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
EYFP	Enhanced yellow fluorescent protein
FBS	Fetal bovine serum
FDA	United States Food and Drug Administration
FGF	Fibroblast growth factor
GABA	Gamma aminobutiric acid
GABA-T	GABA-transaminase
GAD	Glutamic acid decarboxylase
Galc	Galactocerebroside
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAT	GABA transporter
GBZ	Gabazine
GFAP	Glial fibrillary acidic protein
GIRK	G protein-activated inwardly-rectifying K^+ channels
Glu	Glutamate
GPCR	G protein-coupled receptor

GRK	G protein receptor kinase
GSK3β	Glycogen synthase kinase 3 beta
HBSS	Hank´s balanced salt solution
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
hnRNP	Heterogeneous nuclear ribonucleoprotein
Iba1	Ionized calcium binding adaptor molecule 1
IFNβ	Interferon beta
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IMDM	Iscove´s modified Dulbecco´s medium
I.P.	Intraperitoneally
KCTD	Potassium channel tetramerization domain
LINGO	Leucine-rich repeat and immunoglobulin domain-containing-1
LPC	Lysophosphatidylcholine/ lysolecithin
MAG	Myelin-associated glycoprotein
MAO_B	Monoamino oxidase B
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCMS	Myelocortical multiple sclerosis
MCT	Monocarboxylate transporter
MHV	Mouse hepatitis (corona) virus
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
MUS	Muscimol

Myrf	Myelin regulatory factor
NAM	Negative allosteric modulator
NF-L	Neurofilament L
NG2	Neural/glial antigen 2
NGF	Nerve growth factor
NMDAR	N-methyl-D-aspartate receptor
NPC	Neural progenitor cell
NRG	Neuregulin
NSC	Neural stem cell
NSF	NEM-sensitive fusion protein
NT3	Neurotrophin 3
O4	Oligodendrocyte marker O4
OAA	Oxalacetate
Olig2	Oligodendrocyte transcription factor 2
OL	Oligodendrocyte
OPC	Oligodendrocyte precursor cell
P	Postnatal day
PAGE	Polyacrylamide gel electrophoresis
PAM	Positive allosteric modulator
PB	Phosphate buffer
PBS	Phosphate buffer saline
PDGFRα	Platelet-derived growth factor receptor α
PDL	Poly-D-Lysine
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLP	Myelin proteolipid protein
PNS	Peripheral nervous system
PPMS	Primary progressive multiple sclerosis
PRMS	Progressive-relapsing multiple sclerosis

PSA-NCAM	Polysialylated neural cell adhesion molecule
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
RNase	Ribonuclease
ROI	Region of interest
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
SDS	Sodium dodecyl sulfate
S.E.M	Standard error of the mean
SFK	Src family kinase
SPMS	Secondary progressive multiple sclerosis
SSA	Succinic semialdehyde
SSADH	SSA dehydrogenase
T3	Triiodothyronine/ thyroid hormone 3
T4	L-Thyroxine
TBS	Tris buffer saline
TBS-T	Tris buffer saline tween-20
TCA	Tricarboxylic acid cycle
Th	T helper cell
TM	Transmembrane
TMEV	Theiler´s murine encephalomyelitis virus
U	Unit
VFT	Venus flytrap

INTRODUCTION

1. THE GLIAL CELLS

Neurons and glia are the main groups of cells present in the nervous system. Neurons are excitable cells responsible for information processing and transmission. Glia, as the Greek name indicates, has been traditionally considered as the "glue" of the nervous system, providing structural and metabolic support to neurons and creating a proper environment for them. In the central nervous system (CNS), glia can be classified into three different types: astrocytes, which have different functions, including providing metabolic support to axons, recycling neurotransmitters from the synaptic cleft, buffering extracellular potassium concentration and forming the blood brain barrier; microglia, the resident immune cells in the brain, which engulf cellular debris and secrete both pro and anti-inflammatory molecules, and oligodendrocytes (OLs), which produce the myelin sheath around axons, providing protection and metabolic support to neurons and facilitating the saltatory conduction of axon potentials.

The understanding of the role of glial cells in the CNS function has extraordinarily progressed during recent years, and the interest in their study has greatly increased. Glial cells are indispensable for correct neuronal development and functioning, being their ability to respond to changes in the cellular and extracellular environment crucial to the function of the nervous system. The presence of neurotransmitters, their transporters and receptors in glial cells is key to neuron-glia communication (*Baumann and Pham-Dinh, 2001*).

2. OLIGODENDROCYTES

Oligodendrocytes are the myelin forming cells in the vertebrate CNS. They were described by Pío del Río Hortega in 1921 as cells with small cell bodies filled with nuclei containing large amounts of chromatin and a tremendous complexity of cellular extensions without fibers but with cytoplasmic granules (*del Río Hortega, 1921*; *Simons and Nave, 2015*). Although the main role of OLs is the synthesis of myelin around CNS axons, there are also "perineural satellite oligodendrocytes" in close contact with neurons that regulate the microenvironment around them (*Baumann and Pham-Dinh, 2001*). Some other oligodendroglial cells are found in the vicinity of small vessels and are subclassified as "perivascular satellites" (*Simons and Nave, 2015*). Recently, Marques et al. (*2016*) have identified, by single-cell RNA sequencing, 12 subpopulations of oligodendroglial cells

suggesting that, among them, mature OLs are region specific. Thus, OLs are a highly diverse and specialised cell population.

Mature myelinating OLs are postmitotic cells that develop from glial precursors named oligodendrocyte precursor cells (OPCs) which express the platelet-derived growth factor receptor α (PDGFRα) and the proteoglycan NG2. Besides forming OLs during brain development, OPCs remain present in the adult CNS and constitute the main proliferating cell type (*Dawson et al., 2003*). OPCs express a wide variety of receptors and can respond to neuronal derived signals such us neurotransmitters, growth factors or cell adhesion ligands.

Self-renewing neural stem cells (NSCs) in the developing brain produce multipotent, non-self-renewing neural progenitor cells (NPCs), which under specific signals begin to express oligodendrocyte transcription factor 2 (Olig2), triggering the first embryonic wave of specification of OPCs (*Naruse et al., 2017*). Once specified, OPCs migrate away, under control of both repulsive and attractive cues, from the neuroepitheliun of the ventricular/subventricular zone of the brain to the developing white matter, where they proliferate and form an evenly space network of processing-bearing cells (*Simons and Nave, 2015*). While some OPCs find targets and differentiate into myelinating OLs, others remain in the adult brain in a precursor state for replacing OLs at a constant rate of turnover or at an accelerated level for myelin regeneration. However, due to their electrical properties and their participation in synaptic interactions, they may also exert physiological roles in the adult CNS (*Fancy et al., 2011*).

2.1. Oligodendrocyte proliferation

OPC proliferation is regulated by numerous signaling molecules released by neurons or other cell types as astrocytes. Several growth factors act as potent mitogens for OPCs. One of them is the PDGF-AA, which binds to its receptor PDGFRα. This receptor is abundantly expressed in OPCs, and is downregulated as they differentiate into mature OLs (*Calver et al., 1998*). The fibroblast growth factor (FGF) controls OPC proliferation extending the time period over which OPCs are proliferative and preventing their differentiation into myelinating OLs (*Mayer et al., 1993*). Neurotrophin 3 (NT3) and insulin-like growth factor 1 (IGF-1) are also involved in stimulating proliferation of OPCs. However, small hydrophobic signals such as thyroid hormone, retinoic acid or glucocorticoids have been reported to stop OPC proliferation. These signals are proposed to induce the exit from the cell cycle of OPCs and

their differentiation (*Barres et al., 1994*). Moreover, neurotransmitters have been involved in the modulation of OPC proliferation, as they receive both excitatory and inhibitory synaptic inputs mediated by glutamate and γ-aminobutiric acid (GABA) (*Bergles et al., 2000*; *Lin and Bergles, 2004*; *Karadottir et al., 2008*; *Vélez-Fort, 2010*). For that reason, these neurotransmitters are supposed to regulate OPC development.

2.2. Oligodendrocyte differentiation

OPCs go through different stages of cell maturation characterized by changes in the expression pattern of molecular markers as well as changes in their morphology. The sequential expression of developmental markers divides the lineage into distinct phenotypic stages: OPCs or early progenitor cells, immature premyelinating OLs and mature OLs (*Baumann and Pham-Dinh, 2001*; *Simons and Nave, 2015*). Oligodendroglial lineage cells express SOX10 and Olig2 over development, but as OPCs undergo terminal differentiation, they lose the expression of specific progenitor markers as PDGFRα and NG2 and begin to express immature markers as O4 and galactocerebroside (GalC; *Nishiyama et al., 2009*). This change is accompanied by the development of a more complex shape, a loss of synaptic connections and changes in ion channel expression. As they continue progressing in the lineage, they begin to express the earliest myelin-related marker, 2´, 3´-Cyclic nucleotide-3´-phosphodiesterase (CNPase) and adenomatous poliposis coli (APC) marker, also known as CC1. Then, as they are mature enough, they express myelin-related proteins as myelin basic protein (MBP), myelin proteolipid protein (PLP), myelin-associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG; *Miron et al., 2011*) (**Figure 1**).

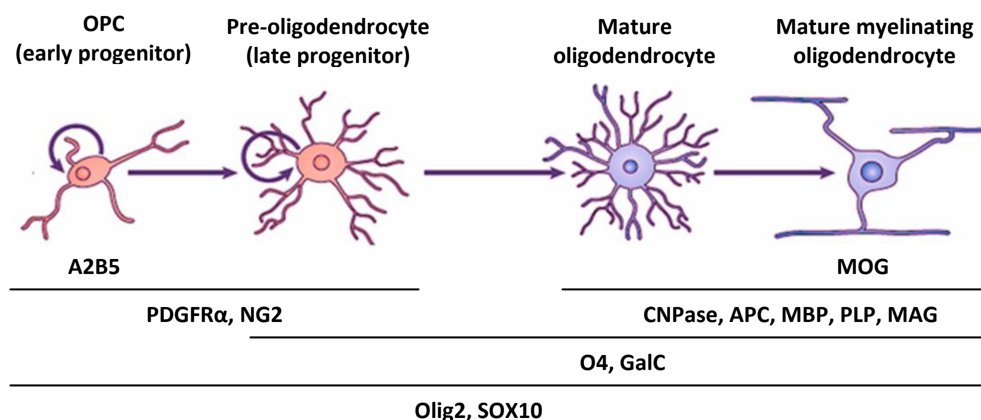

Figure 1. Schematic representation of the developmental stages of the oligodendrocyte lineage with stage-specific markers (adapted from *Nishiyama et al., 2009* and *Traiffort et al., 2016*).

OL development and myelination of axons are highly regulated processes by a variety of events that involve the participation of negative and positive regulators. These include axonal surface ligands, secreted molecules, axonal activity (*Emery, 2010*) as well as glutamate or GABA (*Li et al., 2013*; *Fannon et al., 2015*; *Zonouzi et al., 2015*; *Hamilton et al., 2017*).

Regarding to the negative regulators, many of axonal expressed ligands are involved in preventing OL differentiation or myelination. Among these factors are Jagged, which signals via Notch, PSA-NCAM and the Leucine-rich repeat and immunoglobulin domain-containing-1 (LINGO) (*Emery, 2010*). The binding of Jagged-1, Delta-1 and-Notch-1 generates an intracellular domain that enters in the nucleus and increases the expression of inhibitory transcription factors (*Wang et al., 1998*).

LINGO is a transmembrane protein whose signaling pathways prevent OL differentiation by decreasing Fyn kinase activity and subsequent RhoA signaling (*Mi et al., 2005*). In addition, canonical Wnt signaling has been described as a potent regulator of OL differentiation. Wnt pathway stabilizes β-catenin protein levels, and the constitutive expression of β-catenin impairs OPC differentiation and myelination (*Fancy et al., 2011*). As an example, GSK3β negatively regulates OL differentiation via Wnt signaling pathway by stimulating nuclear translocation of β-catenin (*Azim and Butt, 2011*).

In contrast, a variety of diffusible factors positively regulates OL differentiation. Besides its participation in OPC survival and proliferation, IGF-1 is also involved in OL differentiation and myelination via PI3K/Akt/mTOR signaling pathway (*Flores et al., 2008*). Forced expression of constitutively active Akt or inactivation of phosphatase and tensin homolog (PTEN), results in hypermyelination of axons, which is dependent on the activation of the Akt substrate mTOR (mammalian target of rapamycin; *Fancy et al., 2011*). As mentioned before, triiodothyronine/thyroid hormone 3 (T3) regulates OL differentiation. Although T3 is not ultimately essential for OPCs to differentiate, it plays an important role in the timely and efficient production of myelin during development by accelerating OPC differentiation (*Mitew et al., 2014*).

Transcription factors as Olig2 are intracellular regulators of OPC differentiation. *Mei et al. (2013)* demonstrated that ablation of Olig2 in OPCs postnatally causes an important decrease in OL differentiation, while it has no detrimental effect on the mature cells, suggesting that Olig2 mediates OL differentiation instead of myelination. The transcription

factor Myrf is specifically induced during OL differentiation, mediating the transition of premyelinating OLs to mature myelinating OLs. Knockdown of Myrf in OLs prevents the expression of most myelin genes, and mice in which Myrf function is disrupted within the OL lineage show severe demyelination (*Emery et al., 2009*).

Histone deacetylation is also implicated in OL differentiation. Histone deacetylases (HDACs) form complexes that inhibit expression of transcriptional repressors of differentiation (*Li et al., 2009*).

2.3. Oligodendrocyte functions

The main role of mature OLs is the formation of a myelin sheath around CNS axons, which provides electrical insulation, decreasing the capacitance and increasing the resistance of the axonal membrane. Myelin is essential for rapid action potential propagation, required for the correct functioning of the vertebrate CNS. It increases nerve conduction velocity 20 to 100-fold in comparison with non-myelinated axons. Moreover, it provides fidelity in the transmission of signals to long distances, and restricts axonal energy requirements (*Baumann and Pham-Dinh, 2001*). In contrast to the peripheral nervous system (PNS), where Schwann cells establish a one-to-one cell connection with the axon, OLs are able to myelinate numerous axonal areas, depending on the axon caliber and length. While in the spinal cord some OLs form myelin only around one single large axon with an internode (myelin segment) length of 1500 µm, cells in the cortex and *corpus callosum* generate between 30 and 80 internodes ranging from 20-200 µm (*Snaidero and Simons, 2014*).

Apart from this well-established function, OLs are crucial for providing trophic and metabolic support to axons. They transfer energy metabolites to neurons through cytoplasmic "myelinic" channels and monocarboxylate transporters, which permits fast delivery of metabolites such as pyruvate and lactate, whose metabolism contributes to ATP synthesis in neurons. OLs express specific transporters for glucose-derived metabolites that ensure their shuttling toward neurons in order to fuel their energetic demands (*Philips and Rothstein, 2017*) (**Figure 2**).

As an example, it is well-known that OLs express monocarboxylate transporter 1 (MCT1) and that this transporter mediates metabolic support to axons. In fact, OL-specific MCT1 depletion produces severe axonal injury and motor neuron death *in vivo* (*Lee et al., 2012*). OL accumulate intracellular lactate which can flow through MCT1 into the periaxonal space, where it can be taken up by neurons through MCT2. Furthermore, other metabolites, some proteins, neuronal trophic factors or mRNA are transported as well from OLs to axons possibly through cytoplasmic myelinic channels to support neuronal activity (*Nave and Werner, 2014*).

N-methyl-D-aspartate receptors (NMDARs) also participate in OL-mediated neuronal metabolic support. The OL-neuron lactate shuttle is driven by neuronal activity as glutamate released from neurons binds to oligodendrocyte-NMDARs and produces and increase in glucose uptake and lactate production in these cells (*Saab et al., 2016*).

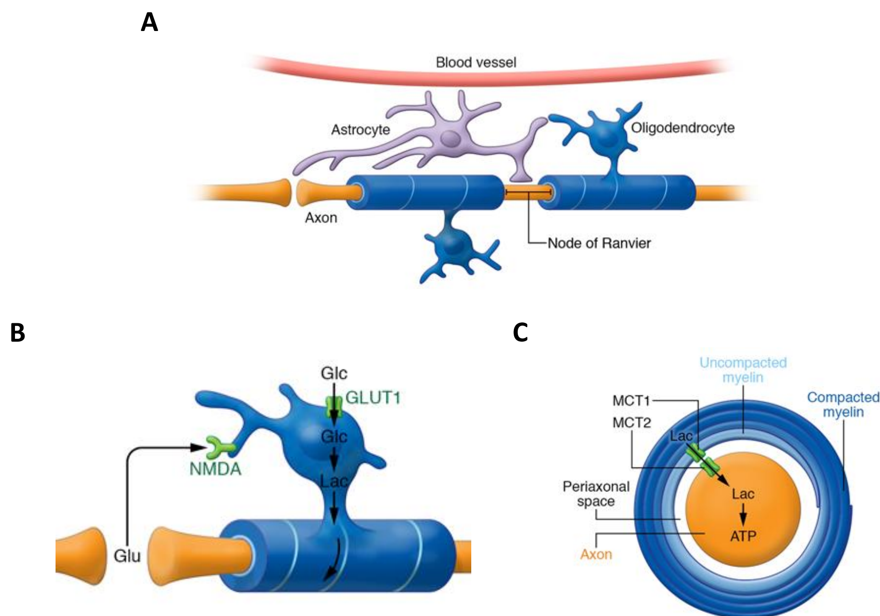

Figure 2. Oligodendrocytes provide metabolic support to axons (adapted from *Philips and Rothstein, 2017*). **A)** Oligodendrocytes and astrocytes contribute to neuronal metabolic support. **B)** Oligodendrocyte-neuron lactate shuttle is driven by neuronal activity. Neuronal released glutamate binds to oligodendrocyte-NMDARs leading to an increase in glucose uptake and its conversion into lactate. **C)** Lactate is transported through MCT1 into the periaxonal space, where is taken up by neurons through MCT2 and processed for ATP synthesis.

2.4. Myelination by oligodendrocytes

The term “myelin” was coined by Rudof Virchow in 1864 and comes from the Greek word “marrow” (myelos), because it is particularly abundant in the core, or marrow, of the brain (*Snaidero and Simons, 2014*). The myelin sheath is separated from the axonal membrane by a narrow extracellular cleft (the periaxonal space), and it is a spiral structure constituted of extensions of the plasma membrane of OLs, which is interrupted by unmyelinated segments rich in voltage-gated Na^+ channels, called nodes of Ranvier (*Baumann and Pham-Dinh, 2001*). This allows the action potential to be regenerated at each node, thus propagating from one node to the next by saltatory conduction. Regarding to its molecular composition, myelin has low water content, a high percentage of lipids, and a small set of proteins that include MBP and PLP (the most abundant), CNPase, MAG and MOG (*Baumann and Pham-Dinh, 2001*; *Simons and Nave, 2015*).

Myelination occurs relatively late in development in a defined temporal sequence. In mice, it starts at birth in the spinal cord and is almost completed at postnatal day 60 (P60) in most brain regions (*Baumann and Pham-Dinh, 2001*). In rats, the peak of myelination takes place around P20 (*Downes and Mullis, 2014*) while in humans occurs during the first year of life and continues into young adulthood, especially in some cortical areas of the brain (*Fields, 2008*). Myelin biogenesis is dynamically regulated by experience and environmental factors both during development and in adult life (*Fields, 2008*; *Makinodan et al., 2012*; *Liu et al., 2012*).

Myelination by OLs is a very well coordinated process that involves the participation of a wide variety of inhibiting and promoting signals coming from axons or other cell types as astrocytes (*Hammond et al., 2014*) or microglia and macrophages (*Miron et al., 2013*). Neuronal activity also influences OL differentiation and myelination (*Gibson et al., 2014*). This complex programme is initiated in response to axon-glia recognition and mediated by membrane bound cell adhesion molecules that allow OLs to extend their processes towards the axon and to start wrapping around it. Then, oligodendroglial cells reorganize their cytoskeleton and increase microfilament polarization and branching (*Simons and Troter, 2007*; *Bauer, 2009*).

Concerning to the axonal-derived signals, polysialylated neural cell adhesion molecule (PSA-NCAM) and the adhesion molecule L1 CAM are expressed on the axonal surface at the beginning of myelination, being removed as the axon become myelinated (*Coman et al., 2005*). In contrast, the Src family kinases (SFKs) are non-receptor tyrosine kinases that integrate neuronal external signals from integrin and growth factor receptors and are involved in OL development. One of its members, Fyn, is expressed in both neurons and glia in the brain (*Colognato et al., 2004*) and its signaling downstream pathways regulate the morphological differentiation of OLs, the recruitment of cytoskeleton components and the local translation of MBP (*White and Krämer-Albers, 2014*) (**Figure 3**).

Fyn kinase activity reaches its maximum peak at the beginning of myelination and Fyn-deficient mice have been reported to show impaired myelination (*Umemori, 1994*; *Sperber et al., 2001*). During OL maturation, Fyn kinase activity is compartimentalized into lipid raft membrane domains with other proteins, to favor their interaction (*White and Krämer- Albers, 2014*). Fyn activates in response of axon-glia contact with the participation of the cell adhesion molecule L1 and extracellular matrix-derived laminin-2 interacting with integrin β1 and contactin-1 OL complex (*White et al., 2008*). At the cellular level, Fyn activation interferes with OL maturation and process outgrowth (*Sperber and Morris, 2001*) as it mediates netrin-1 induced process branching. Netrin 1 binds to Dcc receptors in mature OLs and recruits Fyn to a complex with the receptor intracellular domain and other proteins, resulting in a decrease in RhoA activity and promoting process extension and branching (*Rajasekharan et al., 2009*).

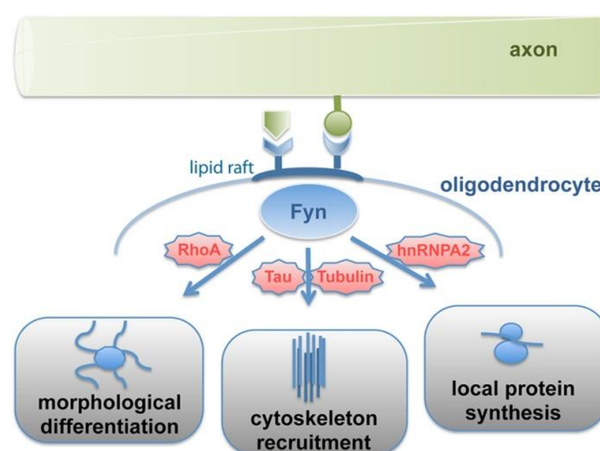

Figure 3. Fyn signaling downstream pathways (adapted from *White and Krämer-Albers, 2014*). Fyn kinase associates with lipid rafts and is activated by axonal signals triggering different signaling pathways that regulate morphological differentiation of OLs, cytoskeleton recruitment and local protein translation, thus controlling myelin formation.

Neuregulins (NRGs), which bind to ErbB tyrosine kinase receptors in OLs, have also been reported to participate in the survival and differentiation of these cells (*Park et al., 2001*). However, its role in myelination still remains under debate as both NRG and ErbB receptor knockout mice show normal myelination (*Brinkmann et al., 2008*). Other signaling pathways, such as PI3K/Akt/mTOR, Erk1/2-MAPK pathways and CAMKIIβ signaling are important regulators of the myelination process (*Flores et al., 2008*; *Guardiola-Díaz et al., 2012*; *White and Krämer-Albers, 2014*).

Neurotransmitters also participate in the regulation of myelination (*Li et al., 2013*; *Hamilton et al., 2017*). OPCs receive both glutamatergic and GABAergic synaptic inputs (*Bergles et al., 2000*; *Lin and Bergles, 2004*; *Karadottir et al., 2008*; *Fannon et al., 2015*; *Zonouzi et al., 2015*) and this communication between individual axons and OPCs promotes myelin formation in electrically active ones. Kukley et al. (*2007*) described that OPCs establish excitatory glutamatergic synapses with unmyelinated axons suggesting that it may play a role in controlling the development of myelination. Moreover, Wake et al. (*2011*) showed that glutamate release from synaptic vesicles promoted myelination by increasing the local synthesis of MBP in OLs. In turn, GABA controls myelination *in vitro* (*Hamilton et al., 2017*), and as the expression and function of $GABA_A$ receptors ($GABA_ARs$) in cultured OLs is driven by axonal cues, GABA signaling may play a relevant role during axon-glia recognition and in myelination (*Arellano et al., 2016*). However, these findings *in vitro* await confirmation *in vivo*.

In addition to the axonal signals, differentiation of OLs and myelination are regulated by transcription factors. The transcription factor cyclic-AMP response element binding protein (CREB) plays an important role in these processes as it acts as a mediator in the c-AMP dependent stimulation of MBP (*Afshari et al., 2001*). However, apart from its role as a cAMP-responsive activator, CREB can be phosphorylated and promote cellular gene expression in response to both extracellular and intracellular signals involving the participation of multiple protein kinases (*Mayr and Montminy, 2001*; *Mao et al., 2007*).

3. MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic, inflammatory and demyelinating disease of the CNS. It was first described clinically by Charcot in 1868 as *Sclérose en plaques*, a term that,

from 1954, became MS in the English literature (**Figure 4**; *Zalc, 2018*). Among the different demyelinating diseases, MS is the most prevalent in young adults, affecting more than 2 million people worldwide (*Reich et al., 2018*) and being more common in women than men (*Ascherio and Munger, 2016*).

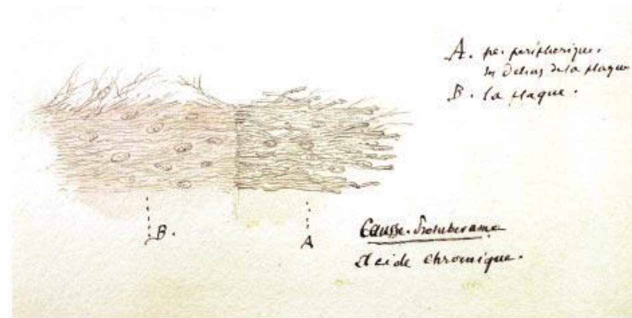

Figure 4. Original drawing from Charcot's notebook illustrating the limit of a lesion in the protuberance with handwritten legends on the right. A: Periphery outside the plaque. B: The plaque. Bottom: Causse (patient's name) Protuberance. Chromic acid. (*Zalc, 2018*).

MS is a heterogeneous neurological disease both clinically and pathologically that causes irreversible physical and mental disability. It is characterized by focal lesions, named sclerotic plaques, with an autoimmune attack to the myelin sheath, leading to OL degeneration and demyelination, gliosis and axonal damage (*Compston and Coles, 2008*; *Dendrou et al., 2015*). Demyelination results in slower conduction velocity or complete failure of transmission, causing neuronal dysfunction and the appearance of diverse signs and symptoms that vary within each affected CNS area. These sclerotic plaques can appear both in the white and grey matter, brain stem, spinal cord, cerebellum or optic nerve (*Reich et al., 2018*) producing motor, sensory and cognitive deficits. Among the symptoms and signs in MS are: optic neuritis, dysarthria, fatigue, dysphagia, spasticity and muscle weakness, paresthesia, tremor, painful spasms, the characteristic Lhermitte´s symptom, which consist of and electrical sensation running down the spine or limbs on neck flexion, and the Uhthoff phenomenon, a transient worsening of symptoms and signs when body temperature increases, as occurs after doing exercise or having a hot bath (*Compston and Coles, 2008*).

Although it is still unknown whether MS has a single or multiple causes, it is widely accepted that both genetic and environmental factors are implicated in the development of the disease (*Huynh and Casaccia, 2013*; *Reich et al., 2018*). Genomewide association studies have identified more than 200 gene variants that increase the risk of MS, being the most significant the human leukocyte antigen (HLA) DRB1*1501 haplotype. Numerous risk alleles are associated with immune-pathway genes, a fact that is consistent with the idea that an

autoimmune mechanism participates in the pathogenesis of the disease (*Reich et al., 2018)*. The environmental risk factors include geographical latitude, vitamin D levels, tobacco exposure, obesity in early life and infection with the Epstein-Barr virus (*Ascherio and Munger, 2016*). Recent studies have also proposed an important influence of gut microbiota in the propensity for development of experimental autoimmune encephalomyelitis (EAE), the mouse model of MS (*Berer et al., 2017*; *Cekanaviciute et al., 2017*), suggesting that individual differences in the composition of the microbiota may affect the immune system by modulating the equilibrium between different subclasses of lymphocytes.

3.1. Etiology of MS

Tissue damage in MS is the consequence of a complex and dynamic interplay between immune cells, glia and neurons. Without a known root cause of MS, there is a debate about whether its origin is extrinsic ("outside-in" theory) or intrinsic ("inside-out" theory) to the CNS and whether the immune cell infiltration precedes or not demyelination and axonal degeneration (*Tsunoda and Fujinami, 2002*; *Matute and Pérez-Cerdá, 2005*; *Reich et al., 2018*) (**Figure 5**).

The most accepted view is that abnormal autoimmune responses, possibly triggered after viral infections, are responsible for the demyelination and the subsequent neurodegeneration that occurs in MS (*Kipp et al., 2012*; *Geginat et al., 2017*). In this "outside-in" theory, activated immune T cells enter the CNS by crossing the blood brain barrier (BBB). Once there, they become autorreactive and destroy myelin directly by releasing toxins and cytokines or indirectly by inducing activation of microglia and recruiting other cell types from the periphery as macrophages, activated B cells, monocytes, dendritic cells and natural killer T cells. Then, myelin loss leads to OL degeneration and axonal injury (*Matute and Pérez-Cerdá, 2005*; *Lopez-Diego and Weiner, 2008*) (**Figure 5A**). This theory supports the use of the EAE murine model to study MS.

On the contrary, the "inside-out" theory defends that OL degeneration is the primary event that takes place in MS. This theory is based on a study of Barnett and Prineas (*2004*) in which they found OL apoptosis and microglial activation in the absence of a clear immune response in lesions from patients who died during or immediately after the onset of a fatal relapse. Different factors as viral infections, viral products like syncytin (*Antony et al., 2004*),

glutamate-derived excitotoxicity (*Matute et al., 2001*; *Werner et al., 2001*), oxidative stress or signals generated during hypoxia can induce OL apoptosis and primary axonal degeneration and, as a consequence, the resulting myelin debris can activate microglia and peripheral immune cells (**Figure 5B**). Thus, the immune response constitutes a secondary event in this model (*Matute and Pérez-Cerdá, 2005*).

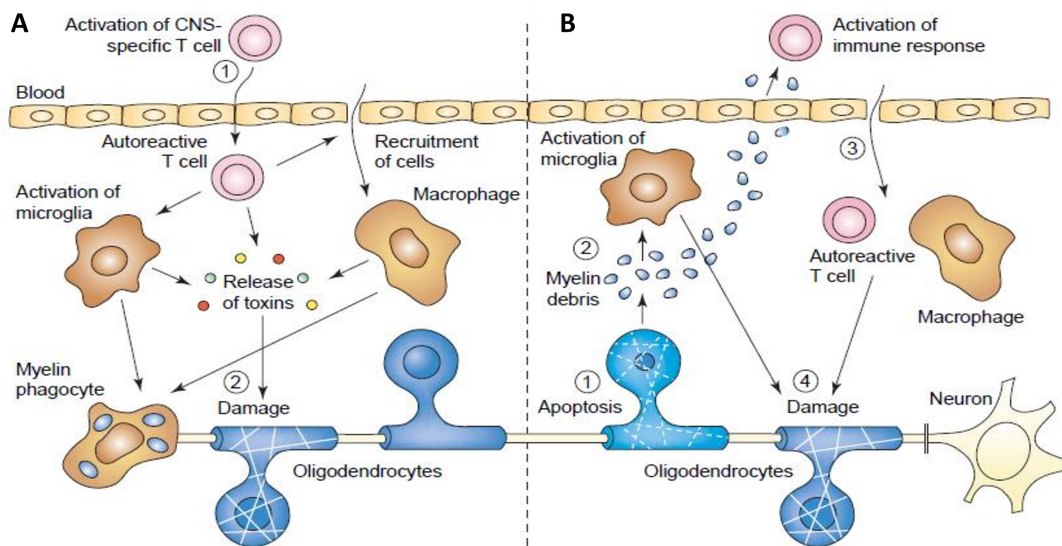

Figure 5. Alternative theories of lesion formation in MS. A) "Outside-in" theory: activated T cells enter the CNS (1) become autorreactive and destroy myelin directly by releasing toxins and cytokines or indirectly by inducing activation of microglia and recruiting macrophages from the periphery. This leads to myelin destruction, OL death and clearance of damaged tissue by phagocytes (2). **(B) "Inside-out" theory**: several factors can induce OL apoptosis (1) and large amounts of myelin debris are generated (2) exceeding the capacity of the phagocytic cells to clear them and triggering inflammation. Consequently, T cells and macrophages invade the CNS (3) and initiate the autoimmune attack of myelin (4) (adapted from *Matute and Pérez-Cerdá, 2005*).

Although the prevailing view is the "outside-in" theory, both models could act simultaneously initiating a cascade of events and contributing to the pathogenesis of the disease.

3.2. MS subtypes

MS is highly heterogeneous in terms of disease course and symptoms, and it is commonly classified into subtypes (*Dendrou et al., 2015*). In 1996, Lublin and Reingold classified MS into four clinical courses defined as relapsing-remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS) and progressive-relapsing MS (PRMS) (*Lublin and Reingold, 1996*). This classification has allowed better communication of

patient´s clinical courses and to define clinical trial populations. However, a better understanding of MS and its pathology will surely lead to a redefinition of this current classification. The revised criteria by Lublin considers disease activity, based on clinical relapse and magnetic resonance imaging (MRI) findings, and disease progression; adding the clinically isolated syndrome (CIS) and removing the PRMS (*Lublin, 2014*). Thus, the current categorization of MS establishes the following subtypes (**Figure 6**):

- Clinically isolated syndrome (CIS): it is the first clinical manifestation and consists of an acute or subacute episode of neurological disturbance attributed to a single white matter lesion. This could be the onset of MS or an isolated clinical event. When CIS is accompanied by MRI abnormalities and cerebrospinal fluid (CSF) criteria, the probability of developing MS is higher. MS can be diagnosed within 3 months of CIS presentation (*Miller et al., 2005*).

- Relapsing-remitting MS (RRMS): it is the most common form, affecting approximately 85% of patients. It is characterized by CIS followed by unpredictable recurring attacks with partial or total neurological recovery. Relapses coincide with focal CNS inflammation and demyelination that are typically distinguishable by MRI as white matter lesions (*Dendrou et al., 2015*; *Filippi et al., 2018*). Normally, disability accumulates with each relapse and most of the patients go on to develop secondary progressive MS.

- Secondary progressive MS (SPMS): in this subtype, inflammatory lesions are no characteristic as progressive and irreversible disability occurs independently of the presence of relapses. Basically, neurological decline is accompanied by CNS atrophy, which consists of a decreased in brain volume as axonal loss progresses. Approximately 80% of RRMS patients develop SPMS (*Dendrou et al., 2015*; *Filippi et al., 2018*).

- Primary-progressive (PPMS): between 10-15% of patients present a gradual, continuous and progressive neurological decline from the onset in absence of relapses (*Dendrou et al., 2015*; *Filippi et al., 2018*).

Apart from that, a recent study by Trapp et al. (*2018*) suggests a new subtype of MS which they called myelocortical MS (MCMS), consisting of spinal cord and cerebral cortex

demyelination without affecting cerebral white matter. In this study, they propose that cortical neuronal loss can occur independently to white matter demyelination.

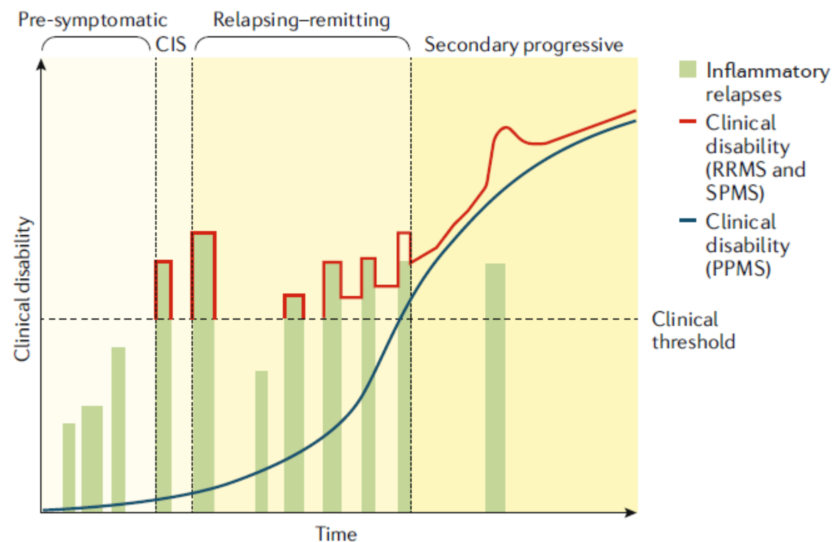

Figure 6. Diagram representing the clinical course of MS showing the progression of disability (*Filippi et al., 2018*).

3.3. Pathophysiology of MS

The pathological hallmark of MS is the accumulation of focal areas of demyelination and OL death in the white and grey matter of the brain and spinal cord known as plaques or lesions (*Filippi et al., 2018*). In the white matter, active lesions are accompanied by inflammation and glial reaction (*Reich et al., 2018*). At the early stages of the disease axons and neurons are preserved but, as it progresses, a gradual axonal loss occurs and correlates with clinical disability. This phenomenon results in brain atrophy with ventricular enlargement (*Dendrou et al., 2015*).

In the early phase of the disease, there is little damage outside focal lesions, termed normal-appearing white matter, although general brain atrophy is also noticed. As the disease progresses, diffuse myelin decrease and axonal injury become evident, resulting in a more pronounced atrophy of the grey and white matter (*Dendrou et al., 2015*) (**Figure 7**).

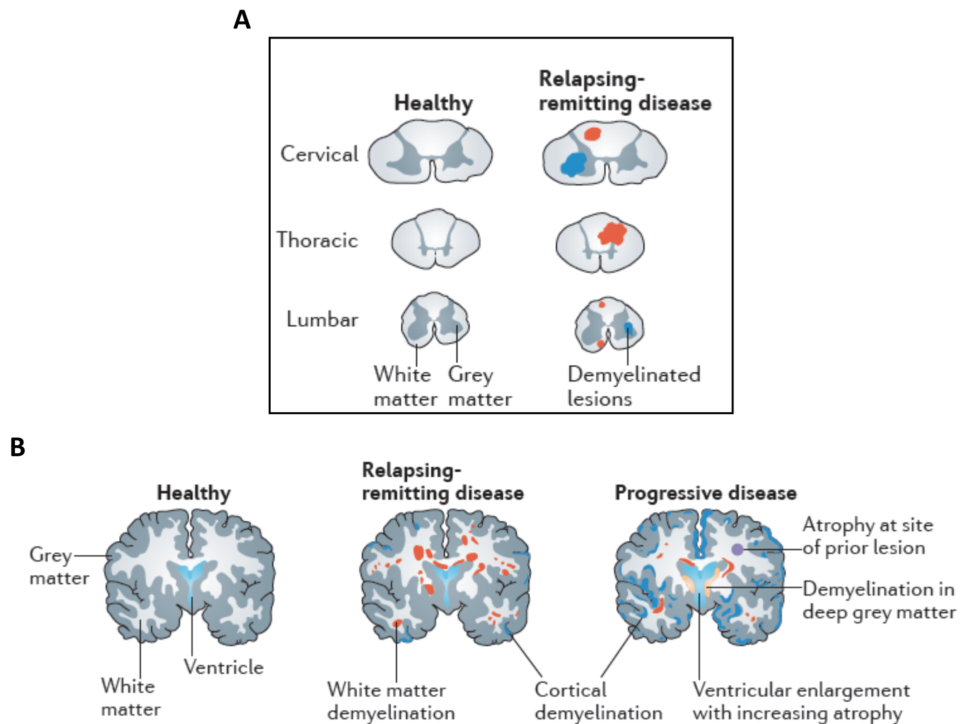

Figure 7. The pathology of MS in the spinal cord (A) and in the brain (B) (transverse spinal cord view and coronal brain view**,** respectively; adapted from *Dendrou et al., 2015*). Red: white matter lesion; dark blue: grey matter lesion.

The disease starts with the breakdown of the BBB, which involves the infiltration of different types of immune cells from the periphery to the CNS. Pro-inflammatory molecules secreted by resident cells and endothelial cells, and leukocyte-mediated injury provoke the disruption of the BBB (*Ortiz et al., 2014*), leading to the infiltration of both helper (CD4+) and cytotoxic (CD8+) T cells, as well as B cells and macrophages (*Filippi et al., 2018; Reich et al., 2018*). While T cells are reactive to myelin antigens, B cells can adopt different roles. Besides secreting CNS-directed autoantibodies, B cells can also present antigens to helper T cells and release cytokines, contributing to exacerbate inflammation (*Michel et al., 2015*). Microglia and astrocytes become activated with the progression of the disease. Astrocytes form multiple glial scars in the white matter lesions. They can enhance inflammation by releasing effector molecules, but, on the other hand, they may also limit damage by removing glutamate, providing metabolic support to axons and maintaining the BBB (*Ludwin et al., 2016*). Thus, there is a complex inflammatory process that is present at all stages of the disease, but is more prominent in the acute than in the chronic phases (*Dendrou et al., 2015*). Activated microglia and macrophages become pathologically indistinguishable. These cells play dual roles as they sometimes mediate inflammation but in other circumstances, promote myelin repair by

clearance of myelin debris (*Aguzzi et al., 2013; Yamasaki et al., 2014*) or contributing to OL differentiation (*Miron et al., 2013*).

Following demyelination, which may be the consequent of the aberrant immune response, the myelin sheath is lost but the underlying axon remains intact. This allows the formation of the regenerative response, which is called remyelination. This process consists of the generation of new myelin sheaths from newly formed OLs, since existing ones with damaged sheaths do not contribute to myelin restoration (*Franklin and ffrench-Constant, 2017*). The MS lesions that undergo remyelination are called shadow plaques (*Franklin, 2002*).

Two immature cell populations have been involved in promoting remyelination. OPCs, which are able to differentiate into mature myelinating OLs, and adult NSCs, which are known to contribute to adult oligodendrogenesis at least in animal models (*Xing et al., 2014*). In response to white matter injury, OPCs migrate at the edge of the lesion, where they enter the cell cycle and expand. Then, they undergo differentiation, a process that concludes with the formation of new myelin sheaths that are thinner and with shorter internodes than those formed during development (*Blakemore, 1974*). It seems that myelin is patched up rather than restored (*Fancy et al., 2011)*. As occurs in myelination during development, remyelination is also strongly influenced by several molecules. Microglia, macrophages and astrocytes are major sources of factors that promote OPC activation, proliferation and migration (*Franklin and ffrench-Constant, 2017*). However, OPCs often fail to differentiate (*Kotter et al., 2006*; *Kuhlmann et al., 2008*) and/or newly formed OLs fail to wrap myelin, and remyelination fails, as occurs in the chronic phases of MS. Consequently, axonal conduction cannot be restored and the supportive role of myelin is lost. This leads to mitochondrial dysfunction and energy deficiency, impaired axonal transport, and ultimately axonal degeneration that can then elicit a secondary immune response (*Franklin and ffrench-Constant, 2017; Reich et al., 2018*) (**Figure 8**).

Furthermore, Franklin (*2002*) proposed a hypothesis for remyelination failure in which the signaling environment becomes “dysregulated”. According to this hypothesis, remyelination fails due to a loss of coordination in this complex and harmonized mechanism (*Fancy et al., 2011*). Whatever happens, remyelination failure represents a severe problem in MS, being the development of drugs that enchance remyelination and attenuate neurodegeneration the major therapeutic challenge.

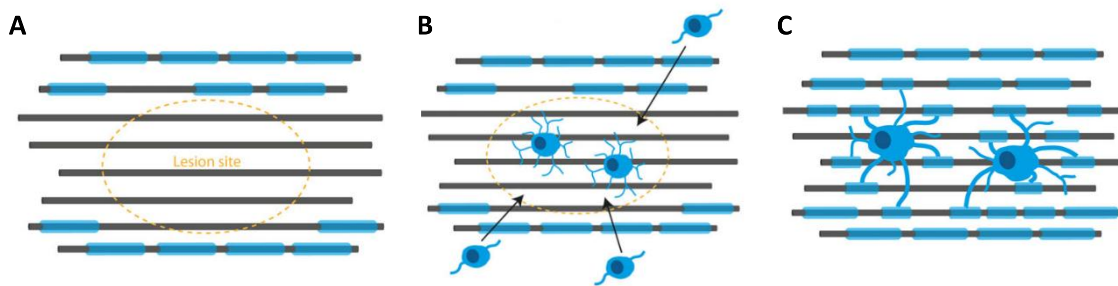

Figure 8. Major steps required for remyelination. Following demyelination **(A)**, OPCs are recruited into the lesion site and then differentiate into myelinating OLs **(B)**. However, they form new myelin sheaths that are thinner and with shorter internodes than those formed during development **(C)**. The remyelination process fails if is not regulated properly (adapted from *Münzel and Williams, 2013*).

3.4. Experimental animal models of MS

The complexity and incomplete understanding of MS requires the use of experimental animal models to further study the disease. Although there are several animal models that reproduce different aspects of MS, none of them recapitulate all the features of its pathophysiology.

3.4.1. Experimental autoimmune encephalomyelitis (EAE)

EAE is the most widely applied animal model of MS and reproduce the inflammatory aspects of the disease. It can be induced by two different protocols, which are the administration of activated T-lymphocytes that act specifically against myelin antigens or, more frequently, by the inoculation of myelin-derived peptides solubilized in Freund´s adjuvant solution (*Palumbo and Pellegrini, 2017*). This produces and immune response against the myelin of the CNS that is predominantly mediated by CD4+ T helper 1 (Th1) and Th17 cells (*Ben et al., 1981; Liblau et al., 1995; Bettelli et al., 2005*), although CD8+ T cells are also present. The addition of the pertussis toxin to the injection mixture facilitates the migration of the lymphocytes across the BBB. Activation of microglia, macrophages and astrocytes also occurs in this model.

The peak of demyelination is reached between 10-15 days after injection and is primarily confined to the spinal cord, whereas in MS inflammatory lesions are mostly found in the brain (*Dendrou et al., 2015*). EAE animals develop a progressive paralysis that starts from the tail and continues to the hind limbs and ultimately the front limbs (*García-Díaz and Estivill-*

Torrús, 2008; Palumbo and Pellegrini, 2017). The clinical course varies with the immunopeptide and the animal strain used. While immunization with MOG peptide in C57BL/6 mice causes a chronic progressive disease (*Slavin et al., 1998*), EAE induced with PLP peptide in SJL/J mice follows a relapsing-remitting course (*Tuohy et al., 1989*).

New transgenic and engineering technologies in combination with advances in imaging are expanding the potential utility of EAE, which is considered a good model to test immunosuppressive therapies. However, it constitutes a reductive model that needs to be use and interpreted with care (*Dendrou et al., 2015*).

3.4.2. Virus-induced inflammatory demyelination

Viral infections may be directly or indirectly involved in the initiation of MS (*Geginat et al., 2017*). Although no MS-specific viral infection have been found, virus induced experimental models of inflammatory demyelination may serve to better understand the basic mechanisms of the disease.

Among the viruses that can produce demyelination in the CNS, the best studied is the Theiler's murine encephalomyelitis virus (TMEV). In this case, chronic demyelinated encephalomyelitis is induced by direct intracerebral infection of the animals with the virus. This leads to an acute encephalomyelitis followed by demyelination mainly in the spinal cord. The disease course and mortality depend on the virulence of the virus strain used and the genetic background of the host animals (*Lassmann and Bradl, 2017*). The lesions are characterized by chronic inflammation, confluent plaques of demyelination, OL apoptosis, axonal damage and remyelination (*Rodriguez et al., 1987*). Inflammatory infiltrates contain a mixture of CD4+ and CD8+ T cells, B cells and plasma cells (*Tsunoda et al., 2007*) and there is also activation of microglia and macrophages. Thus, lesions are similar to those present in MS. Another virus is the mouse hepatitis (corona) virus (MHV), whose JHM strain causes acute encephalitis and chronic demyelination (*Bender and Weiss, 2010*).

3.4.3. Toxin-induced demyelination

Given that primary demyelination is the main pathological hallmark in MS and pre-existing OLs do not contribute to myelin restoration, we need to identify new therapeutic strategies that stimulate OPC differentiation into mature OLs and promote remyelination

(*Lassmann and Bradl, 2017*). However, due to the limitations of studies in human tissue, researchers have focused on animal models to investigate remyelination. Models of toxic demyelination allow the study of the fundamental biology of remyelination without the complicate involvement of an autoimmune process (*Franklin and ffrench-Constant, 2017*). These models are based on the administration of bioactive molecules that specifically targets OLs leading to their degeneration and death, and the subsequent demyelination. Various toxins like cuprizone, lysolecicithin (or lysophosphatidylcholine, LPC) and ethidium bromide efficiently trigger CNS demyelination (*Palumbo and Pellegrini, 2017*).

Cuprizone (bis-cyclohexanone oxalhydrazone) is a neurotoxic copper chelator that impairs cytochrome oxidase activity in OL mitochondria causing apoptosis. The BBB remains intact and no signs of lymphocyte infiltration have been observed (*Kremer et al., 2019*), while microgliosis and astrogliosis are present (*Matsushima and Morell, 2001*). Oral administration of cuprizone in mice results in grey and white matter demyelination, especially in the *corpus callosum* and the superior cerebellar peduncles but not in the spinal cord. After cuprizone removal from diet, spontaneous remyelination occurs via oligodendrogenesis. In contrast, prolonged administration impairs remyelination as in progressive MS (*Palumbo and Pellegrini, 2017*).

Lysolecithin can be injected in several areas of the CNS, as the *corpus callosum* (*Lehto et al., 2017*), the lateral ventricle (*Azim and Butt, 2011*), the optic nerve (*Mozafari et al., 2010*) or the spinal cord (*Tepavcevic et al., 2014*). It induces focal plaques of demyelination due to a direct detergent action against the lipid membrane-rich myelin sheath (*Jeffery and Blakemore, 1995*) and subsequently, OL death. This rapidly leads to OPC recruitment followed by differentiation and remyelination, although the speed and degree of remyelination are age-dependent (*Franklin et al., 2002*; *Crawford et al., 2013*). LPC-induced demyelination occurs independently of immune response and involves the infiltration of T cells, B cells and macrophages, which is suggested to be beneficial for starting remyelination (*Bieber et al., 2003*). Macrophages are proposed to clear the myelin debris that prevents remyelination (*Kotter et al., 2001*). This model allows the study of demyelination and remyelination in different CNS regions without the complication of an autoimmune response and is useful for searching new potential therapies that enhance remyelination, which constitutes the major challenge in MS.

A similar time course of demyelination and remyelination can be attained by injecting ethidium bromide into white matter tracts, causing OL and astrocyte degeneration (*Blakemore, 1982*). Compared to LPC, the last is less toxic for astrocytes and allow demyelination and the subsequent repairing response in a shorter period of time. Hence, both LPC and ethidium bromide constitute an appropriate toxin for inducing local and acute demyelination in the CNS (*Mozafari et al., 2010*).

3.5. Treatments for MS

There is no definitive cure for MS at present, but there are a wide range of pharmacological treatments that attenuate its symptoms and slow down its progression. These treatments are classified into disease-modifying therapies that used to be MS-specific and reduce inflammatory disease activity or relapses frequency; and symptomatic treatments that are not specific for MS and are used for short-term amelioration of the different symptoms of the disease (*Filippi et al., 2018; Dobson and Giovannoni, 2019*).

Disease-modifying treatments (DMTs) are basically immunosuppressant or immunomodulatory therapies that should be administered as soon as possible in order to minimize the risk of disease progression and prevent long-term disability. Thus, injectable interferon beta (IFNβ) and glatiramer acetate have been widely used as first-line treatments, however, they have only moderate clinical effectiveness and poor tolerability due to injection-related adverse reactions as flu or inflammation. The predominant current therapeutic strategy for RRMS is called escalation therapy. It consists of starting with safe but moderately effective DMTs as IFNβ, glatiramer acetate, teriflunomide or dimethylfumarate and then switching to another DMT in patients with intolerable side effects, or to a more effective one in those with new relapses. For patients who do not respond to traditional DMTs, hematopoietic stem cell transplantation might be indicated (*Muraro et al., 2017*). Another strategy is named induction therapy and has appeared due to the need to treat patients earlier with more effective drugs, such as alemtuzumab or ocrelizumab, to prevent the accumulation of CNS damage and clinical disability (*Filippi et al., 2018*). This strategy is indicated for patients with severe and frequent relapses and a higher number of MRI lesions. Fingolimod and natalizumab are also high-efficacy treatments but do not exert a considerable immune reset.

Regarding to progressive disease forms, the anti-CD20 DMTs ocrelizumab and rituximab seem to reduce the risk of disability progression (*Hawker et al., 2009*; *Montalban et*

al., 2017), and are a better option than the cytostatic drug mitoxantrone, which was approved by the US Food and drug administration (FDA) in 2000 but has a limited use due to its cardiotoxic and mutagenic adverse effects (*Hartung et al., 2002*).

Relapses are usually treated with high-dose corticosteroids, but for those that do not respond to this treatment, plasma exchange or intravenous immunoglobulin might be an alternative (*Filippi et al., 2018*).

Symptomatic treatments refer to numerous pharmacological agents and physical therapies prescribed to treat the different symptoms that arise as a consequence of CNS damage. However, for most of them there is no strong evidence of clinical efficacy (*Dobson and Giovannoni, 2019*). Anticholinergic drugs are indicated for bladder dysfunction, although they may induce cognitive impairment. Neuropathic pain is usually treated with gabapentinoids like gabapentin and pregabalin or antidepressants. Opioids are second-line treatments for moderate to severe pain, while cannabinoids can be recommended as a third-line option (*Moulin et al., 2014*). Spasticity is another common symptom of the disease that is targeted with cannabinoids, such as the mucosal spray nabiximol (Sativex) (*Collin et al., 2010*), or other drugs as baclofen, diazepam and tizanidine, although their effectiveness are at stake (*Shakespeare et al., 2003).* The $GABA_B$ receptor ($GABA_BR$) specific agonist baclofen can be administered orally (at higher doses) or intrathecally at lower doses with less adverse effects (*Ertzgaard et al., 2017*). Dalfampridine is a voltage-dependent potassium channel blocker that enhances the transmission of nerve impulses in demyelinated axons improving the walking ability of MS patients (*Goodman et al., 2010*). For the management of fatigue, vitamin D analog alfacalcidol provides clinical benefit (*Achiron et al., 2015*). Physical exercise is also recommended (*Dobson and Giovannoni, 2019*).

Besides that, the major therapeutic challenge is to promote endogenous remyelination by stimulating OPC differentiation and improving the capacity of mature OLs to wrap naked axons. In this sense, there are several remyelinating therapies under clinical trials such as histamine H1 receptor blocker clemastine, the antibody anti-LINGO-1 opicinumab, statins (simvastatin) which are extensively used to treat hypercholesterolemia, vitamin B family coenzyme biotin, the antipsychotic drug quetiapine, the D2/D3 dopamine receptor antagonist domperidone or antisemaphorin 4D (*Cuniffe and Coles, 2019*; *Kremer et al., 2019*).

4. THE GABAERGIC SYSTEM

4.1. GABA synthesis and metabolism

The amino acid GABA is the main inhibitory neurotransmitter in the vertebrate CNS (*Angulo et al., 2008*), though it exerts an excitatory function in the immature brain (*Ben-Ari, 2002*). It is not restricted to the CNS as GABA and its receptors are also expressed in the PNS and in non-neuronal systems such as adrenal glands, kidney, liver, pancreas, stomach and gut, ovary and uterus, semen or urinary bladder (*Erdö, 1992*) as well as in the immune system (*Bhat et al., 2009*).

GABA can be synthesized through two different pathways. On one hand, as firstly demonstrated by Roberts and Frankel (*1950*), it is synthesized by decarboxylation of glutamate (Glu) by glutamic acid decarboxylase (GAD) enzyme. There are two isoforms of GAD, GAD_{65} and GAD_{67}, with 65 and 67 kDa of molecular weight respectively, and with different catalytic and kinetic properties as well as subcellular localization (*Kaufman et al., 1991*). On the other hand, GABA can be produced from putrescine via an alternative pathway that does not involve glutamic acid as an intermediate (*Seiler et al., 1973*). In this case, at least in glial cells, putrescine is first acetylated to monoacetyl putrescine and further degraded to GABA by monoamino oxidase B (MAO_B) enzyme (*Angulo et al., 2008*; *Yoon et al., 2014*). Putrescine is detectable in mouse brain and liver (*Seiler and Askar, 1971*) (**Figure 9**).

Degradation of GABA needs GABA-transaminase (GABA-T), which is a pyridoxal phosphate-dependent enzyme, as well as GAD. It converts GABA to succinic semialdehyde (SSA) by transamination with the co-substrates glutamate and α-ketoglutaric acid (α-KG). Consequently, SSA is oxidized by SSA dehydrogenase (SSADH) to succinate, an intermediary of the tricarboxylic acid cycle (TCA; **Figure 9**) (*Angulo et al., 2008*; *Rowley et al., 2012*). GABA-T is specifically inhibited by vigabatrin, a clinically active antiepileptic drug (*Gram, 1990*).

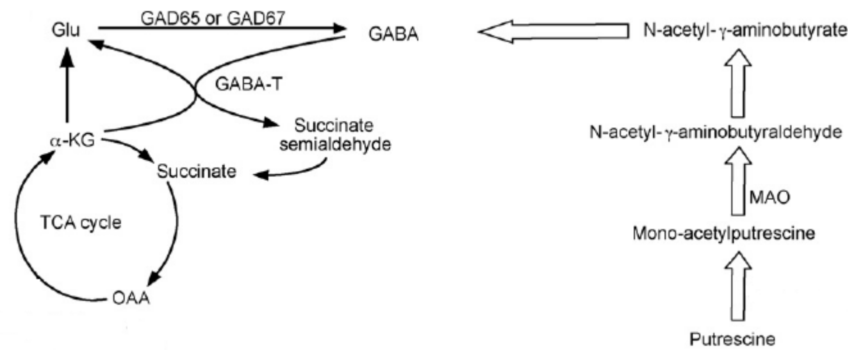

Figure 9. GABA synthesis and GABA metabolism. GABA can be synthesized from glutamate (Glu) through the action of GAD65 or GAD67 or, alternatively from putrescine, which requires the participation of MAO_B enzyme. GABA is metabolized by GABA-transaminase (GABA-T) (adapted from *Angulo et al., 2008*).

4.2. GABA receptors

GABA acts through three major classes of receptors, which have been identified on the basis on their pharmacological and electrophysiological properties: two types of heteropentameric ligand-gated chloride channels, named $GABA_A$ receptors ($GABA_A$Rs) and $GABA_C$ receptors ($GABA_C$Rs); and $GABA_B$ receptors, which are metabotropic or G protein-coupled receptors (GPCRs) (*Enz, 2001*; *Frangaj and Fan, 2018*).

Human ionotropic GABARs are encoded by nineteen genes from the human genome (*Simon et al., 2004*). These include sixteen subunits combined as $GABA_A$Rs ($\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, δ, ε, θ and π) and three ρ subunits that contribute to $GABA_C$Rs, which were originally described in the mammalian retina based on a characteristic pharmacology that did not fit either the “A” or the “B” category (*Polenzani et al., 1991*; *Olsen and Sieghart, 2008*). Compared to $GABA_A$Rs, $GABA_C$Rs have higher sensitivity for GABA, their currents are smaller and they do not seem to desensitize. At the single channel level, they have longer mean open times and smaller chloride conductance (*Enz, 2001*).

$GABA_A$Rs are the targets of numerous clinically important drugs like benzodiazepines (BZs), barbiturates, anesthetics and convulsants that allosterically modulate GABA-induced currents through different binding sites (*Sieghart, 1995*). This fact makes $GABA_A$Rs extremely complex pharmacologically. Knowing the location and structure of these multiple binding sites is required for understanding their modulation by the drugs (*Sieghart, 2015*). $GABA_A$Rs are activated by GABA and the selective agonist muscimol, a natural product from the mushroom

Amanita muscaria; while bicuculline, picrotoxin and gabazine are among its selective antagonists *(Macdonald and Olsen, 1994; Johnston, 2013*).

The existence of such a variety of $GABA_AR$ subunits give rise to a multiplicity of receptor subtypes with different subunit composition and specific pharmacological properties (*Olsen and Sieghart, 2008*), although most $GABA_ARs$ expressed in the brain are composed of 2α, 2β and $1\gamma_2$ subunit. The five subunits form a central chloride channel. Each subunit holds a large N-terminal extracellular domain, four transmembrane domains (TMs) each forming an α-helix, a large intracellular loop between TM3 and TM4, which is the site for several protein interactions and post-translational modifications, and a short extracellular C-terminus (*Schofield et al., 1987*; *Jacob et al., 2008*). A study of $GABA_AR$ subunits by Tretter et al. (*1997*) showed that α and β alternate with each other and are connected by the γ subunit. Moreover, each subunit has a plus (+) and a minus (-) side, being the two GABA-binding sites located at the extracellular β+α- interfaces, and the benzodiazepine one at the extracellular α+γ- interface (*Sieghart, 2015*) (**Figure 10**).

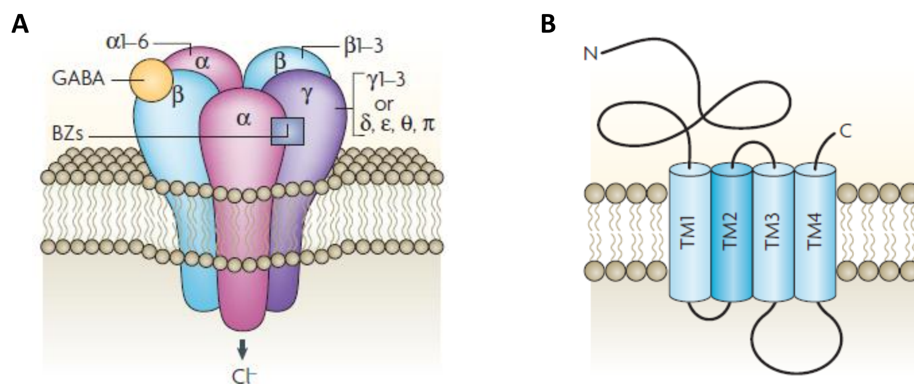

Figure 10. $GABA_A$ receptor structure. A) $GABA_AR$ is composed of five subunits from seven subfamilies **(**α, β, γ, δ, ε, θ and π) that assemble to form a heteropentameric Cl^- permeable channel. Most $GABA_ARs$ expressed in the brain consist of 2α, 2β and $1\gamma_2$ subunit; the γ can be replaced by δ, ε, θ or π. Binding of GABA occurs at the interface between the α and β subunits promoting the opening of the channel and allowing a rapid influx of Cl^- into the cell. BZ binding takes place at the interface between α and γ subunits and potentiates GABA-induced Cl^- influx. **B)** $GABA_AR$ subunits consists of four hydrophobic transmembrane domains (TM1-4), a large extracellular N-terminus which is the site of GABA binding or some psychoactive drugs, a large intracellular domain between TM3 and TM4 that is the site for protein interactions and post-traslational modifications that modulate receptor activity, and a short extracellular C-terminus (adapted from *Jacob et al., 2008*).

$GABA_ARs$ are differentially expressed throughout the brain and mediate fast inhibitory neurotransmission. Binding GABA results in a rapid influx of Cl^- into the cell and BZs binding potentiates this effect. $GABA_ARs$ composed of α (1-3) with β and γ subunits are thought to be localized mainly synaptically, whereas α5βγ and α (4 or 6)βδ are extrasynaptic (*Jacob et al.,*

2008). These receptors are assembled in the endoplasmic reticulum (ER), a process cautiously regulated by classical chaperones. Degradation of homomeric unassembled subunits by ubiquitylation also occurs in this organelle (*Bedford et al., 2001*), and can be regulated by neuronal activity (*Saliba et al., 2007*). After their assembly in the ER, $GABA_A$Rs are trafficked to the Golgi apparatus and divided into vesicles for transportation and insertion into the plasma membrane. This process is promoted by a variety of $GABA_A$R-associated proteins. In neurons, $GABA_A$Rs undergo extensive endocytosis in a clathrin-dependent manner (*Kittler et al., 2000*). Once endocytosed, most of them are recycled back to the plasma membrane over short time frames, while over long time periods they are degraded in lysosomes (*Jacob et al., 2008*).

On the other hand, $GABA_B$ receptors ($GABA_B$Rs), first discovered in 1979 by *Bowery et al.*, are heterodimeric G protein-coupled receptors (GPCR) that mediate slow and prolonged inhibitory transmission. The $GABA_B$ receptor signaling pathways can involve different effector proteins as voltage-gated Ca^{2+} channels, G-protein activated inwardly-rectifying K^+ channels (GIRK) and adenylate cyclase system (*Frangaj and Fan, 2018*), and their downstream effects include blockade of neurotransmitter release and hyperpolarization of neurons (*Bowery et al., 2002*; *Bettler et al., 2004*). However, the precise coupling of $GABA_B$ receptor to the molecular effector can vary depending on the cell type and region analyzed (*Booker et al., 2018*).

Two major $GABA_B$R isoforms, $GABA_{B1}$ and $GABA_{B2}$, have been described (*Bettler et al., 2004*; *Bettler and Tiao, 2006*). In CNS neurons, $GABA_{B1}$ and $GABA_{B2}$ are widely co-expressed and were found to generate functional receptors in a heterodimeric assembly (*Kaupmann et al., 1998*; *Kuner et al., 1999*; *Marshall et al., 1999*). While $GABA_{B1}$ binds orthosteric ligands, $GABA_{B2}$ couples with G protein and contains the allosteric ligand-binding site. The receptors are composed of three domains, a large N-terminal extracellular domain that contains a Venus flytrap-like structure (VFT), a seven-helix transmembrane (TM) domain, and a cytoplasmic tail with a leucine zipper forming a coiled-coil domain (*Benke et al., 2012*; *Frangaj and Fan, 2018*). Two variants of $GABA_{B1}$, $GABA_{B1a}$ and $GABA_{B1b}$ have been identified by molecular cloning (*Kaupmann et al., 1998*). While $GABA_{B1a}$ is predominantly found in the presynaptic terminal, $GABA_{B1b}$ is associated with the postsynaptic one and differ by the presence of two complement control protein (CCP) modules or sushi domains in the N-terminus of $GABA_{B1a}$. The VFT module of each $GABA_B$R subunit contains two domains or lobes called L1 and L2. Binding of an agonist provokes the closing of the $GABA_{B1b}$ VFT, whereas $GABA_{B2}$ VFT is permanently vacant and its

interdomain cleft remains open. Despite these differences, both subunits cooperate with each other to perform signal activation (*Frangaj and Fan, 2018*) (**Figure 11**).

The orthosteric ligand-binding site of $GABA_B$Rs can accommodate various agonists without altering ligand affinity. These orthosteric ligands are usually derivatives of GABA. GABA and the clinical drug *R*-baclofen are two of the best understood $GABA_B$R agonists and they are thought to bind via their carboxylic group to the hydroxyl groups of S246 and Y366 (*Bettler et al., 2004*). On the other hand, there have been described a large range of antagonists with low and high affinities for the $GABA_B$R. The attachment of 3,4-dichlorobenzyl or 3-carboxybenzyl substituents to the existing molecules produced compounds with affinities about 10.000 times higher than previous antagonists, which is the case for CGP55845 (*Bowery et al., 2002*).

The activity of $GABA_B$Rs can be regulated by allosteric modulators binding to the TM domain of the $GABA_{B2}$ subunit. They are classified into three categories: postitive (PAMs), negative (NAMs) and neutral allosteric modulators. The last ones compete with PAMs and NAMs for specific sites without modifying receptor activity. Moreover, Ca^{2+} increases the affinity of GABA to the receptor and enhances G protein activation. However, this allosteric effect is not observed for baclofen (*Galvez et al., 2000*; *Frangaj and Fan, 2018*).

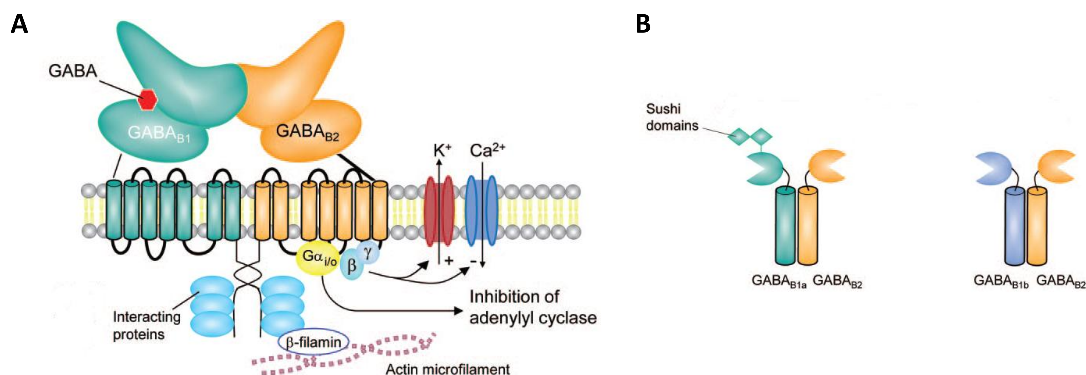

Figure 11. $GABA_B$ receptor structure. A) $GABA_B$Rs are GPCRs with a central core domain constituted of 7 TM helices. They are obligatory heterodimers formed by $GABA_{B1}$ and $GABA_{B2}$ subunits. While $GABA_{B1}$ subunit contains the large extracellular domain that binds GABA, the $GABA_{B2}$ subunit couples the receptor with the effector G protein (predominantly $G_{i/o}$). Their inhibitory effect is via inhibition of adenylyl cyclase and presynaptic Ca^{2+} channels, and activation of postsynaptic K^+ channels. **B)** Detail of the $GABA_B$R subunits. There are two isoforms of $GABA_{B1}$ subunits, named $GABA_{B1a}$ and $GABA_{B1b}$ that differ by the presence of the extracellular sushi domains in the isoform $GABA_{B1a}$ (adapted from *Benarroch. 2012*).

Surface expression of $GABA_BRs$ is regulated by the intracellular domain. The $GABA_{B1}$ subunit is only transported into the cell surface when it is chaperoned by $GABA_{B2}$ in a heterodimeric pair. This subunit is confined in the ER by the membrane protein PRAF2, exiting the organelle only when PRAF2 is displaced by $GABA_{B2}$. Thus, the assembled heterodimer goes to the Golgi apparatus and then to the cell surface, where the receptor can function (*Doly et al., 2016*). $GABA_BRs$ display fast constitutive internalization, being endocytosed *via* the clathrin and dynamin-dependent pathway. They are predominantly recycled back to the plasma membrane and a small fraction is degraded in lysosomes (*Benke et al., 2012*).

Regarding to its desensitization, three different mechanisms have been described. In cerebellar granule cells, G protein receptor kinase (GRK) 4 and 5 associate with the receptors and induce its desensitization in a phosphorylation-independent manner (*Perroy et al., 2003*), while in cortical and hippocampal neurons this process implicates the participation of NEM-sensitive fusion protein (NSF) and PKC (*Pontier et al., 2006*). Some members of the potassium channel tetramerization domain-containing (KCTD) protein family as KCTD-12 or 12b also lead the receptor to desensitization (*Benke et al., 2012*).

4.3. GABA transporters

GABA transporters (GATs) are electrogenic secondary active transport proteins that couple the cotranslocation of 3 Na^+, 1 Cl^- and one GABA molecule across the plasma membrane of neurons and glia (*Willford et al., 2015*). They regulate GABA concentration in synaptic and extrasynaptic regions modulating the GABAergic signaling.

Four GATs with different nomenclature have been cloned from different species. While mouse GATs are named GAT-1, GAT-2, GAT-3 and GAT-4, rat and human are called GAT-1, BGT-1, GAT-2 and GAT-3 (*Rowley et al., 2012*). Besides GABA, BGT-1 also transports betaine, and GAT-2 and GAT-3 β-alanine and taurine (*Liu et al., 1993).* These transporters are composed of 12 TM domains with the N- and C-termini located intracellularly and a potent glycosylation sequence between TM helices III and IV (*Kanner, 1994*).

The regional and cellular distribution of GATs differs within the brain. GAT-1 and GAT-3 are restricted to the brain, while BGT-1 and GAT-2 are also found in peripheral tissues, mainly liver and kidney (*Gadea and López-Colomé, 2001*; *Rowley et al., 2012*). GAT-1 is the most

studied of the GATs and is expressed throughout the brain. Although it is primarily found on presynaptic GABAergic neurons, it is also localized in astrocytic processes in human and monkey cerebral cortices (*Conti et al., 1998*), and in OLs (*Zhang et al., 2014; Fattorini et al., 2017*). GAT-3 is mainly expressed by glial cells, specifically on distal astrocytic processes that are in direct contact with GABAergic terminals however, its expression has also been reported in neurons (*Durkin et al., 1995*; *Minelli et al., 1996*). In the adult rat brain, GAT-2 is strongly expressed in the leptomeninges, choroid plexus and ependyma, while in neurons and astrocytes its expression is weak (*Conti et al., 1999*).

GATs are able to bind GABA and remove it from the extracellular space but, under certain ionic conditions, they can operate in a reverse manner transporting GABA from the cytoplasm back to the extracellular space, as observed in Bergmann glia (*Barakat and Bordey, 2002*) and neurons (*Wu et al., 2003*) (**Figure 12**). GAT-1 transporter can be selectively blocked with anticonvulsant drugs as tiagabine or NNC-711 (*Nielsen et al., 1991*; *Suzdak et al., 1992*). Blocking GABA transport produces an increase in GABA levels in the synaptic cleft, which alleviates pathologies where GABA transmission is reduced. Thus, tiagabine is used as an add-on therapy to treat epilepsy (*Rowley et al., 2012).*

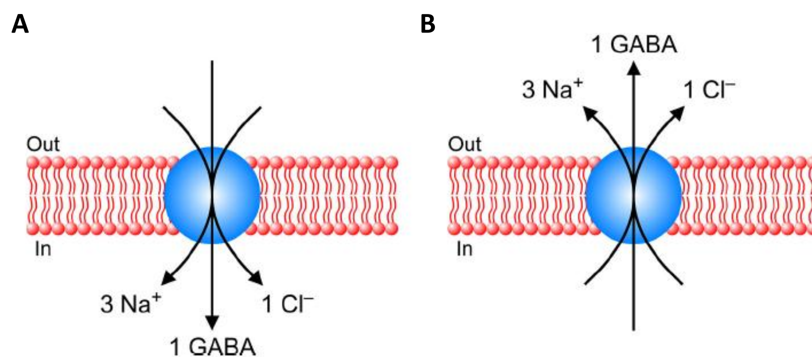

Figure 12. Ion/substrate coupling stochiometry of GABA transporters. A) Forward and **B)** reverse transport mechanisms proposed for these transporters (adapted from *Willford et al., 2015*).

4.4. GABA receptors and transporters in oligodendrocytes

Apart from neurons, OPCs also express functional $GABA_A$Rs (*Hoppe and Kettenman, 1989*; *Von Blankenfeld et al., 1991*; *Kirchhoff and Kettenman, 1992*; *Borges et al., 1995*; *Williamson et al., 1998*; *Lin and Bergles, 2004*; *Cahoy et al., 2008*) however, this expression decreases when they differentiate into OLs (*Von Blankefeld et at., 1991*; *Vélez-Fort et al., 2012*) and it is lost when OLs are cultured in isolation needing the presence of axons to be

maintained (*Arellano et al., 2016*). This means that $GABA_A$R expression in cultured OLs is driven by axonal cues (*Arellano et al, 2016*). Expression of $GABA_B$Rs has also been reported in developing OLs (*Luyt et al., 2007*). On the other hand, colocalization studies between GAT-1 GABA transporter and specific markers for OLs showed that GAT-1 is expressed in both immature and mature OLs in subcortical white matter, and *in vitro* functional assays demonstrated that OLs exhibit GAT-1 dependent GABA uptake (*Fattorini et al., 2017*). These data suggest a specific role for GABA signaling in oligodendroglial lineage development that could be relevant at the initial stages of myelination and/or during axon recognition (*Vélez-Fort et al., 2012*; *Zonouzi et al., 2015*). Moreover, a recent study from Hamilton et al. (*2017*), points at GABA as a potential modulator of oligodendroglial cell number and myelination, and $GABA_B$Rs has been involved in Schwann cell differentiation (*Procacci et al., 2013*). Understanding whether GABA and GABARs are relevant for myelination during development and remyelination in pathological conditions is crucial in the context of demyelinating diseases such as MS, in which reduced GABA levels and dysfunctional GABAergic transmission are associated with cognitive impairment and physical disability (*Cawley et al., 2015*; *Cao et al., 2018*).

HYPOTHESIS AND OBJECTIVES

Demyelination is the main pathological hallmark in multiple sclerosis (MS) but pre-existing oligodendrocytes (OLs) do not contribute to myelin restoration (*Lassmann and Bradl, 2017*). Oligodendrocyte precursor cells (OPCs) are the primary source of remyelination after a demyelinating insult since these cells are able to proliferate, migrate into the lesion and differentiate into myelinating OLs (*Franklin and ffrench-Constant, 2017*). Differentiation of OPCs into mature myelinating OLs is a complex process mediated by neuron-glia interactions that involves the participation of a wide range of molecules such as growth factors and neurotransmitters like GABA (*Zonouzi et al., 2015*; *Hamilton et al., 2017*). We previously reported that the expression and function of $GABA_A$ receptors ($GABA_ARs$) in cultured OLs are regulated by axon-to glia interactions (*Arellano et al., 2016*), suggesting that $GABA_AR$ regulation in these cells is driven by axonal cues. Based on that, we hypothesize that GABA signaling may play a relevant role in OL differentiation and myelination and/or during axon-glia recognition.

The general objective of this Doctoral Thesis has been to study the involvement of the GABAergic system in OPC differentiation as well as in OL myelination and remyelination after demyelinating insults using both *in vitro* and *in vivo* approaches.

For this purpose, we defined the following specific objectives:

1. To study the expression of the major components of the GABAergic system in OLs.

2. To determine the contribution of GABARs to OL differentiation and myelination capacity *in vitro*.

3. To evaluate the involvement of GABARs in myelination during development and in myelin restoration after experimental demyelinating lesions *in vitro*.

4. To analyze the role of GABARs in myelination during development and in remyelination after demyelinating lesions *in vivo*.

MATERIALS AND METHODS

1. ANIMALS

All procedures with animals were carried out with the approval of the internal Animal Ethics Committee of the University of the Basque Country (UPV/EHU) and following the European Communities Council Directive. In particular, all protocols were approved by the "Ethics Committee on Animal Experimentation" (CEEA) which is a collegiate authority in the operational structure of the Ethics Commission for Research and Teaching (CEID) of the University of the Basque Country. Animals were housed in standard conditions with 12 hours light cycle and with *ad libitum* access to food and water. All possible efforts were made to reduce the number of animals used in this study and their suffering.

Experiments were performed in Sprague Dawley rats; C57BL/6 mice; transgenic mice expressing EYFP under the control of the NG2 promoter (NG2-EYFP; *Karram et al., 2008*), a generous gift from Dr. S. Mato (University of the Basque Country, UPV/EHU, Spain) and Dr. J. Trotter (Gutenberg University Mainz, Germany); and transgenic mice expressing fluorescence reporter DsRed under the control of the glial-specific proteolipid protein promoter (PLP-DsRed; *Hirrlinger et al., 2005*), generously provided by Dr. F. Kirchhoff (University of Saarland, Homburg, Germany).

2. *IN VITRO* MODELS

2.1. Rat cortical oligodendrocyte primary culture

Purified OPCs were prepared from mixed glial cultures obtained from the forebrain of newborn (P0-P2) Sprague-Dawley rats according to the protocol of *McCarthy and de Vellis, 1980* with modifications (*Chen et al., 2007*; *Canedo-Antelo et al., 2018*; *Sánchez-Gómez et al., 2018*). Briefly, forebrains were removed from the skulls, and after careful removal of the meninges, cortices were isolated and digested by incubation (15 min, 37°C) in Hank's balanced salt solution (HBSS, without Ca^{2+} and Mg^{2+}) containing 0.25% trypsin and 0.004% deoxyribonuclease (DNAse) (all from Sigma-Aldrich). Then, enzymatic reaction was stopped by adding Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS, Hyclone; both from Gibco) and the cell suspension was centrifuged at 1000 rpm for 5 min. The pellet was resuspended in 1 ml of the same solution and cells were dissociated by passage through needles (21G and 23G), centrifuged and resuspended again in IMDM +

10% FBS. Afterwards, cells were seeded into 75 cm^2 flasks coated with poly-D-lysine (PDL; 1 µg/ml; Sigma-Aldrich) and maintained in culture at 37°C and 5% CO_2 with a medium change every 3 days. After 7 days in culture, flasks were shaken (400 rpm, 1 h, at 37°C) to remove adherent microglia. The remaining OPCs present on top of the confluent monolayer of astrocytes were detached by shaking overnight at 400 rpm. The resulting cell suspension was filtered through a 10 µm pore size nylon mesh and preplated in 100 mm coated Petri dishes (ThermoFisher Scientific) for 30 min at 37°C and 5% CO_2, allowing microglia become firmly attached while OPCs were loosely attached and could be collected with a gentle shaking of the Petri dishes. The collected forebrain OPC cell suspension was filtered again through a 10 µm pore size nylon mesh. Cell number was determined by trypan blue staining (Sigma-Aldrich) with 20 µl of sample and the rest of the cell suspension was centrifuged at 1000 rpm for 10 min and resuspended in a chemically defined medium (OL differentiation medium) consisting of a supplemented (4.5 g/l glucose and 0.11 g/l sodium piruvate) DMEM base with several factors that favor oligodendrocyte survival and development (**Table 1**).

Table 1. Composition of oligodendrocyte differentiation medium.

Reagent	Concentration	Company
DMEM	Base medium	Gibco
Insulin	5 µg/ml	Sigma-Aldrich
Penicillin/streptomycin	100 U/ml	Lonza
Bovine serum albumin (BSA)	100 µg/ml	Sigma-Aldrich
N-acetyl-cysteine	63 µg/ml	Sigma-Aldrich
L-glutamine	2 mM	Sigma-Aldrich
Transferrin	100 µg/ml	Sigma-Aldrich
Progesterone	62.5 ng/ml	Sigma-Aldrich
Sodium selenite	40 ng/ml	Sigma-Aldrich
Putrescine	16 µg/ml	Sigma-Aldrich
Triiodothyronine (T3)	30 ng/ml	Sigma-Aldrich
Thyroxine (T4)	40 ng/ml	Sigma-Aldrich
Ciliary neurotrophic factor (CNTF)	10 ng/ml	Peprotech
Neurotrophin 3 (NT3)	1 ng/ml	Peprotech

Cells were plated onto PDL-coated 14-mm-diameter coverslips in 24-well plates at densities between 4 x 10^3 and 8 x 10^4 cells per well and maintained at 37ºC and 5% CO_2 in

chemically defined OL differentiation medium. The purity of oligodendroglial cultures was confirmed by immunostaining with cell type-specific antibodies. After 1 day in culture, PDGFRα$^+$ OPCs represented 97 ± 5% of the total cells, and after 3 days in OL differentiation medium, at least 98% were MBP$^+$ cells (*Sánchez-Gómez et al., 2018*).

2.2. Rat optic nerve-derived oligodendrocyte primary culture

Primary cultures of optic nerve OLs were prepared from 11-day-old Sprague-Dawley rats as previously described (*Barres et al., 1992*) with modifications (*Sánchez-Gómez et al., 2018*). Optic nerves were extracted and meninges were removed in supplemented (2 µl/ml gentamicin, 1 mg/ml BSA and 2 mM glutamine) HBSS under the microscope. After that, optic nerves were cut in small pieces and digested with 1.25 mg/ml collagenase, 0.125% trypsin and 0.004% DNAse (all from Sigma-Aldrich) for 40 min at 37ºC. Then, enzymatic reaction was stopped by adding 10% FBS in DMEM, and cell suspension was centrifuged at 1000 rpm for 5 min. The pellet was resuspended in 1 ml of the same solution. Mechanical dissociation was performed by passage through needles (23, 25 and 27G), and the resulting cell suspension was filtered through a 40 µm nylon mesh (Millipore). Cell number was determined by trypan blue staining with 10 µl of sample and the rest of the cell suspension was centrifuged at 1000 rpm for 10 min. The obtained pellet was resuspended in the chemically defined medium described before (OL differentiation medium, **Table 1**). Cells were seeded onto PDL-coated 14-mm-diameter coverslips in 24 well plates at a density of 5 x 10^3 cells per well. Cells were maintained at 37°C and 5% CO_2 in OL differentiation medium. After 3-5 days in vitro (DIV), cultures were constituted of at least 98% cells positive for O4 antigen and myelin basic protein (O4$^+$/MBP$^+$). The majority of the remaining cells were positive for the glial fibrillary acidic protein (GFAP) immunostaining. No A2B5$^+$ cells or microglial cells were identified in these cultures (*Alberdi et al, 2002*).

2.3. Dorsal root ganglion neurons-oligodendrocyte precursor cells coculture

Dorsal root ganglion (DRG) neurons were obtained from E15 rat embryos as previously described (*Arellano et al., 2016*). The dissociated cells were resuspended in DRG medium composed of Neurobasal medium (Gibco) supplemented with 10% FBS (Sigma-Aldrich), 50 ng/ml nerve growth factor (NGF; ThermoFisher Scientific) and 2% B27 supplement (Gibco). Cells were plated onto PDL-laminin coated coverslips at a density of 5 x 10^4 cells per coverslip.

DRG neurons were cultured for at least 2 weeks in DRG medium alone with fresh medium added every 3 days.

Isolated OLs (either from optic nerve or cortex) prepared as mentioned before were seeded onto a 2- to 3-week-old DRG neuron culture at a density of 2×10^4 cells/coverslip. The medium was changed to a 50:50 mixture of OL differentiation medium and DRG medium, without NGF, and cocultures were left for 14-21 days in control conditions or in presence of 10 µM or 50 µM GABA.

2.4. Cerebellar organotypic culture

Cultures were elaborated from cerebella of P5-P7 or P11-day-old Sprague Dawley rats and P11-day-old transgenic PLP-DsRed mice according to previously described procedures (*Dusart et al., 1997*; *Doussau et al., 2017*; *Tan et al., 2018*). Briefly, after decapitation cerebella were dissected out and cut with a tissue chopper (McIlwain) into 350 µm parasagittal slices. Meninges were removed and slices were plated onto 0.4 µm pore size Millicell CM culture inserts (Millipore), containing 2-3 slices each. Rat cerebellar slices were maintained in 6-well plates for 13-15 days and mice cerebellar slices for 11 days in culture medium consisting of 50% basal medium with Earle's salt (BME), 25% HBSS, 25% inactivated horse serum (all from Gibco), 5 mg/ml glucose (Panreac), 2 mM L-glutamine (Sigma-Aldrich) and antibiotic-antimycotic solution (100 U/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B; ThermoFisher Scientific) at 37°C in a humidified atmosphere with 5% CO_2. Culture medium was replaced every 2-3 days.

2.4.1. EdU labeling and detection

5-ethynyl-2´-deoxyuridine (EdU; Invitrogen) is a thymidine analogue that is incorporated into the DNA of the cells as they undergo DNA replication (*Chehrehasa et al., 2009*). EdU (10 µM) was added to the organotypic medium on DIV6 for 48 h, to quantify the number of dividing cells between days 6 and 8 *in vitro*. EdU labeling was performed using Click-iT Alexa Fluor 647 Imaging Kit according to the manufacturer's instructions (Invitrogen) before immunofluorescence.

2.4.2. LPC-induced demyelination in cerebellar slices

LPC-induced demyelination experiments were carried out in cerebellar slices from P11 rats and P11 transgenic PLP-DsRed (*Birgbauer et al., 2004*). Slices were maintained for 7 days *in vitro* and incubated for 16 h with 0.5 mg/ml LPC (Sigma-Aldrich). After that, slices were rinsed with culture medium for 10 min and then fresh medium was added. Treatments with GABAergic drugs were performed at the same time or just after LPC-stimulus. Slices were fixed in culture inserts or proteins were extracted 4-6 days after treatment.

2.5. Optic nerve-derived organotypic culture

Cultures were obtained from optic nerves from P11-day-old transgenic PLP-DsRed mice. Optic nerves together with the retina were extracted in order to maintain tissue organization and cellular connections. Meninges and residual tissue were removed in supplemented (2 µl/ml gentamicin, 1 mg/ml BSA and 2 mM glutamine) HBSS under the microscope, and the optic nerve-retina units were maintained in 0.4 µm pore size Millicell CM culture inserts (Millipore), containing one unit each. Explants were maintained in 6-well plates for 3 days in culture medium described before for cerebellar organotypic cultures at 37°C in a humidified atmosphere with 5% CO_2. To favor appropriate feeding of the optic nerve-retina unit, 50 µl of culture medium were added directly over the tissue (*Azim and Butt, 2011*). At the end of culture period, explants were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) for 30 min at room temperature (RT), washed three times in 0.1 M PBS, taken out from the culture inserts and mounted on glass slides with fluorescent mounting medium (Prolong gold antifade reagent).

3. *IN VIVO* MODELS

3.1. Developing Sprague-Dawley rats

P6-day-old Sprague Dawley rat pups were daily intraperitoneally (i.p) injected with vehicle or baclofen (4 mg/kg; Tocris) for 6 or 16 days and processed for immunohistochemistry and electrophysiology. Drugs were diluted in saline solution (0.9% NaCl; Panreac). The volume of injection was 4 ml/kg.

3.2. LPC-induced demyelination in C57BL/6 mice

LPC-induced demyelination was carried out in the spinal cord of 10-week-old female C57BL/6 mice as previously described (*Tepavcevic et al., 2014*). Briefly, demyelinating lesions were induced by a stereotaxic injection of 0.5 µl of 1% LPC in sterile saline solution. Before the surgery, anesthesia was induced by intraperitoneal injection of a solution of ketamine (100 mg/kg; Fatro)/ xylazine (10 mg/kg; Calier). Two longitudinal incisions into *longissimus dorsi* at each side of the vertebral column were performed, and the muscle tissue covering the column was removed. Animals were placed in a stereotaxic frame, the 13th thoracic vertebra was fixed between bars specifically designed for mouse spinal cord surgery, and intravertebral space was exposed by removing the connective tissue. Dura mater was pierced with a 30G needle, and LPC was injected via Hamilton syringe attached to glass micropipette using a stereotaxic micromanipulator. The lesion site was marked with sterile charcoal (Sigma-Aldrich) so that the area of tissue at the lesion center could be unambiguously identified. Following LPC injection, the wound was sutured and the animals were allowed to recover. Buprenorphine (0.1 mg/kg; Dechra) was subcutaneously administered as postoperative analgesic treatment. Five days after surgery, mice were daily i.p injected with vehicle (saline solution) or baclofen (8 mg/kg) for 7 days and processed for immunohistochemistry at the peak of oligodendrocyte differentiation (12 days post-lesion or 12 dpl).

4. SUBSTANCES

The following substances were used: GABA (Ref. A2129), gabazine (Ref. SR-95531) both from Sigma-Aldrich); baclofen (Ref. 0796), CGP55845 (Ref. 1248), muscimol (Ref. 0289) all three from Tocris Bioscience); and PP2 (Src-family kinases inhibitor; Selleckchem).

5. IMMUNOFLUORESCENCE

5.1. Cultured oligodendrocytes

Cells on the coverslips were fixed with 4% PFA in 0.1 M PBS for 20 min at RT and rinsed three times with PBS. Then, fixed cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich), blocked with 4% goat serum (Palex) in 0.1 M PBS for 1 h at RT and incubated with primary antibodies (**Table 2**) overnight at 4ºC in the same solution. After incubation, cells were

washed in PBS and incubated with fluorochrome-conjugated antibodies (**Table 3**) diluted in PBS with 4% goat serum for 1 h at RT. Incubation for 5 min with DAPI (4 µg/ml; Sigma-Aldrich) was used to identify cell nuclei. Cells were washed three times in PBS and mounted on glass slides with fluorescent mounting medium (Prolong gold antifade reagent; ThermoFisher Scientific).

Table 2. Primary antibodies used for immunocytochemistry.

Antibody	Host	Dilution	Company
anti-Olig2	mouse	1:1000	Millipore
anti-Olig2	rabbit	1:800	Millipore
anti-PDGFRα	rabbit	1:200	Santa Cruz
anti-NG2	rabbit	1:200	Millipore
anti-MBP	mouse	1:500	Biolegend
anti-PLP	rabbit	1:100	Abcam
anti-$GABAR_{B1}$	rabbit	1:50	Alomone
anti-$GABAR_{B2}$	rabbit	1:50	Alomone
anti-GAT_1	guinea pig	1:100	Synaptic Systems
anti-GAT_3	rabbit	1:100	Alomone
anti-$GAD_{65/67}$	mouse	1:250	Santa Cruz
anti-MAO_B	rabbit	1:100	Sigma-Aldrich
anti-Ki67	rabbit	1:500	Vector
anti-GABA	mouse	1:7000	*Matute and Streit, 1986*
anti-A2B5 IgM	mouse	1:10	kindly supplied by Dr. Paola Bovolenta (CBMSO, Madrid)
O4 IgM antigen	mouse	1:40	kindly supplied by Dr. Christine Thomson (University of Glasgow)
anti-MBP	mouse	1:100	Covance
anti_NF-L	rabbit	1:200	Cell Signaling

For GABA immunostaining, cells were fixed with 4% PFA and 0.1% glutaraldehyde in 0.1 M PBS for 20 min at RT, permeabilized and blocked as described before and incubated with primary antibody (**Table 2**; *Matute and Streit, 1986*). Specificity of the primary antibody was checked by doing antibody absorption. For that, primary antibody was incubated overnight at

4ºC in blocking solution plus GABA 10 mM. Then, the mix was centrifuged for 5 min at 5000 rmp at 4ºC and supernatant was used for immunocitochemistry.

For immunostaining of A2B5 or the O4 antigen, live cells were incubated for 1 h or 30 min respectively at 37°C with primary antibodies (**Table 2**) in OL differentiation medium. Then, cells were rinsed with 0.1 M PBS, fixed with 4% PFA in PBS as described before, and incubated for 1 h at RT with secondary antibodies (**Table 3**) diluted in PBS containing 4% goat serum. After being rinsed, coverslips were mounted on glass slides.

Table 3. Secondary antibodies used for immunocytochemistry.

Antibody	Host	Dilution	Company
IgG mouse Alexa Fluor 488	goat	1:400	Invitrogen
IgG mouse Alexa Fluor 594	goat	1:400	Invitrogen
IgG rabbit Alexa Fluor 488	goat	1:400	Invitrogen
IgG rabbit Alexa Fluor 594	goat	1:400	Invitrogen
IgG guinea pig Alexa Fluor 647	donkey	1:400	Jackson Labs
IgM mouse Alexa Fluor 488	goat	1:200	Invitrogen
IgM mouse Texas Red Conj.	goat	1:200	Calbiochem

5.2. DRG neurons-OPCs coculture

Cultures were fixed with 4% PFA in PBS as described before, permeabilized with 0.1% Tween 20 and blocked with 5% goat serum in PBS for 30 min at RT. After that, cells were incubated overnight at 4ºC in blocking solution with the primary antibodies mouse anti-MBP, to visualize myelin expression, and rabbit anti-neurofilament-L (NF-L; **Table 2**) to visualize neurites. Then, cells were rinsed and incubated for 2 h at RT with Alexa Fluor 488-conjugated anti-mouse IgG (H+L) and Alexa Fluor 594-conjugated anti-rabbit IgG (H+L) (1:200; Molecular Probes).

5.3. Cerebellar organotypic slices and brain sections

Cerebellar slices in culture inserts were fixed with 4% PFA in 0.1 M PBS for 40 min at RT. After 3 washes in 0.1 M PBS, slices were taken out from the culture inserts and kept in 0.1 M PBS with 0.02% sodium azide (Sigma-Aldrich) at 4ºC. Sprague-Dawley rats and transgenic

mice were anesthetized with 6% chloral hydrate (Panreac) in 0.1 M PBS and transcardially perfused with 4% PFA in 0.1 M phosphate buffer (PB). Brains were extracted and postfixed with the same solution for 3 h at RT, placed in 0.1 M PBS with 0.02% sodium azide at 4ºC and maintain at -20ºC in cryoprotectant solution (30% ethylene glycol (Sigma-Aldrich), 30% glycerol (Merck) and 10% PB 0.4 M in dH_2O). Tissue was cut using a Microm HM650V vibrating blade microtome to obtain coronal 40 µm-thick sections. Cerebellar slices or free-floating vibratome sections were permeabilized with 0.1% Triton X-100 and blocked with 4% goat serum with gentle shaking for 1 h at RT. Then, slices were incubated with primary antibodies (**Table 4**) in blocking solution with 4 or 1% goat serum respectively, overnight at 4ºC with gently shaking**.** After three washes in 0.1 M PBS, with or without 0.1% Triton X-100 respectively, slices were incubated in the same solution as primary antibodies, containing fluorochrome-conjugated secondary antibodies (1:200-1:500; **Table 5**) and DAPI (4 µg/ml) for 1 h at RT with gently shaking. After that, slices were washed three times in 0.1 M PBS with or without 0.1% Triton X-100 respectively, and mounted on glass slides with fluorescent mounting medium (Prolong gold antifade reagent).

5.4. Mice spinal cord tissue samples

LPC-injected mice were anesthetized with 6% chloral hydrate and transcardially perfused with 2% PFA in 0.1 M PB for 20 min. Spinal cords were extracted, additionally post-fixed with the same solution for 30 min at RT, and cryoprotected in 15% sucrose (Panreac) for 48-72 h. Then, tissue samples were embedded in 7% gelatin (Sigma-Aldrich)/ 15% sucrose solution and frozen in isopentane (Honeywell) for 2 min before cryostat cutting. Samples were cut using a cryostat CM3050 S (Leica) at 12 µm.

Spinal cord sections were left at RT for an 1 h, rehydrated in Tris buffer saline (TBS; 20 mM Tris and 1.4 M NaCl in dH_2O, pH 7.6) for 30 min, incubated with absolute ethanol (Sharlab) for 15 min at -20ºC, and blocked and permeabilized with 1% BSA (ThermoFisher Scientific), 5% goat serum and 0.1% Triton X-100 for 30 min at RT. Then, slices were incubated with primary antibodies (**Table 4**) in blocking solution overnight at 4ºC. After three washes in TBS, slices were incubated for 1 h at RT in the same solution as primary antibodies, containing fluorochrome-conjugated secondary antibodies (1:500; **Table 5**).

Table 4. Primary antibodies used for immunohistochemistry.

Antibody	Host	Dilution	Company
anti-$GABAR_{B1}$	rabbit	1:200	Alomone
anti-$GABAR_{B2}$	rabbit	1:200	Alomone
anti-GAT_1	guinea pig	1:500	Synaptic Systems
anti-GAT_3	rabbit	1:100	Alomone
anti-APC (CC1)	mouse	1:200	Calbiochem
anti-Olig2	mouse	1:200-1:1000	Millipore
anti-Olig2	rabbit	1:500-1:1000	Millipore
anti-MBP	mouse	1:1000	Biolegend
anti-MBP	rabbit	1:200	Millipore
anti-MBP	chicken	1:200	Millipore
anti-neurofilament-L	rabbit	1:1000	Cell Signaling
anti-Ki67	rabbit	1:200	Vector
anti-PDGFRα	rat	1:300	BD Biosciences
anti-Iba1	guinea pig	1:200	Synaptic systems

Then, slices were washed three times in TBS, incubated with DAPI for 5 min and washed three times in TBS again. Slices were washed rapidly with Mili-Q H_2O before mounting on glass slides with Fluoromount-G mounting medium (SouthernBiotech).

For APC and Olig2 immunostainning, antigen retrieval was performed by heating the sections in low-pH retrieval buffer (H-3300, Vector Laboratories) for 45 sec using a microwave.

Table 5. Secondary antibodies used for immunohistochemistry.

Antibody	Host	Company
IgG rabbit Alexa Fluor 488	goat	Invitrogen
IgG rabbit Alexa Fluor 594	goat	Invitrogen
IgG mouse Alexa Fluor 488	goat	Invitrogen
IgG mouse Alexa Fluor 594	goat	Invitrogen
IgG2b mouse Alexa Fluor 488	goat	Invitrogen
IgG2b mouse Alexa Fluor 594	goat	Invitrogen
IgG2a mouse Alexa Fluor 546	goat	Invitrogen
IgG chicken Alexa Fluor 647	goat	Invitrogen
IgG rat Alexa Fluor 488	goat	Invitrogen
IgG guinea pig Alexa Fluor 594	goat	Invitrogen
IgG guinea pig Alexa Fluor 488	goat	Invitrogen

5.5. Image acquisition and analysis

Images were acquired using a Zeiss Axioplan2 fluorescence microscope (Department of Neurosciences, UPV/EHU), an Olympus Fluoview FV500 (Analytical and High-Resolution Microscopy Service in Biomedicine, UPV/EHU), a Leica TCS STED SP8 laser scanning confocal microscope and 3DHISTECH panoramic MIDI II digital slide scanner (Achucarro Basque Center for Neuroscience). Same settings were kept for all samples within one experimental group.

Data quantification on isolated OPCs/OLs was performed as follows: $NG2^+$, $A2B5^+$ and $Ki67^+$ cells were counted from at least 10 fields per coverslip in images acquired by using a 20x objective in a Zeiss Axioplan2 fluorescence microscope. MBP^+ cells and values of MBP^+ cells occupied area were derived from at least 15 fields per coverslip using a 20x objective. All these quantifications were obtained by using *ImageJ* software (NIH, Bethesda, MD). Morphology of MBP^+ cells was analyzed by using a *concentric circles* macro (created by Jorge Valero, Achucarro Basque Center for Neuroscience, Spain). Concentric circles at 10 µm intervals emerging from the center of the cell nucleus were created and MBP protein fluorescence signal was quantified after background subtraction. Data were represented as percentage of MBP in soma or periphery respect to MBP total signal for each cell analyzed. All quantifications were performed on a minimum of 2 coverslips per treatment of at least 3 independent

experiments. To avoid the effects of the variations observed in basal differentiation between trials, all experimental conditions were paired with internal control performed in parallel and derived from common pools of cells.

For DRG neurons-OPCs cocultures, MBP expression was analyzed by using *ImageJ* software in 20 random fields per coverslip in images captured with a 20x objective in an Olympus Fluoview FV500. The number of MBP^{+}/$NF\text{-}L^{+}$ segments was counted on a minimum of 30 random fields per coverslip at 40x magnification. All data were showed as percentage respect to non-treated cocultures.

Images from cerebellar organotypic slices of P5-P7 rats, optic nerve explants of PLP-DsRed transgenic mice and cerebellar organotypic slices of PLP-DsRed transgenic mice were acquired by using a 20x objective in a Leica TCS SP8 laser scanning confocal microscope. Cells from rat cerebellar slices were counted blindly along the z-stack. At least three different fields from two slices per experiment were analysed by using *LAS AF Lite* software (Leica). The fluorescence signal corresponding to the PLP-DsRed OLs was quantified by *ImageJ* software and the data were expressed as arbitrary units of fluorescence for each experimental situation. Three independent experiments were included in the analyses. All images shown are projections from z-stacks.

Images of cerebellar organotypic slices of P11 rats and rat brain slices were acquired using a 20x objective (0.8 NA) in a 3DHISTECH panoramic MIDI II digital slide scanner and using a 63x in a Leica TCS STED SP8 laser scanning confocal microscope. Fiber remyelination index was obtained by using *ImageJ* software as Manders´ overlap coefficient of MBP over NF-L staining of at least three different areas from no less than three independent experiments. Cell number from the *corpus callosum* of the brain sections were quantified by using *ImageJ* software. A minimum of 3 different sections per animal were counted from at least 3 rats.

Images of LPC-injected mice spinal cord sections were collected using a 40x objective (1.3 NA) and a 63x objective (1.4 NA) in a Leica TCS SP8 laser scanning confocal microscope and imported to *ImageJ* software. The area lacking MBP staining within the *dorsal funiculus* of the spinal cord (area of demyelination) was delimited as region of interest (ROI) and measured. Cells positive for the markers of interest were counted from at least 3 different slices per animal of as a minimum of 3 mice. Results are presented as total number of positive cells per lesion area measured.

6. PROTEIN EXTRACTS PREPARATION AND DETECTION BY WESTERN BLOTTING

6.1. Oligodendrocyte protein preparation

Cultured cortical OLs were washed twice in cold 0.1 M PBS and scraped in 60 µl of sample buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.002% bromophenol blue and 5.7% β-mercaptoethanol in dH_2O) per treatment (2 wells and 8 x 10^4 cells per well). All the process was performed on ice to avoid protein degradation. After that, lysates were boiled at 99ºC for 8 min.

6.2. Cerebellar slice protein preparation

After 2 days in culture, cerebellar slices from P5-P7-day-old rats were treated with GABAergic drugs for 13 days. Slices from P11-day-old rats were treated as previously described. After treatment, each slice was resuspended in 80 µl of sample buffer and then boiled at 99ºC for 8 min.

6.3. Western blotting

Protein samples were size-separated by SDS-PAGE in 12% Tris-Glycine polyacrylamide gels and Tris-Glycine polyacrylamide gels for the separation of polypeptides from 10-200 kDa (Bio-Rad). Electrophoresis was conducted in a Tris-Glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS in dH_2O, pH 8.3) by using the Criterion cell system (Bio-Rad). Gels were transferred to PVDF membranes by using Trans-Blot® Turbo™ Transfer System (Bio-Rad).

Membranes were blocked for 90 min at RT in blocking solution consisting of Tris buffer saline tween-20 (20 mM Tris, 1.4 M NaCl, 0.05 % Tween-20 in dH_2O, pH 7.6) supplemented with either 5% BSA (Sigma-Aldrich) or 5% Phosphoblocker blocking reagent for phosphorylated protein detection. Then, they were incubated with specific primary antibodies (**Table 6**) in the same solution overnight at 4ºC with gentle shaking. After that, membranes were washed three times in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; Sigma-Aldrich) in blocking solution for 90 min at RT.

Immunoreactive proteins were detected by using enhanced electrochemical luminescence (Supersignal West Dura or Femto; ThermoFisher Scientific) and images were acquired with a ChemiDoc XRS Imaging System (Bio-Rad). Protein bands were quantified by volumetry using *ImageLab* software (version 3.0; Bio-Rad) and were normalized with loading control. At least three independent experiments were analyzed in each case.

Antibodies for phosphorylated proteins were stripped using Restore Western Blot stripping buffer (ThermoFisher Scientific) for 15 min at RT with shaking. Membranes were then washed three times in TBS-T, blocked in 5% BSA and incubated with other primary antibodies.

Table 6. Antibodies for Western blotting.

Antibody	Host	Dilution	Company
anti-PDGFRα	rabbit	1:1000	Santa Cruz
anti-GABAR$_{B1}$	mouse	1:500	Abcam
anti-GABAR$_{B2}$	rabbit	1:200	Alomone
anti-GAT$_3$	rabbit	1:500	Alomone
anti-GAD$_{65/67}$	mouse	1:500	Santa Cruz
anti-MAO$_B$	rabbit	1:500	Sigma-Aldrich
anti-MAG	mouse	1:500	Santa Cruz
anti-CNPase	mouse	1:1000	Sigma-Aldrich
anti-MBP	mouse	1:1000	Biolegend
anti-phospho-Src [Tyr418]	rabbit	1:1000	ThermoFisher Scientific
anti-phospho-CREB (Ser133)	rabbit	1:1000	Cell signaling
anti- Src [36D10]	rabbit	1:1000	Cell Signaling
anti-CREB	rabbit	1:1000	Cell Signaling
anti- GAPDH	mouse	1:1000	Millipore
anti-β-tubulin	mouse	1:5000	Abcam
anti-β-actin	rabbit	1:2000	Sigma-Aldrich

7. RNA EXTRACTION AND QUANTIFICATION

7.1. RNA isolation

Total RNA was extracted from cultured cortical OLs with Trizol reagent (ThermoFisher Scientific) according to the manufacturer´s recommendations (Invitrogen). RNA concentration and integrity were measured by a spectrophotometer Nano Drop™ 2000 (ThermoFisher Scientific).

7.2. Retrotranscription and Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

Strand cDNA synthesis was performed in a 20 µl reaction with 5x buffer (Invitrogen), 0.1 M DTT, random primers (Promega), dNTPs (Invitrogen), RNAase OUT and SuperScript™ III reverse transcriptase (Invitrogen) following manufacturer´s instructions in a Veriti Thermal Cycler (Applied Biosystems). Resulting cDNA samples were diluted in sterile Mili-Q H_2O.

Real-time quantitative PCR (qPCR) reactions were performed by triplicates in 2.95 µl RNAse-free water (Promega), 5.2 µl SYBR-Green (Bio-Rad), 1.35 µl properly diluted primers and 0.5 µl cDNA sample using a Bio-Rad CFX96™ Real-time PCR Detection System. Amplification reactions were optimized (3 min at 95ºC, 40 cycles of 10 sec at 95ºC and 30 sec at 60ºC). Specific primers for $GABA_{B1}$ and $GABA_{B2}$ were obtained from ThermoFisher Scientific and primers for GAPDH and cyclophilin A (CicA) were designed using Primer Express (Applied Biosystems) and PrimerBlast (NIH) (**Table 7**). PCR product specificity was checked by melting curves. Data were normalized to GAPDH and CicA housekeeping genes using a normalization factor obtained in *geNorm* software.

Table 7. Primers used to analyze $GABA_{B1}$ and $GABA_{B2}$ gene expression.

Gene	Forward primer secquence	Reverse primer secquence
Gabbr1	AGATTGTGGACCCCTTGCAC	AGAAAATGCCAAGCCACGTA
Gabbr2	CACCGAGTGTGACAATGCAAA	CCAGATTCCAGCCTTGGAGG
GAPDH	GAAGGTCGGTGTCAACGGATTT	CAATGTCCACTTTGTCACAAGAGA
CicA	CAAAGTTCCAAAGACAGCAGAAAA	CCACCCTGGCACATGAATC

8. CELL VIABILITY ASSAY

Culture OL viability was measured 24 and 72 h after treatment. Cells were incubated with 1 µM calcein-AM (ThermoFisher Scientific) for 30 min at 37ºC in OL differentiation medium and then washed twice in pre-warmed 0.1 M PBS. Emitted fluorescence was measured using a Synergy-HT fluorimeter (Bio-Tek) as indicated by the supplier (485 nm excitation wavelength and 528 nm emission wavelength). All experiments were performed in triplicate. The values provided are the averages of at least three independent experiments.

9. ELECTROPHYSIOLOGY

Compound action potentials (CAPs) were measured in the *corpus callosum* of P25-P42-day-old Sprague Dawley rats treated with vehicle or baclofen (4 mg/kg/day). Animals were anesthetized with isofluorane (Zoetis) and brains were cut in 400 µm-thick coronal sections by using a Leica VT 1200S vibrating blade microtome (Leica Microsystems) at 4ºC in a cutting solution consisting of: 215 mM sucrose, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.6 mM NaH_2PO_4, 20 mM glucose (all from Panreac), 1 mM $CaCl_2$ (Merck), 4 mM $MgCl_2$, and 4 mM $MgSO_4$ (both from Sigma-Aldrich). After that, sections were incubated in a 1:1 mixture of cutting solution and low calcium artificial cerebrospinal fluid (aCSF) for 30 min at 32ºC. Then, sections were maintained in aCFS during the recordings (124 mM NaCl, 2.5 mM KCl, 10 mM glucose, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM $CaCl_2$, and 1.3 mM $MgCl_2$). After that, evoked compound potentials (CAPs) were recorded with a pulled borosilicate glass pipette (≈ 1 MΩ resistance) filled with NaCl 3M by electrically stimulating *corpus callosum* with a bipolar electrode (CE2C55, FHC, USA), stimulation intensities ranging from 30 to 3000 µA (100 µs pulses, Master-8, AMPI, Israel). Conduction velocity values for myelinated (N0), partially myelinated (N1) and unmyelinated (N2) fibers were calculated as the slope of a straight line fitted through a plot of the distance between the recording and stimulating electrodes versus the response latency (time to N0, N1 or N2, respectively) in 4 different distances from both electrodes (500, 1000, 1500 and 2000 µm) subtracting the baseline of the stimulus artifact. Latencies were calculated using custom written routines in pCLAMP 10.0 (Molecular Devices, USA).

10. STATISTICAL ANALYSIS

All data are presented as mean ± S.E.M. Statistical analysis were performed using *GraphPad Prism* statistical software (version 5.0; GraphPad software). Comparisons between multiple experimental groups were made using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. For comparisons between a single experimental group and a control group, we used two-tailed paired or unpaired Student's t-test assuming equal variance. In all instances, statistical differences were considered significant where $p < 0.05$. All the images shown represent the data obtained at least in three independent experiments.

RESULTS

Confidencial por parte de la autora

DISCUSSION

Differentiation of OPCs into mature OLs is a key event for myelination during development and for adult remyelination in the context of demyelinating diseases such as MS (*Franklin and Ffrench-Constant, 2017*; *Kremer et al., 2019*). Failure of OPC differentiation into myelinating OLs or the inability of newly OLs to form new myelin sheaths lead to inefficient remyelination (*Fancy et al., 2011*). Thus, understanding the mechanisms that control oligodendroglial differentiation and myelination is essential for identifying therapeutic strategies that promote myelin repair and limit disability in MS.

OL differentiation and myelination are complex processes mediated by neuron-glial interactions that require the participation of several regulators like neurotransmitters and growth factors. OPCs receive both glutamatergic and GABAergic synaptic inputs (*Bergles et al., 2000*; *Lin and Bergles, 2004*; *Karadottir et al., 2008*; *Fannon et al., 2015*; *Zonouzi et al., 2015*), and oligodendroglial cells express a wide range of neurotransmitter receptors (*Verkhratsky and Steinhaüser, 2000; Butt et al., 2014*). This evidence suggests that these molecules are involved in the regulation of OL development and myelination, as it has been described for ATP, adenosine, glutamate or GABA (*Domercq et al., 2010*; *Li et al., 2013*; *Fannon et al., 2015*; *Zonouzi et al., 2015*; *Hamilton et al., 2017*).

In the present study, we demonstrate that OLs are able to synthesize GABA and express the major components of the GABAergic system, including $GABA_A$Rs, $GABA_B$Rs, GAT-1 and GAT-3 GABA-transporters as well as the two GABA-synthesizing enzymes, $GAD_{65/67}$ and MAO_B. Notably, we show that $GABA_B$R stimulation *in vitro* promotes OPC differentiation enhancing the production of myelin-related proteins. This process requires the phosphorylation of CREB and kinases from the Src family. Interestingly, exogenous GABA and the specific $GABA_B$R agonist baclofen regulate myelination in DRG-OPC cocultures by increasing MBP levels and myelin sheath segments around axons. In cerebellar organotypic slices, $GABA_B$R activation increases myelin protein levels and GABA signaling attenuates LPC-induced demyelination. Finally, we have observed that $GABA_B$R stimulation accelerates OL differentiation *in vivo* in LPC-induced demyelinating lesions in the adult mice spinal cord, as well as during development in the rat *corpus callosum* and cingulate cortex. In addition, conduction velocity of myelinated fibers in the rat *corpus callosum* increases in animals treated with baclofen. These results strongly suggest that GABA signaling mainly through $GABA_B$Rs plays an important role in OL

differentiation and myelination and point at these receptors as a possible therapeutic target to promote remyelination in the context of demyelinating diseases.

1. Oligodendrocytes express the major components of the GABAergic system and synthesize GABA

It is well known that oligodendroglial cells express $GABA_A$ receptors (*Hope and Kettenmann, 1989*; *Von Blankenfeld et al., 1991*; *Berger et al., 1992*; *Cahoy et al., 2008*) and, to a lesser extent, $GABA_B$ receptors (*Luyt et al., 2007*). However, previous studies have reported that $GABA_A$R expression decreases as OPCs differentiate into OLs (*Von Blankenfeld et at., 1991*; *Arellano et al., 2016*) and is lost when these cells are cultured in isolation, needing the presence of axons to be maintained (*Arellano et al., 2016*). In this study, we provide evidence that this is not the case for $GABA_B$Rs as its expression in OLs *in vitro* remains largely stable over time even in absence of axons. Moreover, we have found its presence in oligodendroglial cells from cerebellar organotypic slices, and *in vivo* $GABA_B$R expression occurs in both OPCs ($NG2^+$ cells) and in mature OLs ($CC1^+$ and PLP^+) in the juvenile rodent brain, persisting throughout adulthood (*Serrano-Regal et al., 2019*). We also found $GABA_B$Rs in mature OLs from the mouse spinal cord. These observations are in contrast with that from Charles et al. (*2003*), who did not find colocalization between $GABA_B$Rs and myelin forming oligodendrocytes in the white matter of rat spinal cord. Moreover, it has been reported a downregulation of $GABA_B$Rs in mature OLs (*Luyt et al., 2007*) as well as in pre- and non-myelinating Schwann cells from the sciatic nerve (*Corell et al., 2015*) but the mechanisms that regulate $GABA_B$R expression are likely different between central and PNS. However, and in line with the findings reported in the current study, *Corell et al.* observed that GABA, via $GABA_B$Rs, is a positive regulator of myelination without affecting Schwann cell proliferation.

In this study, we demonstrate $GABA_B$R mRNA and protein expression in mature OLs cultured in isolation as well as *in vivo* in the juvenile and adult rodent CNS (*Serrano-Regal et al., 2019*). Nevertheless, the specific composition of $GABA_B$Rs in OLs still needs to be determined in order to better understand their function in these glial cells. In this sense, while $GABA_B$R-mediated mechanisms in neurons have been well characterized, function of $GABA_B$Rs in OLs is less well understood (*Xu et al., 2014; Booker et al., 2018*). In order to resolve this question, we developed electrophysiological recordings and calcium imaging assays on OLs expecting to detect coupling between $GABA_B$Rs and Kir3 and L-type calcium channels,

described for other cells types. However, we could not get results that confirmed this coupling (data not shown). In this line, a recent paper describes that $GABA_BRs$ are present on dendritic membranes of CA1 somatostatin interneurons, but do not activate the canonical Kir3 signaling cascade, while pyramidal neurons do it (*Booker et al., 2018*). Our data suggest that $GABA_BRs$ in OLs may signal through alternative effectors, whose nature remains elusive.

Regarding GABA transporters, *Fattorini et al.* (*2017*) described that OLs express a functional GAT-1 GABA transporter. Here we show that they also express GAT-3 GABA transporter and again, the expression of both transporters is stable over time as we have detected them at all stages of maturation in cultured OLs. Moreover, we demonstrate the presence of both transporters in mature OLs of the juvenile rat brain (*Serrano-Regal et al., 2019*). The presence of GAT-1 and GAT-3 in these cells is not unexpected as GAT-1 may contribute to the effects of GABA on OLs by regulating GABA levels, either by taking up GABA or by releasing it, if operating in the reverse mode as described in neurons (*Wu et al., 2003*) and Bergmann glia (*Barakat and Bordey, 2002*). In turn, GAT-3 might cooperate with GAT-1 in the regulation of GABA homeostasis in OLs, however its precise contribution requires further investigation.

GABA can be synthesized through two different pathways, either from glutamate decarboxylation by $GAD_{65/67}$ enzyme, or alternatively, from putrescine by the action of MAO_B (*Angulo et al., 2008*; *Yoon et al., 2014*). Consistent with databases (*Zhang et al., 2014*), we have observed that OLs express both $GAD_{65/67}$ and MAO_B. Additionally, we have detected GABA itself in OPCs, $NG2^+$ cells and OLs at different stages of maturation by immunostaining and in OL culture medium by ELISA (data not shown). These findings suggest that OLs synthesize GABA possibly for a proper differentiation and maturation (*Serrano-Regal et al., 2019*) and confirm previous observations by *Barres et al (1990),* who demonstrated GABA synthesis via putrescine in primary OPC cultures. In agreement with that, Bergmann glia and Schwann cells have the ability to produce GABA and respond to this neurotransmitter, suggesting a putative autocrine/paracrine signaling with additional roles in axon-glia communication (*Yoon et al., 2014*; *Corell et al., 2015*). Determining if OLs are able to release GABA at sufficient concentrations to activate GABARs may help to answer this question.

2. GABA via $GABA_B$Rs regulates oligodendrocyte differentiation and myelination *in vitro*

Different studies point at GABA as one of the modulators of oligodendroglial cell proliferation, differentiation and myelination (*Zonouzi et al., 2015*; *Hamilton et al., 2017*). *Hamilton et al.* (*2017*) suggested that endogenous GABA, via $GABA_A$Rs, reduces the number of oligodendroglial cells and, therefore, negatively modulates myelination *in situ* in cortical slices. In contrast, these authors report that activating or blocking $GABA_B$Rs has no effect on the number of OLs, the later in agreement with our data. In turn, we have observed in DRG-OPC cocultures that exogenous GABA and the specific $GABA_B$R agonist baclofen regulate myelination by increasing MBP levels and myelin sheath segments around axons. These effects are likely direct, since GABA stimulation of isolated OPCs accelerates their differentiation into mature OLs by promoting an increase in MBP levels in the periphery of the cells, suggesting that these molecules are promoting local translation of MBP in distal areas. In addition, chronic treatment with baclofen in cultured OLs enhances the production of the myelin proteins MAG and MBP, an event prevented by the $GABA_B$R specific antagonist CGP55845. On the other hand, selective activation of $GABA_A$Rs does not change the levels of these myelin proteins (*Serrano-Regal et al., 2019*). Thus, our results indicate that potentiation of MBP expression and OL differentiation by GABA is mainly mediated by $GABA_B$Rs, as observed in Schwann cells (*Procacci et al., 2013*; *Corell et al., 2015*) and in a similar way as NMDA receptor-mediated OPC differentiation (*Li et al., 2013*; *Lundgaard et al., 2013*).

To elucidate the underlying mechanisms of the $GABA_B$R-induced OL differentiation, we investigated whether this effect is due to an increase in OPC proliferation. We observed that neither GABA nor baclofen have an impact on OPC proliferation *in vitro*. This result is at odds with the idea that GABA decreases OPC proliferation as suggested by *Hamilton et al.* (*2017*). However, it is important to note that dissociated *in vitro* cultures might behave differently than a more integral preparation such as organotypic slices, where different type of cells express GABARs and interact with each other.

3. Baclofen-induced MBP upregulation *in vitro* requires the participation of Src-family kinases and the phosphorylation of CREB

We then explored the downstream molecular mechanisms underlying GABA$_B$R-induced OL differentiation. On one hand, we tested whether GABA$_B$R activation initiated Fyn kinase signaling, a known integrator of neuronal signals that regulates the morphological differentiation of OLs, the recruitment of cytoskeleton components and the MBP local translation (*White and Krämer-Albers, 2014*). Indeed, GABA$_B$R stimulation mediates chemotaxis and cytoskeletal rearrangement, which is dependent on signaling via PI3-K/Akt/Src kinases, and the modulation of this pathway may be potentially used to regulate cellular responses to injury and disease (*Barati et al., 2015*). Our data show that chronic treatment with baclofen in rat OPC cultures induces Src-phosphorylation. Additionally, GABA$_B$-R-induced OL differentiation is blocked by the Src-family kinases inhibitor PP2, suggesting that Src-family acts downstream of GABA$_B$R to favor OL differentiation and local translation of MBP in OL distal processes (*Serrano-Regal et al., 2019*). Similarly, activation of Src and phospho-FAK kinases promotes Schwann cell development and maturation, though these events lie under control of GABA$_A$R-dependent mechanisms (*Melfi et al., 2017*).

On the other hand, CREB is a mediator in the stimulation of MBP expression and plays an important role in OL differentiation and myelination (*Afshari et al., 2001*). Its phosphorylation is regulated by several extracellular and intracellular signals involving the participation of multiple protein kinases (*Mao et al., 2007*). Moreover, stimulation of GABA$_B$Rs with baclofen mediates CREB upregulation in cultured mouse cerebellar granule neurons (*Zhang et al., 2015*). Thereby, we then analyzed CREB phosphorylation and we found that treatment with baclofen induces rapid CREB phosphorylation, an effect that is prevented in the presence of the GABA$_B$R antagonist CGP55845. These results corroborates that baclofen-induced MBP upregulation involves CREB activation.

4. GABAergic signaling modulates oligodendrocyte differentiation in organotypic cultures

GABA is an inhibitory neurotransmitter, however in the immature brain plays an excitatory role during a specific time-window. This fact may be relevant for neuronal growth

and synapse formation (*Ben-Ari, 2002*). The relationship between neuronal activity and oligodendroglial cell function impacts on myelin and axon dynamics and the signals involved in this communication are crucial for circuit formation and maturation. In addition, this is a key event for network functioning (*Habermacher et al., 2019*), as depletion of OLs at postnatal day 1 affects the establishment of cerebellar circuitries in mice (*Doretto et al., 2011*). Myelination has a prominent peak during postnatal development, and considering that OPCs receive glutamatergic and GABAergic synaptic inputs (*Bergles et al., 2000*; *Lin and Bergles, 2004*; *Karadottir et al., 2008*; *Fannon et al., 2015*; *Zonouzi et al., 2015*), signaling through GABARs might be important for OPC development and differentiation as well as for OLs to initiate myelination of axons. This has been reported for $GABA_A$Rs (*Zonouzi et al., 2015*; *Hamilton et al., 2017*), but participation of $GABA_B$Rs in these events is not yet completely known. To answer this question, we analyzed the impact of the GABARs-mediated signaling on OL differentiation and myelination during *in vitro* development using cerebellar organotypic cultures. We observed that $GABA_B$R stimulation with baclofen promotes an increase in the myelin-related proteins MAG, CNPase and MBP that is not observed neither when $GABA_A$Rs are stimulated with muscimol nor when both $GABA_A$Rs and $GABA_B$Rs are activated with exogenous GABA. In the latter, the non-detection of the same effect of that promoted by baclofen may be due to a dual activation of $GABA_A$ and $GABA_B$Rs by GABA, promoting activation of several signaling cascades that could mediate opposing actions and mask the upregulation of myelin proteins observed with baclofen alone. Surprisingly, we found an increase in CNPase levels when slices were treated with the $GABA_B$R antagonist CGP55845, which seems to be contradictory to what is mentioned before. However, $GABA_B$Rs in organotypic slicesare present in oligodendroglia and other cells and CGP55845 is not cell-specific. This fact may have a different impact on oligodendroglial cells, in which CNPase is crucial during development for process formation and branching (*Raasakka and Kursula, 2014*), but the exact mechanism has not been evaluated yet.

On the other hand, in optic nerve organotypic explants from PLP-DsRed, we observed an increase in DsRed fluorescent signal in nerves treated with baclofen versus controls. Altogether, these results suggest that specific stimulation of $GABA_B$Rs with baclofen also upregulates the expression of the mature oligodendrocyte marker PLP in organotypic cultures.

Using a similar approach as in dissociated cultures, we observed in cerebellar organotypic slices that neither GABAR activation with GABA or baclofen, nor $GABA_A$R blocking

with gabazine induce changes in PDGFRα levels, indicating that the OPC population does not change under these conditions. In addition, EdU labeling did not reveal changes in OL proliferation neither with GABA nor with the specific agonists for each GABAR. This is in contrast to the study from *Hamilton et al.* (*2017*), who proposed that endogenous GABA via $GABA_A$Rs reduces the number of total oligodendroglial cells. However, the fact that they used cortical slices could underlie this apparent contradiction, since cells from different CNS areas may behave and interact in a different manner regarding GABA signaling. In this sense, OPCs are functionally heterogeneous among brain regions and different ages because they have different expression of ion channels (*Spitzer et al., 2019*). Apart from that, we observed a reduction in the number of mature OLs and total oligodendroglial cells in the cerebellar slices treated with muscimol, which indicates that $GABA_A$R activation at this time point may be decreasing OPC proliferation and their subsequent differentiation into mature OLs. This late observation is in accordance with the proposal from *Hamilton et al.* In agreement with this, *Zonouzi et al.* (*2015*) also suggest that GABAergic signaling via $GABA_A$Rs regulates OPC proliferation *in vivo* in a mouse model of diffuse white matter injury.

5. GABAergic signaling modulates LPC-induced myelin damage and restores MAG levels in cerebellar organotypic cultures

As cerebellar organotypic cultures constitute an excellent model to study remyelination (*Tan et al., 2018*), we next performed LPC-experimental demyelination in cerebellar slices in order to analyze the impact of the GABAergic drugs in this issue. We carried out two different experimental designs. First, we analyzed the effect of the GABAergic modulators after the demyelinating insult, specifically focusing on remyelination. Our results show that in the presence of baclofen, MAG protein levels recover after LPC-induced demyelination, which points at baclofen as a possible modulator of remyelination However, we did not find a restoration of MBP with any of the treatments. Moreover, we analyzed association of MBP and neurofilament, but we did not find any significant effect either. Thus, although we did not observe a recovery of MBP levels with these approaches, we propose to further investigate this question modifying times and drug concentrations and analyzing in detail other parameters, as the nodes of Ranvier and the state of other proteins present in myelin, as PLP or MOG.

In the second experimental design, we applied the drugs together with LPC, and we observed that activation of GABARs with GABA, baclofen or muscimol ameliorates LPC-induced decrease of MAG and MBP myelin proteins. On the other hand, in cerebellar slices from transgenic PLP-DsRed mice, no loss of PLP induced by LPC occurred in the presence of baclofen, gabazine and GABA+gabazine. The latter suggests that activation of $GABA_BR$ or block of $GABA_AR$ modulates myelin restoration after a demyelinating insult. Blocking $GABA_ARs$ with gabazine could provoke that endogenous GABA, present in cerebellar organotypic preparations, acts preferably via $GABA_BRs$, reinforcing its role in OL differentiation and myelination. Hamilton and colleagues (*2017*) indicated a similar possibility to justify the effect of endogenous GABA release on oligodendrocyte development in cerebral cortical slices, where blocking $GABA_ARs$ with gabazine increased the number of oligodendrocyte lineage cells. They suggested that GABA could be release from OPCs or astrocytes, as both synthesize GABA from putrescine using MAO_B (*Barres et al., 1990; Yoon et al., 2014*). In addition, they postulated that gabazine could also act on neurons, increasing spiking frequency and promoting the release of substances that affect oligodendroglial population. Finally, they concluded that a combination of both events might explain their results, and point that the difference in behavior from the cerebellar OPCs studied by *Zonouzi et al. (2015*) could reflect the difference in the brain area studied (*Hamilton et al., 2017*), an interpretation that could be extended to the results of our work.

6. Baclofen promotes *in vivo* OL differentiation and accelerates postnatal myelination in rats

We performed *in vivo* studies in rats to further analyze the effect of $GABA_BR$ activation in OL differentiation and myelination during development. Immunohistochemical studies at P12 revealed an increase in OL differentiation in the corpus callosum of baclofen-treated rats compared to controls, which is in accordance with our results *in vitro* and supports the role of $GABA_BR$ as a key regulator of OL differentiation. On the other hand, total number of oligodendroglial cells remained unchanged after baclofen treatment. Despite *Luyt et al.* (*2017*) described an increase in OPC proliferation *in vitro* after baclofen exposure, our results *in vivo* are in agreement with those obtained *in vitro* (*Serrano-Regal et al., 2019*) and with the observations by *Corell et al.* (*2015*), who reported that $GABA_BR$ activation with baclofen does not affect developmental Schwann cell proliferation.

In contrast to the results above, we did not observe an increase in the number of mature OLs in the presence of baclofen in more advanced stages of development (P34-P42). Considering that mature OLs mostly arise during the first postnatal weeks (*Habermacher et al., 2019*), our data indicates that baclofen appears to be more effective in sizing the oligodendrocyte population. Thus, the lack of changes in the number of mature OLs at advanced stages of development may be due to a strong reduction in the differentiation rate at these ages. In contrast, we found an enhancement in MBP fluorescence intensity between P34-P42, both in the *corpus callosum* and the cingulate cortex. This latter result reflects that myelination is potentiated by $GABA_BR$ activation since it modulates the peak of myelination, which in rats takes place around P20 (*Downes and Mullis, 2014*). Altogether, these results suggest that GABA signaling specifically through $GABA_BRs$ accelerates OL differentiation and MBP production during development. Improving myelination during development is crucial to deal with neurological and psychological disorders that appear caused by white matter disturbances (*Travis et al., 2019*).

Interestingly, we found a side effect after *in vivo* administration of baclofen. Animals treated with this drug exhibited a slower gain of weight body during development (data not shown) which may be due to the systemic effects of GABA signaling as it is known that GABAR expression is not restricted to the nervous system. Purwana et al. (*2014*) demonstrated that GABA modulates glucose homeostasis. In this study, GABA treatment increases β-cell mass in a mouse model of diabetes transplanted with human islets. This fact was associated with increased insulin circulating levels and reduced blood glucose levels, and was attributed to both $GABA_A$ and $GABA_BRs$ involving the activation of the PI3K-Akt signaling pathway and the stimulation of CREB. Thus, this reduction in the body weight observed in our experiment may be explained by the modulation of glucose homeostasis exerted by GABARs. Another possible explanation may be that myelination is a costly process in terms of energy, as enough lipids and proteins are produced in order to wrap many membranes around axons. This energetic investment may require more food intake or burning body fat (*Harris and Attwell, 2012*). Considering that, baclofen-treated animals could have an unbalance in their metabolism, burning more calories than what they intake.

As myelin allows fast propagation of nerve impulses (*Baumann and Pham-Dinh, 2001*) and the *corpus callosum* is the biggest white matter tract in the brain, we checked the functional implications of the baclofen-mediated upregulation of MBP during development. To

that end, we recorded compound action potentials (CAPs) of the *corpus callosum* fibers in order to determine if treatment with baclofen modified its conduction velocity. The *corpus callosum* is widely used to study white matter function under physiological and pathological conditions. It contains both myelinated and unmyelinated axons and constitutes a great structure to characterize the electrophysiological pattern of white matter. Two types of phase peaks are present in CAP traces. The early peak (N1) represents the conduction of myelinated axons, whereas the late peak (N2) represents unmyelinated fibers. The heterogeneity of the CAPs responses between the rostral and the caudal part of the *corpus callosum* is due to the different proportion of myelinated and non-myelinated axons (*Mu et al., 2019*). This argument matches with the fact that in our study we have observed the appearance of a third peak in CAPs recordings in the first place (N0). Thus, we can classify the fibers in myelinated (N0), partially myelinated (N1) and unmyelinated (N2). Our data show that $GABA_B$R activation with baclofen decreases the latency of myelinated fibers (N0) meaning that its conduction velocity is increased, whereas the conduction of N1 and N2 fibers remain unchanged. Thus, although further analysis including electron microscopy may help to confirm this hypothesis, we hypothesize that the increase in the conduction velocity produced by baclofen is associated with a higher myelination ratio. This result is consistent with the effects of baclofen modulating OL differentiation and myelination, and reinforces the role of $GABA_B$R activation in myelination *in vivo*.

7. $GABA_B$R activation promotes OPC differentiation without modifying proliferation *in vivo* after LPC-induced demyelination

Given that primary demyelination is the main pathological hallmark of MS and pre-existing OLs do not contribute to myelin restoration (*Franklin and ffrench-Constant, 2017*), it is necessary to develop therapies that stimulate OPC differentiation into mature OLs and promote remyelination (*Lassmann and Bradl, 2017*). With this goal in mind, we decided to assess the potential role of baclofen in promoting OPC differentiation after LPC-induced demyelination *in vivo* in the mouse spinal cord. This model drives toxic demyelination without the complication of an autoimmune response (*Franklin and ffrench-Constant, 2017*). LPC injection induces focal plaques of demyelination due to its detergent action against the myelin sheath (*Jeffery and Blakemore, 1995*) and it is a useful experimental setting to study OL differentiation (*Azim and Butt, 2011*). Thus, after treating LPC-injected mice with baclofen, we performed immunohistological studies at day 12th post-lesion, which coincides with the peak

of OPC differentiation. Our results show that whereas the number of OPCs and oligodendroglial cells remain unchanged, treatment with baclofen increases OPC differentiation into mature OLs. As pre-existing OLs do not contribute to remyelination following toxin-induced demyelination (*Crawford et al., 2016*), these mature OLs derived from OPCs withinthe lesion. This result indicates that at that stage baclofen is able to induce OPC differentiation but not OPC recruitment whose peak takes place at 7 dpl. Therefore, although the specific contribution of baclofen to remyelination needs to be evaluated later in time -at the peak phase of remyelination (21 dpl)- this result shows the potential role of baclofen in promoting OPC differentiation and the initial stages of remyelination in a pathological context. This observation highlights the relevance of the GABAergic metabotropic signaling in modulating OL differentiation and myelination under pathological conditions, as described earlier for $GABA_A$Rs (*Zonouzi et al., 2015*; *Kalakh and Mouihate, 2019*).

Finally, microglial cells are key to the inflammatory response that occurs in the damaged area after LPC-induced demyelination (*Bieber et al., 2003*). Thus, we also evaluated the efficacy of baclofen in modulating the inflammatory response triggered by these glial cells, so we analyzed changes in microglial cell number. At 12 dpl, treatment with baclofen did not induce changes in microglia, indicating that the treatment may be out of the time window in which baclofen exerts its effects. Nonetheless, we need analyze additional parameters to further assess the potential of baclofen in modulating microglial inflammatory response, as changes in cellular morphology, release of pro and anti-inflammatory molecules or phagocytic capacity of these cells to engulf myelin debris.

Overall, the present study introduces an important role for the GABAergic system via $GABA_B$Rs in oligodendroglial cell differentiation and myelination. In particular, it will be relevant to study the role of GABA in demyelinating diseases, such as MS, in which reduced GABA levels are associated with cognitive impairment and physical disability (*Cawley et al., 2015*; *Cao et al., 2018*). Since baclofen is already used as a treatment for spasticity in MS (*Stevenson 2014*; *Ertzgaard et al., 2017*), elucidating its exact role in OL differentiation and myelination may reinforce its clinical use. In addition, understanding their mechanisms of action may be relevant for the development of new therapies that promote remyelination under pathological conditions.

CONCLUSIONS

Confidencial por parte de la autora

REFERENCES

Achiron A, Givon U, Magalashvili D, Dolev M, Liraz Zaltzman S, Kalron A, Stern Y, Mazor Z, Ladkani D, Barak Y (2015) Effect of alfacalcidol on multiple sclerosis-related fatigue: a randomized, double-blind placebo-controlled study. *Mult Scler* 21: 767-75.

Afshari FS, Chu AK, Sato-Bigbee C (2001) Effect of cyclic AMP on the expression of myelin basic protein species and myelin proteolipidprotein in committed oligodendrocytes: differential involvement of the transcription factor CREB. *J Neurosci Res* 66: 37-45.

Aguzzi A, Barres BA, Bennet ML (2013) Microglia: scapegoat, saboteur, or something else? *Science* 339: 156-161.

Alberdi E, Sánchez-Gómez MV, Marino A, Matute C (2002) Ca(2+) influx through AMPA or kainate receptors alone is sufficient to iniciate excitotoxicity in cultured oligodendrocytes. *Neurobiol Dis* 9: 234-243.

Angulo MC, Le Meur K, Kozlov AS, Charpak S, Audinat E (2008) GABA, a forgotten gliotransmitter. *Prog Neurobiol* 86: 297-303.

Antony JM, van Marle G, Opii W, Butterfield DA, Mallet F, Yong VW, Wallace JL, Deacon RM, Warren K, Power C (2004) Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination. *Nat Neurosci* 7: 1088-95.

Arellano RO, Sánchez-Gómez MV, Alberdi E, Canedo-Antelo M, Chara JC, Palomino A, Pérez-Samartín, Matute C (2016) Axon-to-glia interaction regulates GABAA receptor expression in oligodendrocytes. *Mol Pharmacol* 89: 63-74.

Ascherio A, Munger KL (2016) Epidemiology of multiple sclerosis: from risk factors to prevention-an update. *Semin Neurol* 36: 103-14.

Azim K, Butt AM (2011) GSK3β negatively regulates oligodendrocyte differentiation and myelination *in vivo*. *Glia* 59: 540-553.

Bhat R, Axtell R, Mitra A, Miranda M, Lock C, Tsien RW, Steinman L (2010) Inhibitory role for GABA in autoimmune inflammation. *Proc Natl Acad Sci USA* 107: 2580-5.

Barati MT, Lukenbill J, Wu R, Rane MJ, Klein JB (2015) Cytoskeletal rearrangement and Src and PI-3K-dependent Akt activation control $GABA_B$R-mediated chemotaxis. *Cell Signal* 27: 1178-1185.

Barakat L, Bordey A (2002) GAT-1 and reversible GABA transport in Bergmann glia in slices. *J Neurophysiol* 88: 1407-19.

Barnett MH, Prineas JW (2004) Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann Neurol* 55: 458-68.

Barres BA, Koroshetz WJ, Swartz KJ, Chun LL, Corey DP (1990) Ion channel expression by white matter glia: the O-2A glial progenitor cell. *Neuron* 4:507-24.

Barres BA, Hart IK, Coles HS, Burne JF, Voyvodic JT, Richardson WD, Raff MC (1992) Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70: 31-46.

Barres BA, Raff MC (1994) Control of oligodendrocyte number in the developing rat optic nerve. *Neuron* 12: 935-942.

Bauer NG, Richter-Landsberg C, Ffrench-Constant C (2009) Role of the oligodendroglial cytoskeleton in differentiation and myelination. *Glia* 57: 1691-705.

Baumann N, Pham-Dinh D (2001) Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev* 81: 871-927.

Bedford FK, Kittler JT, Muller E, Thomas P, Uren JM, Merlo D, Wisden W, Triller A, Smart TG, Moss SJ (2001) GABA(A) receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein Plic-1. *Nat Neurosci* 4: 908-16.

Bender SJ, Weiss SR (2010) Pathogenesis of murine coronavirus in the central nervous system. *J Neuroimmune Pharmacol* 5: 336-54.

Ben-Ari Y (2002) Excitatory actions of gaba during development: the nature of the nurture. *Nat Rev Neurosci* 3: 728-39.

Benarroch EE (2012) GABAB receptors: structure, functions, and clinical implications. *Neurology* 78: 578-84.

Benke D, Zemoura K, Maier PJ (2012). Modulation of cell surface GABA(B) receptors by desensitization, trafficking and regulated degradation. *World J Biol Chem* 3: 61-72.

Ben-Nun A, Wekerle H, Cohen IR (1981) The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur J Immunol* 11: 195-9.

Berer K, Gerdes LA, Cekanaviciute E, Jia X, Xiao L, Xia Z, Liu C, *et al*. (2017) Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proc Natl Acad Sci USA* 114: 10719-10724.

Berger T, Walz W, Schnitzer J, Kettenmann H (1992) GABA-and glutamate-activated currents in glial cells of the mouse corpus callosum slice. *J Neurosci Res* 31: 21-27.

Bergles DE, Roberts JD, Somogyi P, Jahr CE (2000) Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus. *Nature* 405: 187-191.

Bettelli E, Kuchroo VK (2005) IL-12- and IL-23-induced T helper cell subsets: birds of the same feather flock together. *J Exp Med* 201: 169-71.

Bettler B, Kaupmann K, Mosbacher J, Gassmann M (2004) Molecular structure and physiological functions of GABAB receptors. *Physiol Rev* 84: 835–867.

Bettler B, Tiao JY (2006) Molecular diversity, trafficking and subcellular localization of GABAB receptors. *Pharmacol Ther* 110: 533-543.

Bieber AJ, Kerr S, Rodriguez M (2003) Efficient central nervous system remyelination requires T cells. *Ann Neurol* 53: 680-4.

Birgbauer E, Rao TS, Webb M (2004) Lysolecithin induces demyelination in vitro in a cerebellar slice culture system. *J Neurosci Res* 78: 157-66.

Blakemore WF (1982) Ethidium bromide induced demyelination in the spinal cord of the cat. *Neuropathol Appl Neurobiol* 8: 365-75.

Blakemore WF (1994) Pattern of remyelination in the CNS. *Nature* 249: 577- 578.

Booker SA, Loreth D, Gee AL, Watanabe M, Kind PC, Wyllie DJA, Kulik A, Vida I (2018) Postsynaptic $GABA_B$Rs inhibit L-type calcium channels and abolish long-term potentiation in hippocampal somatostatin interneurons. *Cell Rep* 22: 36-43.

Borges K, Wolswijk G, Ohlemeyer C, Kettenmann H (1995) Adult rat optic nerve oligodendrocyte progenitor cells express a distinct repertoire of voltage-and ligand-gated ion channels. *J Neurosci Res* 40: 591-605.

Bowery NG, Doble A, Hill DR, Hudson AL, Shaw JS, Turnbull MJ (1979) Baclofen: a selective agonist for a novel type of GABA receptor proceedings. *Br J Pharmacol* 67: 444P-445P.

Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ (2002). International Union of Pharmacology. XXXIII. Mammalian gamma-aminobutyric acid(B) receptors: structure and function. *Pharmacol Rev* 54: 247-264.

Brinkmann BG, Agarwal A, Sereda MW, Garratt AN, Müller T, Wende H, Stassart RM, *et al*. (2008) Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system. *Neuron* 59: 581-95.

Butt AM, Fern RF, Matute C (2014) Neurotransmitter signaling in white matter. *Glia* 62: 1762-79.

Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, *et al*. (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J Neurosci* 28: 264-278.

Calver AR, Hall AC, Yu WP, Walsh FS, Heath JK, Betsholtz C, Richardson WD (1998) Oligodendrocyte population dynamics and the role of PDGF in vivo. *Neuron* 20: 869-882.

Canedo-Antelo M, Serrano MP, Manterola A, Ruiz A, Llavero F, Mato S, Zugaza JL, Pérez-Cerdá F, Matute C, Sánchez-Gómez MV (2018) Inhibition of casein kinase 2 protects oligodendrocytes from excitotoxicity by attenuating JNK/p53 signaling cascade. *Front Mol Neurosci* 11: 333.

Cao G, Edden RAE, Gao F, LiH, Gong T, Chen W, Liu X, Wang G, Zhao B (2018) Reduced GABA levels correlate with cognitive impairment in patients with relapsing-remitting multiple sclerosis. *Eur Radiol* 28: 1140-1148.

Cawley N, Solanky BS, Muhlert N, Tur C, Edden RA, Wheeler-Kingshott CA, Miller DH, Thompson AJ, Ciccarelli O (2015). Reduced gamma-aminobutyric acid concentration is associated with physical disability in progressive multiple sclerosis. *Brain* 138: 2584-2595.

Cekanaviciute E, Yoo BB, Runia TF, Debelius JW, Singh S, Nelson CA, Kanner R, *et al*. (2017) Gut bacteria from multiple sclerosis patients modulate human T cells and exacerbate symptoms in mouse models. *Proc Natl Acad Sci USA* 114: 10713-10718.

Charles KJ, Deuchars J, Davies CH, Pangalos MN (2003) GABA B receptor subunit expression in glia. *Mol Cell Neurosci* 24: 214-223.

Chehrehasa F, Meedeniya AC, Dwyer P, Abrahamsen G, Mackay-Sim A (2009) EdU, a new thymidine analogue for labeling proliferating cells in the nervous system. *J Neurosci Methods* 177: 122:30.

Chen Y, Balasubramaniyan V, Peng J, Hurlock EC, Tallquist M, Li J, Lu QR (2007) Isolation and culture of rat and mouse oligodendrocyte precursor cells. *Nat Protoc* 2: 1044-1051.

Collin C, Ehler E, Waberzinek G, Alsindi Z, Davies P, Powell K, Notcutt W, *et al*. (2010) A double-blind, randomized, placebo-controlled, parallel-group study of Sativex, in subjects with symptoms of spasticity due to multiple sclerosis. *Neurol Res* 32: 451-9.

Colognato H, Ramachandrappa S, Olsen IM, ffrench-Constant C (2004) Integrins direct Src family kinases to regulate distinct phases of oligodendrocyte development. *J Cell Biol* 167: 365-75.

Coman I, Barbin G, Charles P, Zalc B, Lubetzki C (2005) Axonal signals in central nervous system myelination, demyelination and remyelination. *J Neurol Sci* 233: 67-71.

Compston A, Coles A (2008) Multiple sclerosis. *Lancet* 372: 1502-17.

Conti F, Melone M, De Biasi S, Minelli A, Brecha NC, Ducati A (1998) Neuronal and glial localization of GAT-1, a high-affinity gamma-aminobutyric acid plasma membrane transporter, in human cerebral cortex: with a note on its distribution in monkey cortex. *J Comp Neurol* 396: 51-63.

Conti F, Zuccarello LV, Barbaresi P, Minelli A, Brecha NC, Melone M (1999) Neuronal, glial and epitelial localization of gamma-aminobutyric acid transporter 2, a high-affinity gamma-aminobutyric acid plasma membrane transporter, in the cerebral cortex and neighboring structures. *J Comp Neurol* 409: 482-94.

Corell M, Wicher G, Radomska KJ, Daglikoca ED, Godskesen RE, Fredriksson R, Benedikz E, Magnaghi V, Fex Svenningsen A (2015) GABA and its B-receptor are present at the node of Ranvier in a small population of sensory fibers, implicating a role in myelination. *J Neurosci Res* 93: 285-295.

Crawford AH, Chambers C, Franklin RJ (2013) Remyelination: the true regeneration of the central nervous system. *J Comp Pathol* 149: 242:54.

Crawford AH, Tripathi RB, Foerster S, McKenzie I, Kougioumtzidou E, Grist M, Richardson WD, Franklin RJ (2016) Pre-existing mature oligodendrocytes do not contribute to remyelination following toxin-induced spinal cord demyelination. *Am J Pathol* 186: 511-6.

Cuniffe N, Coles A (2019) Promoting remyelination in multiple sclerosis. *J Neurol*. doi: 10.1007/s00415-019-09421-x.

Dawson MR, Polito A, Levine JM, Reynolds R (2003) NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. *Mol Cell Neurosci* 24: 476-488.

del Río-Hortega P (1921) La glía de escasas radiaciones (oligodendroglia) [Glia with many processes (oligodendroglia)]. *Trab Lab Histol Patol* 1-15: 1-43.

Dendrou CA, Fugger L, Friese MA (2015) Immunopathology of multiple sclerosis. *Nat Rev Immunol* 15: 545-58.

Dobson R, Giovannoni G (2019) Multiple sclerosis – a review. *Eur J Neurol* 26: 27-40.

Doly S, Shirvani H, Gäta G, Meye FJ, Emerit MB, Enslen H, et al. (2016) GABAB receptor cell-surface export is controlled by and endoplasmic reticulum gatekeeper. *Mol Psychiatry*: 21:480-90.

Domercq M, Pérez-Sanmartín A, Aparicio D, Alberdi E, Pampliega O, Matute C (2010) P2X7 receptors mediate ischemic damage to oligodendrocytes. *Glia* 58: 730-740.

Doretto S, Malerba M, Ramos M, Ikrar T, Kinoshita C, De Mei C, Tirotta E, Xu X, Borrelli E (2011) Oligodendrocytes as regulators of neuronal networks during early postnatal development. *PLoS One* 6: e19849.

Doussau F, Dupont JL, Neel D, Schneider A, Poulain B, Bossu JL (2017) Organotypic cultures of cerebellar slices as a model to investigate demyelinating disorders. *Expert Opin Drug Discov* 12: 1011-1022.

Downes N, Mullins P (2014) The development of myelin in the brain of the juvenile rat. *Toxicol Pathol* 42: 913-22.

Durkin MM, Smith KE, Borden LA, Weinshank RL, Branchek TA, Gustafson EL (1995) Localization of messenger RNAs encoding three GABA transporters in rat brain: an in situ hybridization study. *Brain Res Mol Brain Res* 33: 7-21.

Dusart I, Airaksinen MS, Sotelo C (1997) Purkinje cell survival and axonal regeneration are age dependent: an in vitro study. *J Neurosci* 17: 3710-26.

Emery B, Agalliu D, Cahoy JD, Watkins TA, Dugas JC, Mulinyawe SB, Ibrahim A, Ligon KL, Rowitch DH, Barres BA (2009) Myelin gene regulatory factor is a critical transcriptional regulator required for CNS myelination. *Cell* 138: 172-85.

Emery B (2010) Regulation of oligodendrocyte differentiation and myelination. *Science* 330: 779-82.

Enz R (2001) GABA(C) receptors: a molecular view. *Biol Chem* 382: 1111-22.

Erdö SL (1992) Non-neuronal GABA systems: an overview. In: Erdö SL (eds) GABA outside de CNS. *Springer*, Berlin, Heidelberg.

Ertzgaard P, Campo C, Calabrese A (2017) Efficacy and safety of oral baclofen in the management of spasticity: a rationale for intrathecal baclofen. *J Rehabil Med* 49: 193-203.

Fancy SP, Chan JR, Baranzini SE, Franklin RJ, Rowitch DH (2011) Myelin regeneration: a recapitulation of development? *Annu Rev Neurosci* 34: 21-43.

Fannon J, Tarmier W, Fulton D (2015) Neuronal activity and AMPA-type glutamate receptor activation regulates the morphological development of oligodendrocyte precursor cells. *Glia* 63: 1021-1035.

Fattorini G, Melone M, Sánchez-Gómez MV, Arellano RO, Bassi S, Matute C, Conti F (2017) GAT-1 mediated GABA uptake in rat oligodendrocytes. *Glia* 65: 514-522.

Fields RD (2008) White matter in learning, cognition and psychiatric disorders. *Trends Neurosci* 31: 361-70.

Filippi M, Bar-Or A, Piehl F, Preziosa P, Solari A, Vukusic S, Rocca MA (2018) Multiple sclerosis. *Nat Rev Dis Primers* 4: 43.

Flores AI, Narayanan SP, Morse EN, Shick HE, Yin X, Kidd G, Avila RL, Kirschner DA, Macklin WB (2008) Constitutively active Akt induces enhanced myelination in the CNS. *J Neurosci* 28: 7174-83.

Frangaj A, Fan QR (2018) Structural biology of $GABA_B$ receptor. *Neuropharmacology* 136: 68-79.

Franklin RJ (2002) Why does remyelination fail in multiple sclerosis? *Nat Rev Neurosci* 3: 705-14.

Franklin RJ, Zhao C, Sim FJ (2002) Ageing and CNS remyelination. *Neuroreport* 13: 923-8.

Franklin RJM, Ffrench-Constant C (2017) Regenerating CNS myelin-from mechanisms to experimental medicines. *Nat Rev Neurosci* 18: 753-769.

Gadea A, López-Colomé AM (2001) Glial transporters for glutamate, glycine, and GABA: II. GABA transporters. *J Neurosci Res* 63: 461-8.

Galvez T, Urwyler S, Prézeau L, Mosbacher J, Joly C, Malitschek B, Heid J, Brabet I, Froestl W, Bettler B, Kaupmann K, Pin JP (2000). Ca(2+) requirement for high-affinity gamma-aminobutyric acid (GABA) binding at GABA(B) receptors: involvement of serine 269 of the GABA(B)R1 subunit. *Mol Pharmacol* 57: 419-426.

García-Díaz B, Estivill-Torrús G (2008) Modelos de experimentación animal para la investigación en esclerosis múltiple. *Revista española de esclerosis múltiple* 7: 5-19.

Geginat J, Paroni M, Pagani M, Galimberti D, De Francesco R, Scarpini E, Abrignani S (2017) The enigmatic role of viruses in multiple sclerosis: molecular mimicry or disturbed immune surveillance? *Trends Immunol* 38: 498-512.

Gibson EM, Purger D, Mount CW, Goldstein AK, Lin GL, Wood LS, Inema I, Miller SE, Bieri G, Zuchero JB, Barres BA, Woo PJ, Vogel H, Monje M (2014) Neuronal activity promotes oligoendrogenesis and adaptive myelination in the mammalian brain. *Science* 344: 1252304.

Goodman AD, Brown TR, Edwards KR, Krupp LB, Schapiro RT, Cohen R, Marinucci LN, Blight AR, MSF204 Investigators (2010) A phase 3 trial of extended release oral dalfampridine in multiple sclerosis. *Ann Neurol* 68: 494-502.

Gram L (1990) Vigabatrin. *Dam & Gram (Eds) Comprehensive epileptology*: 631-640.

Guardiola-Diaz HM, Ishii A, Bansal R (2012) Erk1/2 MAPK and mTOR signaling sequentially regulates progression through distinct stages of oligodendrocyte differentiation. *Glia* 60: 476-86.

Habermacher C, Angulo MC, Benamer N (2019) Glutamate versus GABA in neuron-oligodendroglia communication. *Glia*, 1-15, doi: 10.1002/glia.23618.

Hamilton NB, Clarke LE, Arancibia-Carcamo IL, Kougioumtzidou E, Matthey M, Karadottir R, Whiteley L, Bergersen LH, Richardson WD, Atwell D (2017) Endogenous GABA controls oligodendrocyte lineage cell number, myelination and CNS internode length. *Glia* 65: 309-321.

Hammond TR, Gadea A, Dupree J, Kerninon C, Nait-Oumesmar B, Aguirre A, Gallo V (2014) Astrocyte-derived-endothelin-1 inhibits remyelination through notch activation. *Neuron* 81: 588-602.

Harris JJ, Attwell D (2012) The energetics CNS white matter. *J Neurosci* 32: 356-71.

Hartman BK, Agrawal HC, Agrawal D, Kalmbach S (1982) Development and maturation of central nervous system myelin: comparison of immunohistochemical localization of proteolipid protein and basic protein in myelin and oligodendrocytes. *Proc Natl Acad Sci USA* 79: 4217-20.

Hartung HP, Gonsette R, König N, Kwiecinski H, Guseo A, Morrissey SP, Krapf H, Zwingers T, Mitoxantrone in Multiple Sclerosis Study Group (MIMS) (2002) Mitoxantrone in progressive multiple sclerosis: a placebo-controlled, double-blind, randomized, multicentre trial. *Lancet* 360: 2018-25.

Hawker K, O´Connor P, Freedman MS, Calabresi PA, Antel J, Simon J, Hauser S, Waubant E, Vollmer T, Panitch H, Zhang J, Chin P, Smith CH, OLYMPUS trial group (2009) Rituximab in patients with primary progressive multiple sclerosis: results of a randomized double-blind placebo-controlled multicenter trial. *Ann Neurol* 66: 460-71.

Hirrlinger PG, Scheller A, Braun C, Quintela-Schneider M, Fuss B, Hirrlinger J, Kirchhoff F (2005) Expression of reef coral fluorescent proteins in the central nervous system of transgenic mice. *Mol Cell Neurosci* 30: 291-303.

Hoppe D, Kettenmann H (1989) GABA triggers a Cl^- efflux from cultured mouse oligodendrocytes. *Neurosci Lett* 97: 334-339.

Huynh JL, Casaccia P (2013) Epigenetic mechanisms in multiple sclerosis: implications for pathogenesis and treatment. *Lancet Neurol* 12: 195-206.

Jacob TC, Moss SJ, Jurd R (2008) $GABA_A$ receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat Rev Neurosci* 9: 331-343.

Jeffery ND, Blakemore WF (1995) Remyelination of mouse spinal cord axons demyelinated by local injection of lysolecithin. *J Neurocytol* 24: 775-781.

Johnston GA (2013) Advantages of an antagonist: bicuculline and other GABA antagonists. *Br J Pharmacol* 169: 328-36.

Kalakh S, Mouihate A (2019) Enhanced remyelination during late pregnancy: involvement of the GABAergic system. *Sci Rep* 9: 7728.

Kanner BI (1994) Sodium-coupled neurotransmitter transport: structure, function and regulation. *J Exp Biol* 196: 237-49.

Karadottir R, Hamilton NB, Bakiri Y, Atwell D (2008) Spiking and nonspiking classes of oligodendrocyte precursor glia in CNS white matter. *Nat Neurosci* 11: 450-456.

Karram K, Goebbels S, Schwab M, Jennissen K, Seifert G, Steinhäuser C, Nave KA, Trotter J (2008) NG2-expressing cells in the nervous system revealed by the NG2-EYFP-knockin mouse. *Genesis* 46: 743-757.

Kaufman DL, Houser CR, Tobin AJ (1991) Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. *J Neurochem* 56: 720-3.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B. (1998) GABAB-receptor subtypes assemble into functional heteromeric complexes. *Nature* 396: 683-687.

Kipp M, van der Star B, Vogel DY, Puentes F, van der Valk P, Baker D, Amor S (2012) Experimental in vivo and in vitro models of multiple sclerosis: EAE and beyond. *Mult Scler Relat Disord* 1: 15-28.

Kirchhoff F, Kettenmann H (1992) GABA triggers a [Ca2+]i increase in murine precursor cells of the oligodendrocyte lineage. *Eur J Neurosci* 4: 1049-1058.

Kittler JT, Delmas P, Jovanovic JN, Brown DA, Smart TG, Moss SJ (2000) Constitutive endocytosis of GABAA receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. *J Neurosci* 20: 7972-7.

Kotter MR, Setzu A, Sim FJ, Van Rooijen N, Franklin RJ (2001) Macrophague depletion impairs oligodendrocyte remyelination following lysolecithin-induced demyelination. *Glia* 35: 204-12.

Kotter MR, Li WW, Zhao C, Franklin RJ (2006) Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. *J Neurosci* 26: 328-32.

Kremer D, Akkermann R, Küry P, Dutta R (2019) Current advancements in promoting remyelination in multiple sclerosis. *Mult Scler* 25: 7-14.

Kuhlmann T, Miron V, Cui Q, Wegner C, Antel J, Brück W (2008) Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. *Brain* 131: 1749-58.

Kukley M, Capetillo-Zarate E, Dietrich D (2007) Vesicular glutamate release from axons in white matter. *Nat Neurosci* 10: 311-20.

Kuner R, Köhr G, Grünewald S, Eisenhardt G, Bach A Kornau HC (1999) Role of the heteromer formation in GABAB receptor function. *Science* 283: 74-77.

Lassmann H, Bradl M (2017) Multiple sclerosis: experimental models and reality. *Acta Neuropathol* 133: 223-244.

Lee Y, Morrison BM, Li Y, Lengacher S, Farah MH, Hoffman PN, Liu Y, Tsingalia A, Jin L, Zhang PW, Pellerin L, Magistretti PJ, Rothstein JD (2012) Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* 487: 443-8.

Lehto LJ, Albors AA, Sierra A, Tolppanen L, Eberly LE, Mangia S, Nurmi A, Michaeli S, Gröhn O (2017) Lysophosphatidyl choline induced demyelination in rat probed by relaxation along a fictitious field in high rank rotating frame. *Front Neurosci* 11: 433.

Li C, Xiao L, Liu X, Yang W, Shen W, Hu C, Yang G, He C (2013) A functional role of NMDA receptor in regulating the differentiation of oligodendrocyte precursor cells and remyelination. *Glia* 61: 732-749.

Li H, He Y, Richardson WD, Casaccia P (2009) Two-tier transcriptional control of oligodendrocyte differentiation. *Curr Opin Neurobiol* 19: 479-85.

Liblau RS, Singer SM, McDevitt HO (1995) Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmuned disease. *Immunol Today* 16: 34-8.

Lin SC, Bergles DE (2004) Synaptic signaling between GABAergic interneurons and oligodendrocyte precursor cells in the hippocampus. *Nat Neurosci* 7: 24-32.

Liu QR, López-Corcuera B, Mandiyan S, Nelson H, Nelson N (1993) Molecular characterization of four pharmacologically distinct gamma-aminobutyric acid transporters in mouse brain. *J Biol Chem* 268: 2106-12.

Liu J, Dietz K, DeLoyht JM, Pedre X, Kelkar D, Kaur J, Vialou V, Lobo MK, Dietz DM, Nestler EJ, Dupree J, Casaccia P (2012) Impaired adult myelination in the prefrontal cortex of socially isolated mice. *Nat Neurosci* 15: 1621-3.

Lopez-Diego RS, Weiner HL (2008) Novel therapeutic strategies for multiple sclerosis- a multifaceted adversary. *Nat Rev Drug Discov* 7: 909-25.

Lublin FD, Reingold SC (1996) Defining the clinical course of multiple sclerosis: results of an international survey. National multiple sclerosis society (USA) advisory committee on clinical trials of new agents in multiple sclerosis. *Neurology* 46: 907-11.

Lublin FD (2014) New multiple sclerosis phenotypic classification. *Eur Neurol* 72 Suppl 1:1-5.

Ludwin SK, Rao VTs, Moore CS, Antel JP (2016) Astrocytes in multiple sclerosis. *Mult Scler* 22: 1114-24.

Lundgaard I, Luzhynskaya A, Stockley JH, Wang Z, Evans KA, Swire M, Volbracht K, Gautier HO, Franklin RJ, Charles Ffrench-Constant, Attwell D, Káradóttir RT (2013) Neuregulin and BDNF induce a switch to NMDA receptor-dependent myelination by oligodendrocytes. *PLoS Biol* 11: e1001743.

Luyt K, Slade TP, Dorward JJ, Durant CF, Wu Y, Shigemoto R, Mundell SJ, Váradi A, Molnár E (2007) Developing oligodendrocytes express functional $GABA_{(B)}$ receptors that stimulate cell proliferation and migration. *J Neurochem* 100: 822-840.

Macdonald RL, Olsen RW (1994) GABAA receptor channels. *Annu Rev Neurosci* 17: 569-602.

Magnaghi V, Ballabio M, Cavarretta ITR, Wolfgang W, Lambert JJ, Zucchi, Melcangi RC (2004) GABAB receptors in Schwann cells influence proliferation and myelin protein expression. *Eur J Neurosci* 19: 2641-2649.

Makinodan M, Rosen KM, Corfas G (2012) A critical period for social experience-dependent oligodendrocyte maturation and myelination. *Science* 337: 1357-60.

Mao LM, Tang Q, Wang JQ (2007) Protein kinase C-regulated cAMP response element-binding protein phosphorylation in cultured rat striatal neurons. *Brain Res Bull* 72: 302-8.

Marques S, Zeisel A, Codeluppi S, van Bruggen D, Mendanha Falcão A, Xiao L, Li H, *et al*. (2016) Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system. *Science* 352: 1326-1329.

Marshall FH, Jones KA, Kaupmann,K Bettler B (1999) GABAB receptors - the first 7TM heterodimers. *Trends Pharmacol Sci* 20: 396-399.

Matsushima GK, Morell P (2001) The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain Pathol* 11: 107-16.

Matute C, Streit P (1986) Monoclonal antibodies demonstrating GABA-like immunoreactivity. *Histochem* 86:147-57.

Matute C, Alberdi E, Domercq M, Pérea-Cerdá F, Pérez-Samartín A, Sánchez-Gómez MV (2001) The link between excitotoxic oligodendroglial death and demyelinating diseases. *Trends Neurosci* 24: 224-30.

Matute C, Pérez-Cerdá F (2005) Multiple sclerosis: novel perspectives on newly forming lesions. *Trends Neurosci* 28: 173-5.

Mayer M, Bogler O, Noble M (1993) The inhibition of oligodendrocyte differentiation of O-2A progenitors caused by basic fibroblast growth factor is overridden by astrocytes. *Glia* 8: 12-19.

Mayr B, Montminy M (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* 2: 599-609.

McCarthy KD, de Vellis J (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 85: 890-902.

Mei F, Wang H, Liu S, Niu J, Wang L, He Y, Etxebarria A, Chan JR, Xiao L (2013) Stage-specific deletion of Olig2 conveys opposing functions on differentiation and maturation of oligodendrocytes. *JNeurosci* 33: 8454-8462.

Melfi S, Montt Guevara MM, Bonalume V, Ruscica M, Colciago A, Simoncini T, Magnaghi V (2017) Src and phosphor-FAK kinases are activated by allopregnanolone promoting Schwann cell motility, morphology and myelination. *J Neurochem* 141: 165-178.

Mi S, Miller RH, Lee X, Scott ML, Shulag-Morskaya S, Shao Z, Chang J, *et al*. (2005) LINGO-1 negatively regulates myelination by oligodendrocytes. *Nat Neurosci* 8: 745-51.

Michel L, Touil H, Pikor NB, Gommerman JL, Prat A, Bar-Or A (2015) B cells in the multiple sclerosis central nervous system: trafficking and contribution to CNS-compartmentalized inflammation. *Front Immunol* 6: 636.

Miller D, Barkhof F, Montalban X, Thompson A, Filippi M (2005) Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis. *Lancet Neurol* 4: 281-8.

Minelli A, DeBiasi S, Brecha NC, Vitellaro Zuccarello L, Conti F (1996) GAT-3, a high-affinity GABA plasma membrane transporter, is localized to astrocytic processes, and it is not confined to the vicinity of GABAergic synapses in the cerebral cortex. *J Neurosci* 16: 6255-6264.

Miron VE, Kuhlmann T, Antel JP (2011) Cells of the oligodendroglial lineage, myelination and remyelination. *Biochim Biophys Acta* 1812: 184-93.

Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, van Wijngaarden P, Wagers AJ, Williams A, Franklin RJM, Ffrench-Constant C (2013) M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nat Neurosci* 16: 1211-1218.

Mitew S, Hay CM, Peckham H, Xiao J, Koenning M, Emery B (2014) Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. *Neuroscience* 276: 29-47.

Montalban X, Hauser SL, Kappos L, Arnold DL, Bar-Or A, Comi G, de Seze J, *et al*. (2017) Ocrelizumab versus placebo in primary progressive multiple sclerosis. *N Engl J Med* 376: 209-220.

Moulin D, Boulanger A, Clark AJ, Clarke H, Dao T, Finley GA, Furlan A, *et al.* (2014) Pharmacological management of chronic neuropathic pain: revised consensus statement from the Canadian Pain Society. *Pain Res Manag* 19: 328-35.

Mozafari S, Sherafat MA, Javan M, Mirnajafi-Zadeh J, Tiraihi T (2010) Visual evoked potentials and MBP gene expression imply endogenous myelin repair in adult rat optic nerve and chiasm following local lysolecithin induced demyelination. *Brain Res* 1351: 50-6.

Mu J, Li M, Wang T, Li X, Bai M, Zhang G, Kong J (2019) Myelin damage in diffuse axonal injury. *Front Neurosci* 13: 217.

Münzel EJ, Williams A (2013) Promoting remyelination in multiple sclerosis-recent advances. *Drugs* 73: 2017-29.

Muraro PA, Pasquini M, Atkins HL, Bowen JD, Farge D, Fassas A, *et al.* (2017) Long-term outcomes after autologous hematopoietic stem cell transplantation for multiple sclerosis. *JAMA Neurol* 74: 459-469.

Naruse M, Ishizaki Y, Ikenaka K, Tanaka A, Hitoshi S (2017) Origin of oligodendrocytes in mammalian forebrains: a revised perspective. *J Physiol Sci* 67: 63-70.

Nave KA, Werner HB (2014) Myelination of the nervous system: mechanisms and functions. *Annu Rev Cell Dev Biol* 30: 503-33.

Nielsen EB, Suzdak PD, Andersen KE, Knutsen LJ, Sonnewald U, Braestrup C (1991) Characterization of tiagabine (NO-328), a new potent and selective GABA uptake inhibitor. *Eur J Pharmacol* 196: 257-66.

Nishiyama A, Komitova M, Suzuki R, Zhu X (2009) Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. *Nat Neurosci* 10: 9-22.

Olsen RW, Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and fuction. Update. *Pharmacol Rev* 60: 243-60.

Ortiz GG, Pacheco-Moisés FP, Macías-Islas MÁ, Flores-Alvarado LJ, Mireles-Ramírez MA, González-Renovato ED, Hernández-Navarro VE, Sánchez-López AL, Alatorre-Jiménez MA (2014) Role of the blood-brain barrier in multiple sclerosis. *Arch Med Res* 45: 687-97.

Palumbo S, Pellegrini S (2017) Experimental *in vivo* models of multiple sclerosis: state of the art. *Codon Publications*: 173-183.

Park SK, Miller R, Krane I, Vartanian T (2001) The erbB2 gene is required for the development of terminally differentiated spinal cord oligodendrocytes. *J Cell Biol* 154: 1245-58.

Perroy J, Adam L, Qanbar R, Chénier S, Bouvier M (2003) Phosphorylation-independent desensitization of $GABA_B$ receptor by GRK4. *EMBO J* 22: 3816-3824.

Philips T, Rothstein JD (2017) Oligodendroglia: metabolic supporters of neurons. *J Clin Invest* 127: 3271-3280.

Polenzani L, Woodward RM, Miledi R (1991) Expression of mammalian gamma-aminobutyric acid receptors with distinct pharmacology in Xenopus oocytes. *Proc Natl Acad Sci USA* 88: 4318-22.

Pontier SM, Lahaie N, Ginham R, St-Gelais F, Bonin H, Bell DJ, *et al*. (2006) Coordinated action of NSF and PKC regulates GABAB receptor singnaling efficacy. *EMBO J* 25: 2698-709.

Procacci P, Ballabio M, Castelnovo LF, Mantovani C, Magnaghi V (2013) GABA-B receptors in the PNS have a role in Schwann cells differentiation? *Front Cell Neurosci* 6:68.

Purwana I, Zheng J, Li X, Deurloo M, Son DO, Zhang Z, Liang C, *et al*. (2014) GABA promotes human β-cell proliferation and modulates glucose homeostasis. *Diabetes* 63: 4197-205.

Raasakka A, Kursula P (2014) The myelin membrane-associated enzyme 2´3´-cyclic nucleotide 3´-phosphodiesterase: on a highway to structure and function. *Neurosci Bull* 30: 956-66.

Rajasekharan S, Baker KA, Horn KE, Jarjour AA, Antel JP, Kennedy TE (2009) Netrin 1 and Dcc regulate oligodendrocyte process branching and membrane extension via Fyn and RhoA. *Development* 136: 415-26.

Reich DS, Lucchinetti CF, Calabresi PA (2018) Multiple Sclerosis. *N Engl J Med* 378: 169-180.

Roberts E, Frankel S (1950) gamma-Aminobutyric acid in brain: its formation from glutamic acid. *J Biol Chem* 187: 55-63.

Rodriguez M, Oleszak E, Leibowitz J (1987) Theiler´s murine encephalomyelitis: a model of demyelination and persistence of virus. *Crit Rev Immunol* 7: 325-65.

Rowley NM, Madsen KK, Schousboe A, Steve White H (2012) Glutamate and GABA synthesis, release, transport and metabolism as targets for seizure control. *Neurochem Int* 61: 546-58.

Saab AS, Tzvetavona ID, Trevisiol A, Baltan S, Dibaj P, Kusch K, Möbius W, *et al*. (2016) Oligodendroglial NMDA receptors regulate glucose import and axonal energy metabolism. *Neuron* 91: 119-32.

Saliba RS, Michels G, Jacob TC, Pangalos MN, Moss SJ (2007) Activity-dependent ubiquitination of GABA(A) receptors regulates their accumulation at synaptic sites. *J Neurosci* 27: 13341-51.

Sánchez-Gómez MV, Serrano MP, Alberdi E, Pérez-Cerdá F, Matute C (2018) Isolation, expansión and maturation of oligodendrocyte lineage cells obtained from rat neonatal brain and optic nerve. *Methods Mol Biol* 1791: 95-113.

Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA *et al*. *Nature* 328: 221-7.

Seiler N, Askar A (1971) A micro method for the quantitative estimation of putrescine in tissues. *J Chromatogr* 62: 121-7.

Seiler N, al-Therib MJ, Kataola K (1973) Formation of GABA from putrescine in the brain of fish (Salmo irideus Gibb.). *J Neurochem* 20: 699-708.

Serrano-Regal MP, Luengas-Escuza I, Bayón-Cordero L, Ibarra-Aizpurua N, Alberdi E, Pérez-Samartín A, Matute C, Sánchez-Gómez MV (2019) Oligodendrocyte differentiation and myelination is potentiated via $GABA_B$ receptor activation. *Neuroscience*, Jul 23. pii: S0306-4522(19)30488-9. doi: 10.1016/j.neuroscience.2019.07.014.

Shakespeare DT, Boggild M, Young C (2003) Anti-spasticity agents for multiple sclerosis. *Cochrane Database Syst Rev* (4): CD001332.

Sieghart W (1995) Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol Rev* 47: 181:234.

Sieghart W (2015) Allosteric modulation of GABAA receptors via multiple drug-binding sites. *Adv Pharmacol* 72: 53-96.

Simon J, Wakimoto H, Fujita N, Lalande M, Barnard EA (2004) Analysis of the set of GABA(A) receptor genes in the human genome. *J Biol Chem* 279: 41422-35.

Simons M, Trotter J (2007) Wrapping it up: the cell biology of myelination. *Curr Opin Neurobiol* 17: 533-40.

Simons M, Nave KA (2015) Oligodendrocytes: myelination and axonal support. *Cold Spring Harb Perpect Biol* 8: a020479. doi: 10.1101/cshperspect.a020479.

Slavin A, Ewing C, Liu J, Ichikawa M, Slavin J, Bernard CC (1998) Induction of a multiple sclerosis-like disease in mice with an immunodominant epitope of myelin oligodendrocyte glycoprotein. *Autoimmunity* 28: 109-20.

Snaidero N, Simons M (2014) Myelination at a glance. *J Cell Sci* 127: 2999-3004.

Sperber BR, McMorris FA (2001) Fyn tyrosine kinase regulates oligodendroglial cell development but is not required for morphological differentiation of oligodendrocytes. *J Neurosci Res* 63: 303-12.

Sperber BR, Boyle-Walsh EA, Engleka MJ, Gadue P, Peterson AC, Stein PL, Scherer SS, McMorris FA (2001) A unique role for Fyn in CNS myelination. *J Neurosci* 21: 2039-47.

Spitzer SO, Sitnikov S, Kamen Y, Evans KA, Kronenberg-Versteeg D, Dietmann S, de Faria O Jr, Agathou S, Káradóttir RT (2019) Oligodendrocyte progenitor cells become regionally diverse and heterogeneous with age. *Neuron* 101: 459-471.

Stevenson VL (2014) Intrathecal baclofen in multiple sclerosis. *Eur Neurol* 72: 32-4.

Suzdak PD, Frederiksen K, Andersen KE, Sorensen PO, Knutsen LJ, Nielsen EB (1992) NNC-711, a novel potent and selective gamma-aminobutyric acid uptake inhibitor: pharmacological characterization. *Eur J Pharmacol* 224: 189-98.

Tan GA, Furber KL, Thangaraj MP, Sobchishin L, Doucette JR, Nazarali AJ (2018) Organotypic cultures from the adult CNS: a novel model to study demyelination and remyelination ex vivo. *Cell Mol Neurobiol* 38: 317-328.

Tepavcevic V, Kernion C, Aigrot MS, Meppiel E, Mozafari S, Arnould-Laurent R, Ravassard P, Kennedy TE, Nait-Oumesmar B, Lubetzki C (2014) Early netrin-1 expression impairs central nervous system remyelination. *Ann Neurol* 76: 252-68.

Traiffort E, Zakaria M, Laouarem Y, Ferent J (2016) Hedgehog: a key signaling in the development of the oligodendrocyte lineage. *J Dev Biol* 4. doi: 10.3390/jdb4030028.

Trapp BD, Vignos M, Dudman J, Chang A, Fisher E, Staugaitis SM, Battapady H, *et al*. (2018) Cortical neuronal densities and cerebral white matter demyelination in multiple sclerosis: a retrospective study. *Lancet Neurol* 17: 870-884.

Travis KE, Castro MRH, Berman S, Dodson CK, Mezer AA, Ben-Shachar M, Feldman HM (2019) More than myelin: probing white matter differences in prematurity with quantitative T1 and diffusion MRI. *Neuroimage Clin* 22: 101756.

Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant GABAA receptor subtype. *J Neurosci* 17: 2728-37.

Tsunoda I, Fujinami RS (2002) Inside-out versus outside-in models for virus induced demyelination: axonal damage triggering demyelination. *Springer Semin Immunopathol* 24: 105-25.

Tsunoda I, Libbey JE, Fujinami RS (2007) TGF-beta1 supresses T cell infiltration and VP2 puff B mutation enchances apoptosis in acute polioencephalitis induced by Theiler´s virus. *Neuroimmunol* 190: 80-89.

Tuohy VK, Lu Z, Sobel RA, Laursen RA, Lees MB (1989) Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. *J Immunol* 142: 1523-7.

Umemori H, Sato S, Yagi T, Aizawa S, Yamamoto T (1994) Initial events of myelination involve Fyn tyrosine kinase signalling. *Nature* 367: 572-6.

Velez-Fort M, Maldonado PP, Butt AM, Audinat E, Angulo MC (2010) Postnatal switch from synaptic to extrasynaptic transmission between interneurons and NG2 cells. *J Neurosci* 30: 6921-6929.

Vélez-Fort M, Audinat E, Angulo MC (2012) Central role of GABA in neuron-glia interactions. *Neuroscientist* 18: 237-250.

Verkhratsky A, Steinhaüser C (2000) Ion channels in glial cells. *Brain Res Rev* 32: 380-412.

Von Blankenfeld G, Trotter J, Kettenmann H (1991) Expression and developmental regulation of a GABAA receptor in cultured murine cells of the oligodendrocyte lineage. *Eur J Neurosci* 3: 310-316.

Wake H, Lee PR, Fields RD (2011) Control of local protein synthesis and initial events in myelination by action potentials. *Science* 333: 1647-51.

Wang S, Sdrulla AD, diSibio G, Bush G, Nofziger D, Hicks C, Weinmaster G, Barres BA (1998) Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* 21: 63-75.

Werner P, Pitt D, Raine CS (2001) Multiple sclerosis: altered glutamate homeostasis in lesions correlates with oligodendrocyte an axonal damage. *Ann Neurol* 50: 169-80.

White R, Gonsior C, Krämer-Albers EM, Stör N, Hüttelmaier S, Trotter J (2008) Activation of oligodendroglial Fyn kinase enhances translation of mRNAs transported in hnRNPA2-dependent RNA granules. *J Cell Biol* 181: 579-86.

White R, Krämer-Albers EM (2014) Axon-glia interaction and membrane traffic in myelin formation. *Front Cell Neurosci* 7: 284.

Willford SL, Anderson CM, Spencer SR, Eskandari S (2015) Evidence for a revised ion/substrate coupling stoichiometry of GABA transporters. *J Membr Biol* 248: 795-810.

Williamson AV, Mellor JR, Grant AL, Randall AD (1998) Properties of GABA(A) receptors in cultured rat oligodendrocyte progenitor cells. *Neuropharmacology* 37: 859-873.

Wu Y, Wang W, Richerson GB (2003) Vigabatrin induces tonic inhibition via GABA transporter reversal without increasing vesicular GABA release. *J Neurophysiol* 89: 2021-34.

Xing YL, Röth PT, Stratton JA, Chuang BH, Danne J, Ellis SL, Ng SW, Kilpatrick TJ, Merson TD (2014) Adult neural precursor cells from the subventricular zone contribute significantly to oligodendrocyte regeneration and remyelination. *J Neurosci* 34: 14128-46.

Xu C, Zhang W, Rondard P, Pin JP, Liu J (2014) Complex GABAB receptor complexes: how to generate multiple functionally distinct units from a single receptor. *Front Pharmacol* 5:12.

Yamasaki R, Lu H, Butovsky O, Ohno N, Rietsch AM, Cialic R, Wu PM, *et al*. (2014) Differential roles of microglia and monocytes in the inflamed central nervous system. *J Exp Med* 211: 1533-49.

Yoon BE, Woo J, Chun YE, Chun H, Jo S, Bae JY, An H, Min JO, Oh SJ, Han KS, Kim HY, Kim T, Kim YS, Bae YC, Lee CJ (2014) Glial GABA, synthesized by monoamine oxidase B, mediates tonic inhibition. *J Physiol* 592: 4951-68.

Zalc B (2018) One hundred and fifty years ago Charcot reported multiple sclerosis as a new neurological disease. *Brain* 141: 3482-3488.

Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O' Keeffe S, Phatnani HP, *et al*. (2014) An RNA-sequencing transcriptome and splicing database of glia, neurons and vascular cells of the cerebral cortex. *J Neurosci* 34: 11929-11947.

Zhang W, Xu C, Tu H, Wang Y, Sun Q, Hu P, Hu Y, Rondard P, Liu J (2015) GABAB receptor unregulates fragile X mental retardation protein expression in neurons. *Sci Rep* 5: 10468.

Zonouzi M, Scafidi J, Li P, McEllin B, Edwards J, Dupree JL, Harvey L, Sun D, Hübner CA, Cull-Candy SG, Farrant M, Gallo V (2015) GABAergic regulation of cerebellar NG2 cell development is altered in perinatal white matter injury. *Nat Neurosci* 18: 674-682.