University of Crete
School of Medicine

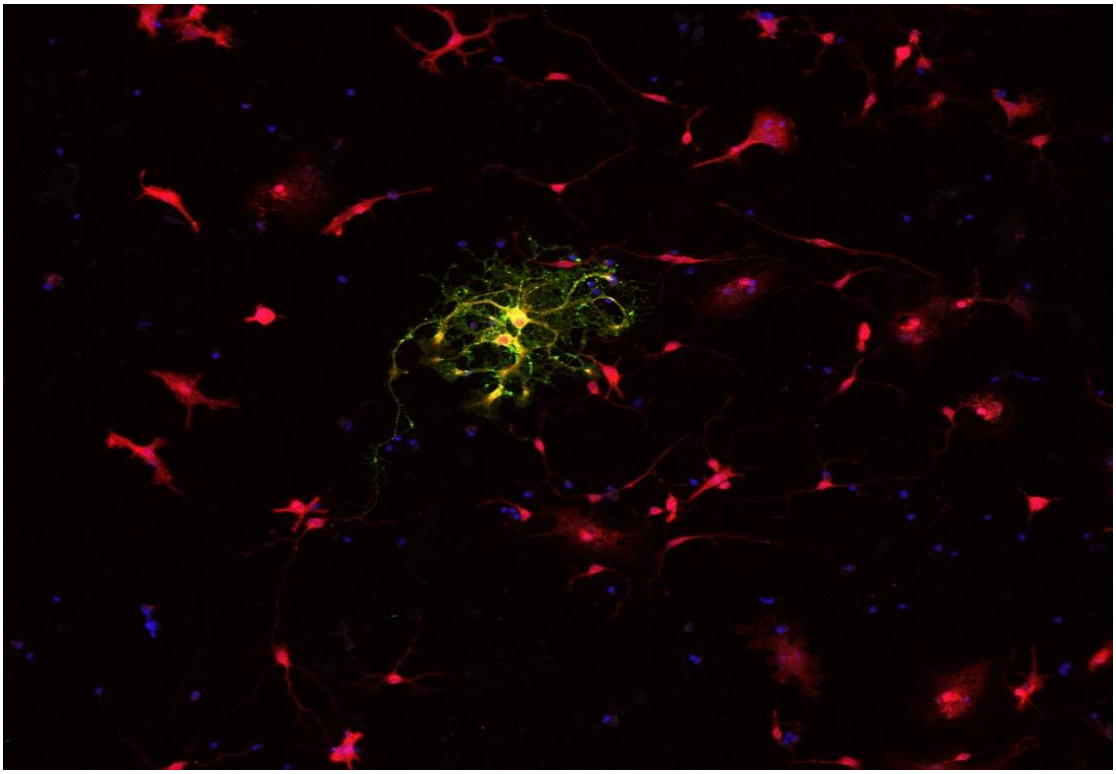
Institute of Molecular Biology & Biotechnology (IMBB)

Graduate Program in Neuroscience

Study of myelinophagy in oligodendrocytes

MSc Thesis

Niki Ktena



Laboratory of Basic Neuroscience

Group Leader: Prof. Domna Karagogeos

Supervisor: Dr. Maria Savvaki

Heraklion, August 2018

Κλείνοντας αυτόν τον κύκλο σπουδών -και εμπειριών- θα ήθελα να ευχαριστήσω όλους τους ανθρώπους που ήταν δίπλα μου όλο αυτό το διάστημα. Για αρχή το μεγαλύτερο ευχαριστώ το οφείλω από κοινού στην κα Καραγωγέως και την κα Σαββάκη (!) …και βασικά δεν ξέρω ποια απ' τις δύο να ευχαριστήσω πρώτη!

Κα Καραγωγέως σας ευχαριστώ για όλα αυτά τα χρόνια που είστε δίπλα μου, για όλη την εμπιστοσύνη που μου δείξατε απ' την αρχή και συνεχίζετε να μου δείχνετε, για όλες τις συμβουλές, όλη την καθοδήγηση και την υποστήριξη σε ό,τι κι αν σας χρειάστηκα, όποτε σας το ζήτησα..

Για το Μαράκι μου δεν ξέρω ειλικρινά για τι απ' όλα να την πρωτοευχαριστήσω… Σε ευχαριστώ, λοιπόν, για όλα όσα πέρασαν και κυρίως για όλα όσα θα έρθουν! Σ' ευχαριστώ που μου δίνεις την ευκαιρία να συνεχίσω να δουλεύω μαζί σου, επειδή είσαι το καλύτερο «αφεντικό» και νοιώθω πολύ τυχερή που σε βρήκα!

Στη συνέχεια, οφείλω ένα τεράστιο ευχαριστώ στη Βασιλική Νικολετοπούλου, για αρχή για τους 3 μήνες που με δέχτηκε στο εργαστήριο της, για όλα όσα μου έμαθε, για όλη την καθοδήγηση και όλες τις πολύτιμες συμβουλές όλο αυτό το διάστημα!

Τον κ. Χαραλαμπόπουλο, που μου έκανε την τιμή να είναι στην τριμελή μου επιτροπή, αλλά και όλα όσα μου δίδαξε μέσα απ' τα μαθήματα.

Τον Ηλία γιατί είναι ο καλύτερος lab partner που θα μπορούσα να έχω και κάνει την κάθε μέρα στο εργαστήριο απλά υπέροχη!

Τον κ. Κώστα γιατί δεν μπορώ ούτε να φανταστώ το εργαστήριο χωρίς αυτόν..

Τη Ζουζάνα και την Κατερίνα για όλο το κλίμα στο εργαστήριο και όλες τις συζητήσεις, καθώς και τα υπόλοιπα παιδιά του εργαστηρίου (την Κλειώ, την Αγγελική Κ.Σ., το Νίκο, το Μανώλη και όσους/όσες έρχονται και φεύγουν) γιατί ο καθένας μας έχει κάτι να δώσει στον άλλο.

Όλα τα παιδιά απ' το εργαστήριο της Βασιλικής (και ιδιαίτερα την Εμμανουέλα και τη Θεοδώρα) για όλη την άψογη συνεργασία και όλη τη βοήθεια όποτε χρειάστηκε!

Όλες και όλους τις καθηγήτριες και τους καθηγητές των «Νευροεπιστημών» για όλη την υπέροχη δουλειά που έκαναν και συνεχίζουν να κάνουν, παρά τις δυσκολίες.

Τέλος θα ήθελα να ευχαριστήσω όλες/όλους τις/τους φίλες/ους μου που ήταν και συνεχίζουν να είναι δίπλα μου όλα αυτά τα χρόνια. Τανάκι, Βαγγ, Γιάννη, Μήτσο και Βικάκι μου σε εσάς πάει εξτρά ευχαριστώ που με ανέχεστε! Και κυρίως την Αγγελική και τη Μαρία –όχι γιατί απείλησαν πως θα σκίσουν την πτυχιακή μου αν δεν τις ευχαριστήσω- αλλά επειδή χωρίς αυτές η ζωή μου και η κάθε μου μέρα δε θα μπορούσε ποτέ να είναι τόσο όμορφη!

Και φυσικά τους γονείς μου και την αδερφή μου που με έμαθαν να κυνηγώ τα όνειρά μου και γιατί πάντα με στηρίζουν σε όλες μου τις επιλογές!

Σας ευχαριστώ ειλικρινά όλους και όλες σας!

Χωρίς εσάς δε θα είχα καταφέρει τίποτα από όλα αυτά…

TABLE OF CONTENTS

ABSTRACT

Autophagy comprises a conserved lysosome-dependent catabolic pathway, facilitating degradation of cytoplasmic proteins and damaged organelles. Through its role in energy production and in maintaining cellular homeostasis, autophagy is crucial during development as shown in many tissues and organisms. Its dysregulation has been linked to several disorders, including neurodegenerative diseases, and more recently demyelinating disorders affecting mainly the PNS.

Myelin, produced by oligodendrocytes (OLs) in the CNS, provides mammals with an evolutionary advantage that insulates the axon, provides trophic support and ensures the rapid and efficient propagation of action potentials along its length. Its disruption, namely demyelination, may occur as a consequence of aging, from genetic alterations in genes encoding myelin proteins (dysmyelination) or from an inflammatory response against myelin producing cells, as is the case in Multiple Sclerosis (MS).

The present study aimed to shed light on the significance of autophagy in the myelinating glia in the central nervous system (CNS). More specifically, we examined the role of autophagy in both oligodendrocyte precursor cells and mature oligodendrocytes, using in vitro approaches.

In the first part of this study, we showed that autophagy is active in OPCs and that its pharmacological inhibition causes translocation of the autophagy cargo receptor p62 to the nucleus and increased proliferation. Subsequently, in the second part of the study, we proved that autophagy is also active in mature oligodendrocytes and inhibition of autophagy leads to severe morphological defects in the myelin sheath of these cells. These observations strongly support that autophagy is an essential mechanism for central myelin formation and maintenance and propose the principle that the progression of myelination in the CNS requires the involvement of a fully functional autophagic machinery.

ΠΕΡΙΛΗΨΗ

Η αυτοφαγία αποτελεί ένα συντηρημένο καταβολικό μονοπάτι, το οποίο μεσολαβεί στην αποδόμηση κυτταροπλασματικών πρωτεϊνών και ελαττωματικών οργανιδίων. Μέσω του ρόλου της στην παραγωγή ενέργειας και στη διατήρηση της κυτταρικής ομοιόστασης, συνιστά ένα σημαντικό μηχανισμό, κατά την ανάπτυξη, για πληθώρα οργάνων και οργανισμών. Η απορρύθμισή της έχει συνδεθεί με διάφορες διαταραχές, περιλαμβανομένων νευροεκφυλιστικών ασθενειών καθώς και απομυελινωτικών νοσημάτων, κυρίως του Περιφερικού Νευρικού Συστήματος (ΠΝΣ).

Η μυελίνη είναι η λιποειδής ουσία, η οποία παρέχει στα σπονδυλωτά το εξελικτικό πλεονέκτημα της μόνωσης του άξονα, διασφαλίζει τη ραγδαία και αποτελεσματική διάδοση των ηλεκτρικών ώσεων κατά μήκος του και παρέχει τροφική στήριξη. Στο ΚΝΣ παράγεται από μια κατηγορία γλοιακών κυττάρων, τα ολιγοδενδροκύτταρα. Η μοριακή βάση της απομυελίνωσης, όπως ονομάζεται η καταστροφή της μυέλινης, δεν έχει μέχρι σήμερα διευκρινιστεί ενώ οι απομυελινωτικές ασθένειες πλήττουν ένα μεγάλο τμήμα του παγκόσμιου πληθυσμού.

Στόχος της συγκεκριμένης διατριβής ήταν η μελέτη του ρόλου της αυτοφαγίας στα ολιγοδενδροκύτταρα. Ειδικότερα, επιχειρήσαμε να μελετήσουμε το ρόλο της αυτοφαγίας τόσο σε πρόδρομα, όσο και σε ώριμα ολιγοδενδροκύτταρα.

Στο πρώτο μέρος της παρούσας εργασίας, αποδείξαμε ότι η αυτοφαγία είναι ενεργή στα πρόδρομα ολιγοδενδροκύτταρα και ότι η φαρμακολογική αναστολή της οδηγεί στη μετακίνηση του αυτοφαγικού υποδοχέα p62 στον πυρήνα των συγκεκριμένων κυττάρων και σε αύξηση της πολλαπλασιαστικής τους ικανότητας. Στη συνέχεια, στο δεύτερο τμήμα της μελέτης, φάνηκε ότι η αυτοφαγία είναι ενεργή και στα ώριμα ολιγοδενδροκύτταρα και η αναστολή της συνεπάγεται σοβαρών μορφολογικών αλλοιώσεων στο έλυτρο μυελίνης των κυττάρων αυτών. Τα συγκεκριμένα ευρήματα υποστηρίζουν την υπόθεση μας ότι η αυτοφαγία αποτελεί έναν απαραίτητο μηχανισμό για την παραγωγή και τη διατήρηση της μυελίνης και προτείνουν ότι η πρόοδος της μυελίνωσης στο Κεντρικό Νευρικό Σύστημα (ΚΝΣ) απαιτεί τη μεσολάβηση ενός πλήρως λειτουργικού αυτοφαγικού μηχανισμού.

INTRODUCTION

Autophagy

Autophagy is an evolutionarily conserved cellular degradation process that targets cytoplasmic materials including cytosol, macromolecules and unwanted organelles. In other words, it could be described as the process of cellular "self-eating" by which intracellular materials are delivered to the lysosome for degradation. Although, initially autophagy was regarded as a survival response to stress, recent studies have revealed its significance in cellular and organismal homeostasis, development and immunity.

As autophagy cargoes journey towards the lysosomal lumen, there are membrane barriers for hydrophilic molecules and large structures that cannot freely cross lipid bilayers. To overcome these barriers, three major types of autophagy are used: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Mizushima and Komatsu, 2011). Microautophagy refers to the invagination of the lysosomal or endosomal membrane, resulting in the direct engulfment of substrates that are subsequently degraded by lysosomal proteases (Li et al., 2012). Chaperone-mediated autophagy is distinct from macroautophagy and microautophagy because cargo is not sequestered within a membrane de-limited vesicle. Instead, proteins targeted by chaperone-mediated autophagy contain a KFERQ-like pentapetide motif that is recognized by the cytosolic chaperone heat shock cognate 70 kDa protein (HSC70); HSC70 promotes the translocation of these targets across lysosomal membranes into the lysosomal lumen via the lysosomal-associated membrane protein 2A (LAMP2A) receptor (Cuervo and Wong, 2014). Finally, macroautophagy is most widely investigated and best known among the three pathways and the type of autophagy this study focus on. When macroautophagy (hereafter simply referred to as "autophagy") is induced, an isolation membrane encloses a portion of cytoplasm, forming a characteristic double-membraned vesicle termed the autophagosome. The autophagosome then fuses with the lysosome to form an autolysosome, the contents of which are then degraded by lysosomal enzymes (Mizushima and Komatsu, 2011).

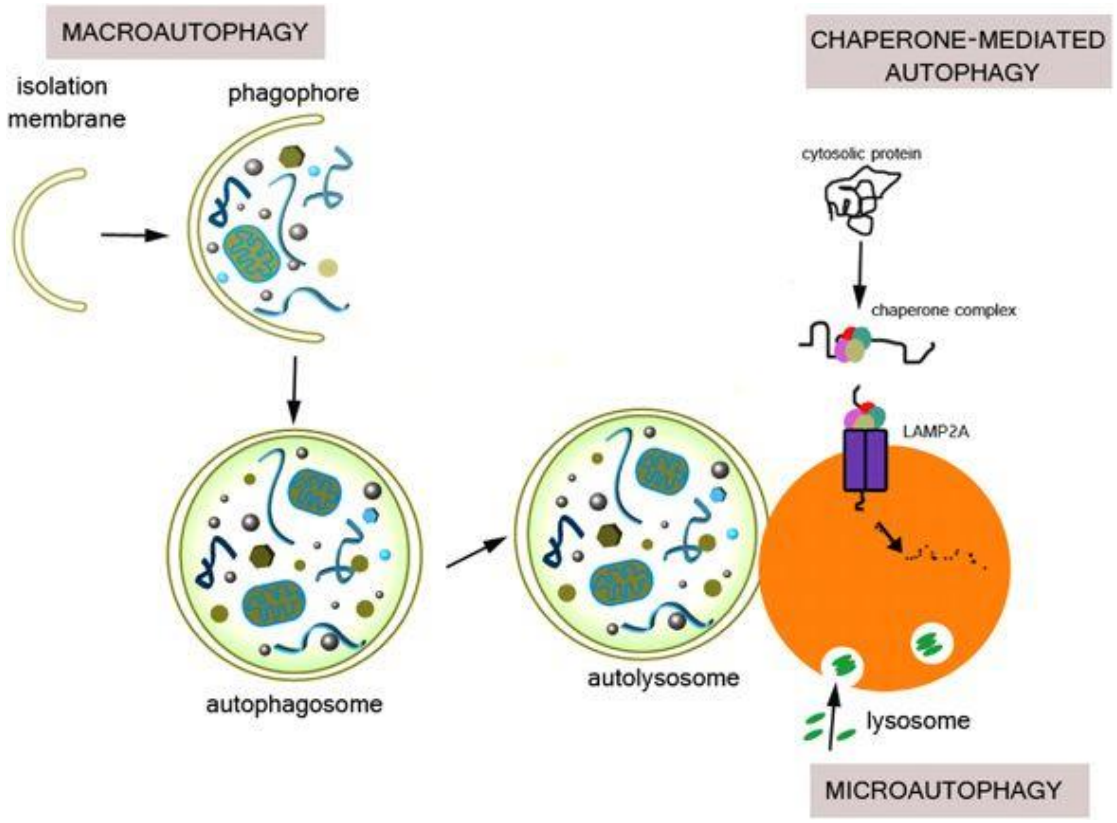


Figure 1. The different types of autophagy.

(a) Macroautophagy is characterized by the sequestration of structures targeted for destruction into double-membrane vesicles called autophagosomes.

(b) During chaperone-mediated autophagy, proteins carrying the KFERQ-like sequence are recognized by the Hsc70 chaperone, which then associates with the integral lysosome membrane protein LAMP-2A, leading to the translocation of the bound protein into the lysosome interior for degradation.

(c) Microautophagy entails the recruitment of targeted components in proximity with the lysosomal membrane, which subsequently invaginates and pinches off.

(Nikoletopoulou et al., 2015)

Autophagic machinery

One of the most exciting topics in the field of autophagy today is understanding the molecular details of autophagosome biogenesis. The formation of the phagophore and its subsequent maturation to become the autophagosome is a transient event, but extremely dynamic, involving multiple protein complexes, that participate in the

different stages of autophagy, and the mobilization of substantial membrane reserves.

Mechanistically, the stages of autophagy can be summarized into four events: initiation, expansion of the autophagosome membrane, fusion and cargo degradation/recycling, each step requiring the orchestration of several ATG genes (Lee, 2012) (Figure 2).

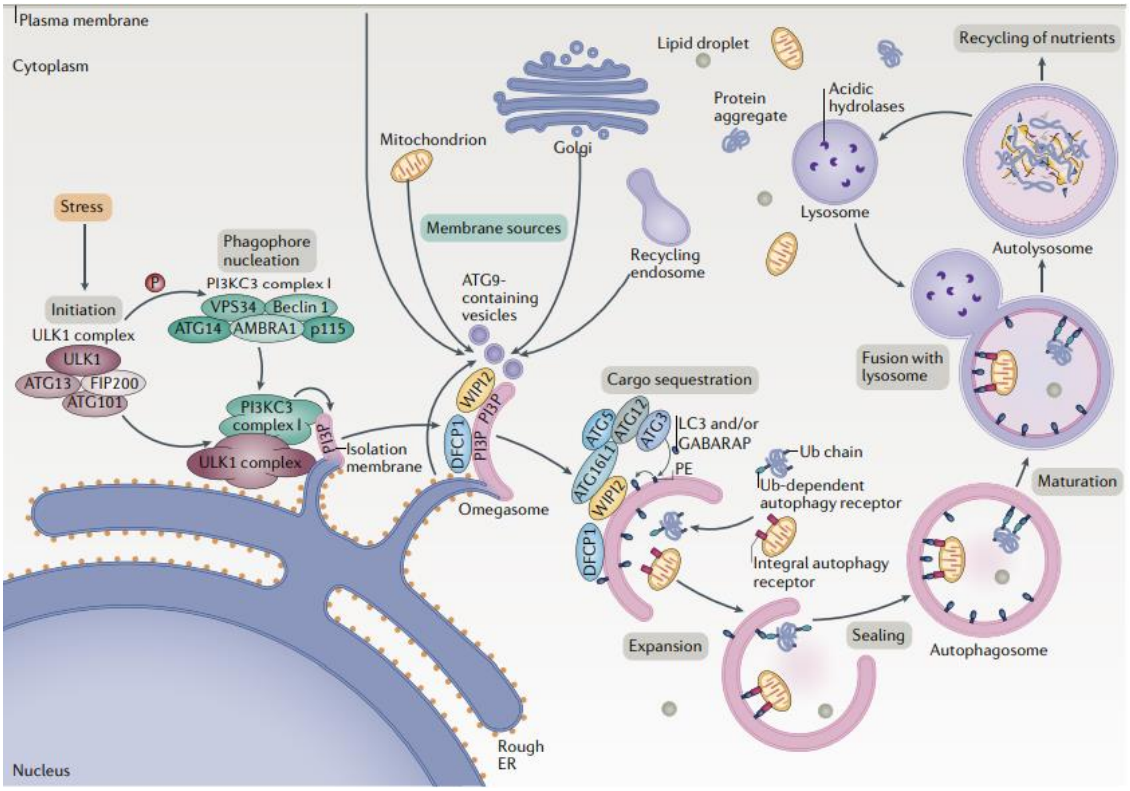


Figure 2. Overview of the autophagy process.

Autophagy proceeds through a series of steps, which include induction, phagophore nucleation, elongation and fusion of the autophagosome with the lysosome for degradation of the sequestered cargoes.

(Dikic and Elazar, 2018).

Initiation and nucleation

Autophagy occurs at basal levels in most cells, however, autophagy may be induced as a response to a stressor change in the extracellular environment of a cell, and the

target of rapamycin complex 1 (TORC1) is one of the signaling pathways that plays a primary role in sensing the shift in nutrient availability. Nutrient starvation, initiates an intracellular signaling cascade by discontinuing TORC1 stimulation, resulting in the activation of key downstream targets, un-coordinated 51 (UNC-51)-like kinase1 (ULK1), ATG13 and focal adhesion kinase family interacting protein of 200 kD (FIP200), which form part of a complex –called ULK1 complex- that initiates autophagy (Kamada et al., 2000). Assembly of this complex is crucial for autophagy because it plays a role in recruiting other Atg proteins to the phagophore assembly site (PAS) and activating downstream targets through phosphorylation (Suzuki et al., 2007). In autophagy, nucleation refers to the process of mobilizing a small group of molecules to the PAS; the phagophore is the active sequestering compartment of autophagy. Once autophagy is activated, it begins with the formation of the phagophore, the origin of which remains under debate. Although evidence supports the idea that nucleation of the isolation membrane occurs at a distinct site emanating from the endoplasmic reticulum (ER), termed the omegasome (Axe et al., 2018), other sources of membrane contribute to autophagosome formation, including ER–Golgi intermediate compartments, ER– mitochondria junctions, mitochondria, endosomes and the plasma membrane. Taken together, these studies highlight the complexities of autophagy initiation in mammals.

Phagophore expansion

In part, the nucleation process may be viewed as an amplification event that results in the further recruitment of proteins that are needed for phagophore expansion. In the expansion stage, the ATG12–ATG5–ATG16 complex is recruited to the autophagosome membrane where it facilitates the lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3; also known as LC3) with phosphatidylethanolamine (PE). Cytosolic form of LC3, LC3-I, is generated by cleavage of pro-LC by ATG4B and further processed by ATG7 and ATG3 to be conjugated to PE (LC3-II) (Tanida et al., 2002). LC3 is the chief mammalian homologue of yeast Atg8, which is required for the expansion of the isolation membrane and LC3-II specifically

associates with autophagosome membranes, therefore its levels correlate with the number of autophagosomes.

Autophagosome maturation and fusion

A characteristic feature of autophagy is the formation of double-membraned vesicles known as autophagosomes, which correspond to the mature form of the phagophore (Baba et al., 1994).

Following expansion and sealing of the phagophore, the autophagosome undergoes maturation, which involves gradual clearance of ATGs from the nascent autophagosome outer membrane and recruitment of machinery responsible for lysosomal delivery (microtubule-based kinesin motors) and machinery that mediates fusion with the lysosome. Upon completion of the autophagosome, it targets to and then fuses with the vacuole. This fusion allows the release of the inner autophagosome vesicle into the vacuole lumen where it is now termed an autophagic body.

Cargo degradation and recycling

After the cargo is delivered inside the vacuole, the autophagic body membrane is degraded by a putative lipase, Atg15 (Teter et al., 2001), followed by cargo degradation by resident hydrolases. Once degraded, the resulting macromolecules are released back into the cytosol through various permeases including Atg22 (Yang et al., 2006).

The role of autophagy in neurodegenerative disease

Underscoring the importance of the autophagic process, growing evidence suggests that mutations in autophagy-related genes have pathogenic roles in various human

pathological conditions including cancer, inflammatory diseases and neurodegenerative diseases (Mizushima and Komatsu, 2011).

Focusing on neurodegenerative diseases, the importance of this quality control mechanism is obvious if someone take into account that that nerves are postmitotic tissues , where autophagy is important for the removal of aggregated proteins and, therefore, for protecting the cells from the toxic effects of dysfunctional proteins that cannot be diluted via cell division (Mizushima et al., 2008). In aggrement with this statement, the phenotype of multiple nervous system-specific knockout mouse models that have been generated to allow analyses of the roles of autophagy in neuronal function, point to reduced survival, early-onset, progressive neurodegeneration across broad areas of the brain the accumulation of intra-neuronal aggregates in case of autophagic deletion in neuronal cells and glia (Hara et al., 2006; Komatsu et al., 2006, 2007). The accumulation of these aggregates in otherwise normal mice suggests that autophagy plays a key role in removing aggregate-prone proteins. And vice versa, the correlation between autophagy and neurodegeneration was further supported by the major pathological phenotype of most late-onset neurodegenerative diseases, meaning the presence of intraneuronal aggregates of misfolded proteins, which are substrates for autophagic degradation (Menzies et al., 2015). Furthermore, identification of disease-associated genes and investigations into their functions reveal that many affect autophagy. The last years lots of neurodegenerative diseases have been associated with autophagy, among which are Alzheimer's disease, tauopathies, Parkinson's disease, polyglutamine disorders, amylotrophic lateral sclerosis (ALS), hereditary spastic paraplegias (HSPs), Lafora disease and Charcot-Marie-Tooth (CMT) disease. And although the autophagy pathway is really complex, with multiple steps and models of regulation, many efforts have been made in order to highlight how autophagy defects at various stages of the pathway may be linked to neurodegenerative diseases (Figure 3).

All these data provide increasing evidence for the physiological importance of autophagy in neuronal health, raising the possibility that autophagy upregulation may be a promising therapeutic strategy for some neurodegenerative diseases.

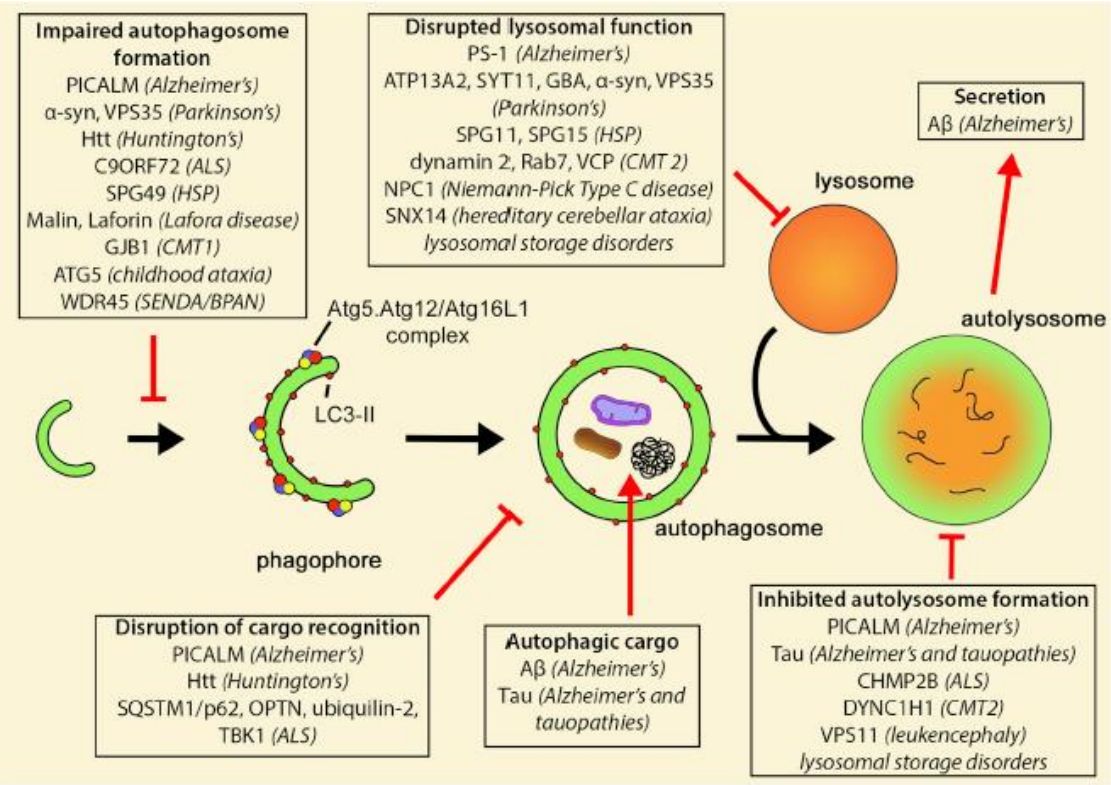


Figure 3. Intersections between Autophagy and Disease-Associated Genes.
An increasing number of genes associated with neurodegenerative diseases have now been implicated in autophagy function. These genes act at a number of different steps throughout the autophagic process, from early steps of autophagosome formation through autolysosome formation. Their proposed sites of action are indicated, along with the neurodegenerative disease with which they are associated.
(Menzies et al., 2017)

Autophagic selectivity

As previously mentioned, autophagy was originally believed to non-selectively sequester and degrade cytoplasmic material. However, it is increasingly being appreciated that autophagy can also be a selective process, resulting in the targeted engulfment of specific cargoes such as mitochondria, peroxisomes and ribosomes, and protein aggregates etc. Thus, non-selective (bulk) autophagy is important for starvation adaptation, whereas selective autophagy may be more important for maintaining homeostasis of cytosolic proteins and organelles, recognized as a critical housekeeping pathway even in nutrient-rich conditions, although these two categories are not mutually exclusive (Mizushima and Komatsu, 2011).

Nevertheless, how can autophagy be selective? Selective autophagy is usually mediated by autophagy cargo receptors that bind cargo earmarked with degradation signals, most commonly ubiquitin in mammals, through their ubiquitin-binding domain (UBD). These receptors also commonly possess a motif called the LC3 interacting region (LIR), which mediates their binding to Atg8 (LC3 in mammals) isoforms present on newly developing autophagosomes. As a result, autophagy cargo receptors act as molecular bridges that capture ubiquitylated proteins targeted for degradation by the autophagy pathway and complement the UPS. For example, the autophagy cargo receptors p62 (also known as SQSTM1), NBR1 (next to BRCA1 gene 1 protein) and histone deacetylase 6 (HDAC6) all promote the autophagic clearance of protein aggregates in a process known as aggrephagy, which is dependent on both the UBD and LIR2 (Rogov et al., 2014).

Several cargo-specific autophagic subtypes have been described: for example, the removal of aggregated proteins (aggrephagy), damaged mitochondria (mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy), endoplasmic reticulum (reticulophagy), lipid droplets (lipophagy), pathogens (xenophagy) etc. (Figure 4).

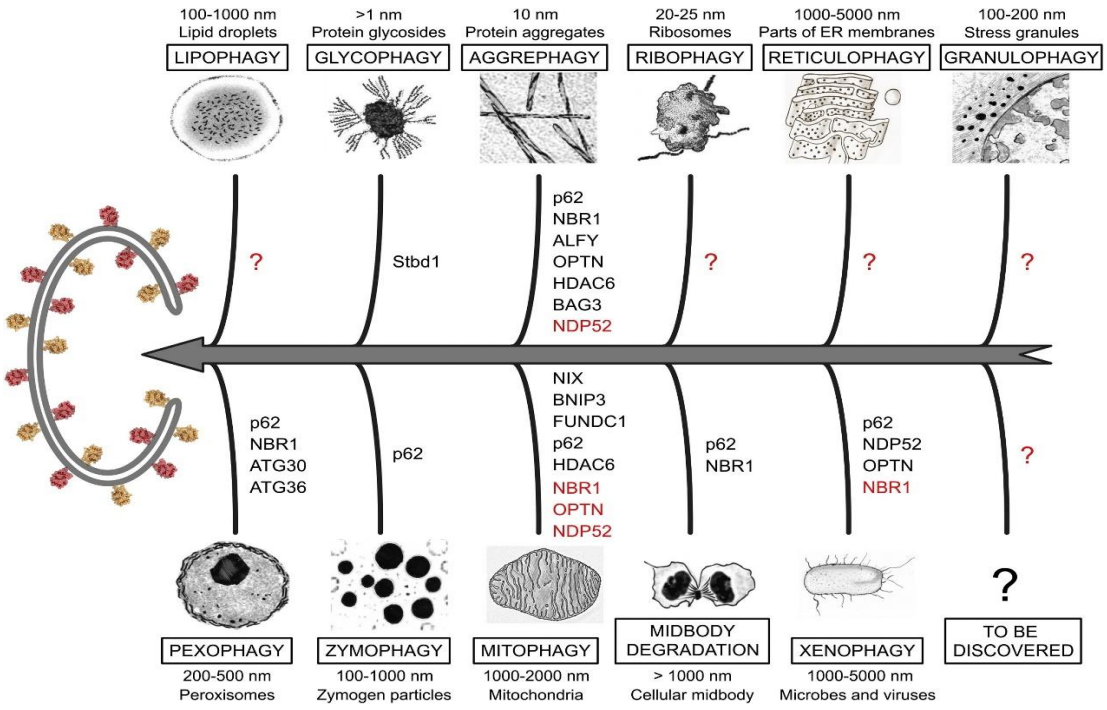


Figure 4. Types of Selective Autophagy in Mammalian Cells.

Subcellular structures are specifically targeted for lysosomal degradation by autophagy. Depending on the cargo, the processes are named differently: mitophagy for the specific elimination of mitochondria; ribophagy for ribosomes; and lipophagy for the degradation of lipid droplets etc. Established (black) and putative (red) selective autophagy receptors for the respective processes are listed. Question marks indicate as-of-yet unidentified receptor proteins. (Rogov et al., 2014).

Myelinophagy

Expanding this list of forms of selective autophagy, Gomez-Sanchez et al. (2015) a couple of years ago, showed that in response to nerve injury, autophagy is responsible for clearing away damaged myelin within Schwann cells in the peripheral nervous system (PNS), a process that they term “myelinophagy”.

The peripheral nervous system has the considerable ability to regenerate axons after nerve injury. A prerequisite for successful repair is the degradation and clearance of axonal and myelin fragments in areas distal to the injury site, a process known as Wallerian degeneration (Simons et al., 2014). In nerves undergoing Wallerian degeneration, myelin breaks down and fragments into small ovoid-like structures at the Schmidt-Lanterman clefts (funnel-shaped incisures in the myelin). These fragmented pieces of damaged myelin were thought to be cleared away by phagocytosis both by Schwann cells and by invading macrophages (Hirata and Kawabuchi, 2002).

However, in this issue, Gomez-Sanchez and colleagues now show that macroautophagy is essential for myelin breakdown and clearance in Schwann cells after nerve injury (Figure 5). Specifically, they revealed that autophagy was up-regulated by myelinating Schwann cells after nerve injury, myelin debris was present in autophagosomes, and pharmacological and genetic inhibition of autophagy impaired myelin clearance. These findings were particular remarkable for a series of reasons. Primarily, they managed to show that it is macroautophagy and not phagocytosis that is essential for myelin breakdown and clearance in Schwann cells after nerve injury. Above all, however, they introduced a form of autophagy, which

in contrast to other cargo-selective variants of macroautophagy that degrade damaged intracellular components, describes a process of how a plasma membrane is degraded.

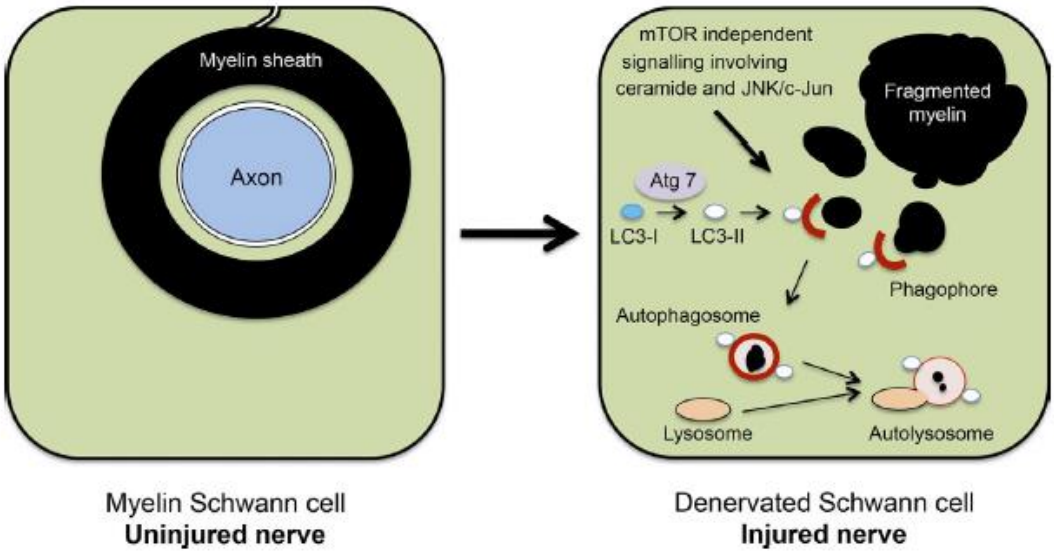


Figure 5. Outline of myelinophagy.

(left) A transverse section through a myelin Schwann cell in an uninjured nerve. Note that the myelin sheath is in direct continuity with the Schwann cell membrane and an integral component of the Schwann cell. (right) A myelin Schwann cell after nerve injury and axonal degeneration. Note that the myelin sheath has broken up into myelin fragments lying in the Schwann cell cytoplasm. The proposed role of autophagy in digesting these fragments is illustrated. (Gomez-Sanchez et al., 2015).

Myelin Composition and Architecture

To make clear all of the above, a closer look at myelin architecture is necessary.

Myelin sheath is a lipid-rich membrane surrounding most large axons in the mammalian nervous system, which promotes rapid conduction of nerve impulses and protects against axonal damage. Myelin sheaths form during development and consist of compacted spiral wraps of membrane that are supplied by oligodendrocytes in the CNS and Schwann cells in the PNS (Figure 6).

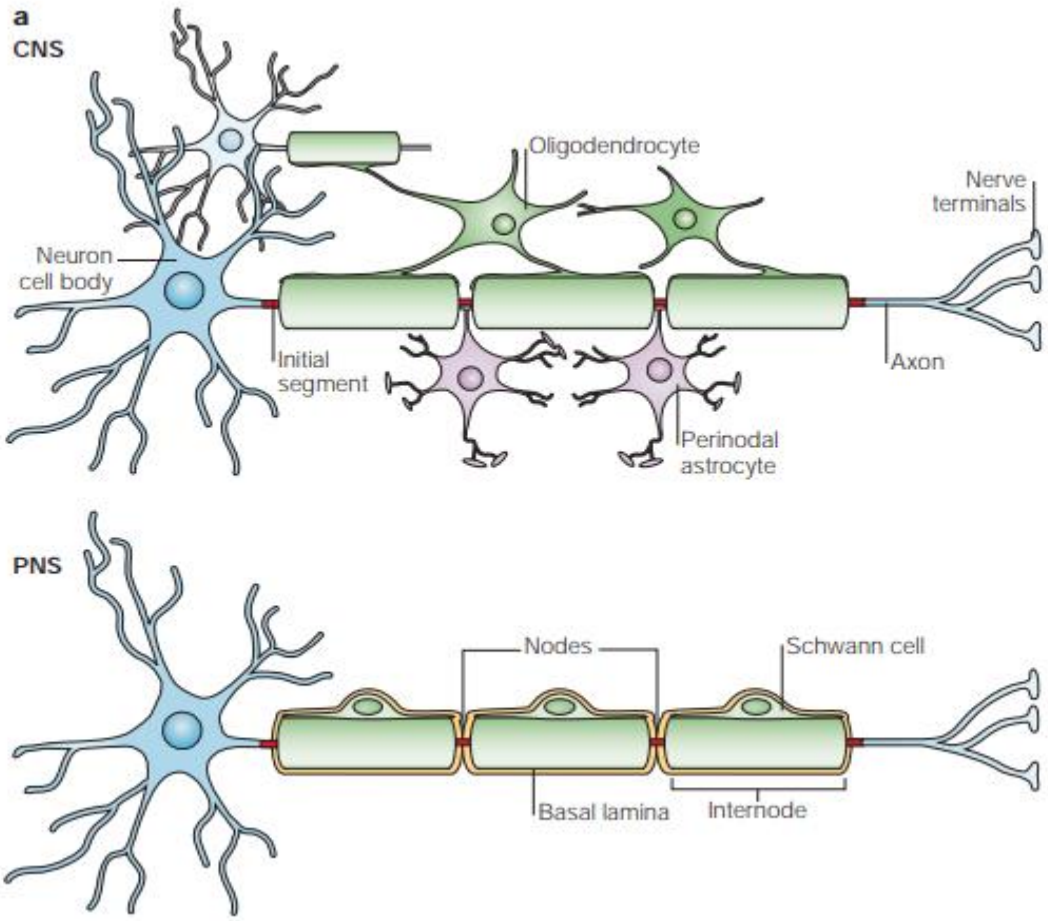


Figure 6. Structure of myelinated axons.

Myelinating glial cells, oligodendrocytes in the central nervous system (CNS) or Schwann cells in the peripheral nervous system (PNS), form the myelin sheath by enwrapping their membrane several times around the axon. Myelin covers the axon at intervals (internodes), leaving bare gaps — the nodes of Ranvier. Oligodendrocytes can myelinate different axons and several internodes per axon, whereas Schwann cells myelinate a single internode in a single axon. (Poliak and Peles, 2003).

Myelin sheath consists of repeating units of double bilayers separated by 3- to 4-nm-thick aqueous layers that alternate between the cytoplasmic and extracellular faces of cell membranes (Inouye and Kirschner, 1988) (Figure 7A). One of the biochemical characteristics distinguishing myelin from other biological membranes is its high lipid-to-protein ratio. It is found that dehydrated myelin is composed of 75–80% lipid and 20–25% protein by weight, compared with ≈50% of most other cell membranes

(Williams and Deber, 1993). The lipids are assembled concomitantly with myelin-specific membrane proteins, the most abundant in CNS being the intrinsic (integral) membrane protein, myelin proteolipid protein (PLP) and the extrinsic (peripheral) myelin basic protein (MBP) while myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), and 2'3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) are quantitatively minor constituents. Myelin basic protein (MBP) constitutes 20–30% of total protein by weight and is located only between the two cytoplasmic faces, where it acts as an intermembrane adhesion protein. The three main classes of lipids comprising CNS myelin are cholesterol, glycosphingolipids (derivatives of galactosylceramides [GalCer] and glucosylceramides [GlcCer]), and phospholipids (PLs): these myelin lipid classes have long been known to have constant molar proportions of 2:1:2 in CNS (Norton and Autilio, 1966; Fewester et al., 1976) (Figure 7B). The asymmetric distribution of lipid composition on the cytoplasmic and extracellular faces likely also plays an important role (Inouye and Kirschner, 1988 b).

The myelin sheath acts as an electrical insulator, forming a capacitor surrounding the axon, which allows for faster and more efficient conduction of nerve impulses than unmyelinated nerves. It represents a key determinant for saltatory nerve conduction, in which neuronal action potentials are restricted to nodes of Ranvier increasing conduction speed 20–100-fold as compared with non-myelinated axons (Nave and Werner, 2014).

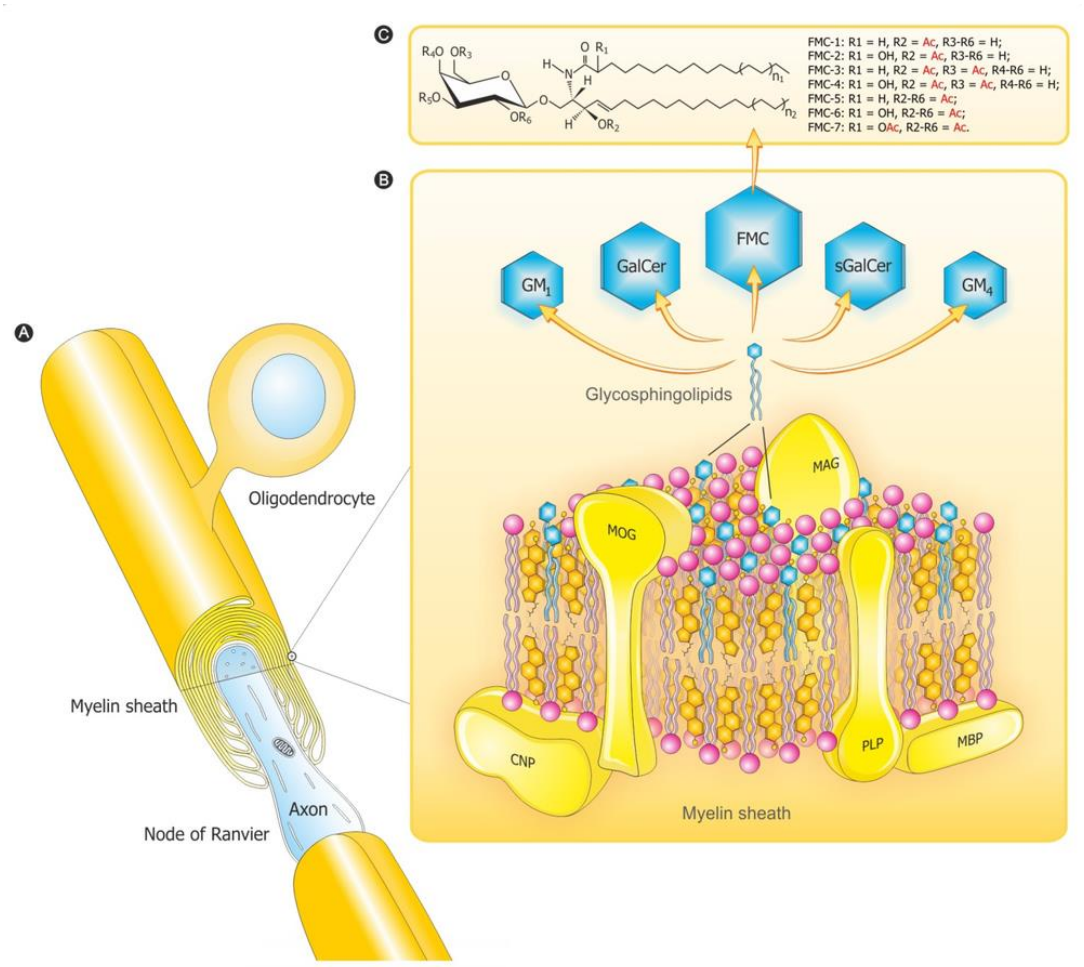


Figure 7. A composite diagram summarizing features of CNS myelin.

(A) architecture; (B) 3D-molecular composition and conformation-based assembly and (C) the unique sphingosine 3-O-acetylated-GalCer GL series. The diagram depicts arrangement of complex lipids (cholesterol, PLs and GLs) and most abundant proteins (PLP, MBP).

(Podbielska et al., 2013).

Oligodendrocyte Differentiation

As mentioned before, myelin sheaths are made by oligodendrocytes in the CNS and by Schwann cells in the PNS. A single oligodendrocyte can generate multiple myelin sheaths, up to 80 separate myelin sheaths in fact, although approximately 10–20 is more usual, whereas an individual Schwann cell only makes one.

Oligodendrocytes are a category of glial cells that generate CNS myelin. Oligodendroglial precursor cells (OPCs; also known as oligodendrocyte progenitor

cells or oligodendrocyte precursor cells) are considered the main source for generating mature oligodendrocytes and myelin during development. They are generated in sequential waves within specific regions of the ventral and the dorsal neuroepithelium of the spinal cord and brain before they migrate and disperse into the CNS (Kessaris et al., 2006; Crawford et al., 2014).

The highly migratory and proliferative OPC is identified by its expression of the NG2 proteoglycan and the platelet-derived growth factor receptor alpha (PDGFRa). OPCs differentiate through a premyelinating stage to become the mature myelinating cell, which generates the myelin internode, and thereby interacts with axons to organize the nodal, paranodal, and juxtaparanodal regions of myelinated axons. Progression through the oligodendrocyte lineage is tightly regulated by a multitude of intrinsic and extrinsic cues, which control myelination both spatially and temporally during development and after demyelination. These signals include growth factors, protein kinases, and extracellular matrix molecules, all of which influence epigenetic modifications, transcriptional and translational regulation, and the actin cytoskeleton in oligodendrocytes (Bauer et al., 2009). While the vast majority of OPCs differentiate into myelinating oligodendrocytes, a proportion of OPCs remains undifferentiated and persists as quiescent cells within the adult CNS. They comprise 5–8% of the total cell population and are distributed throughout gray and white matter (Nishiyama et al., 2009; Chang et al., 2000) (Figure 8).

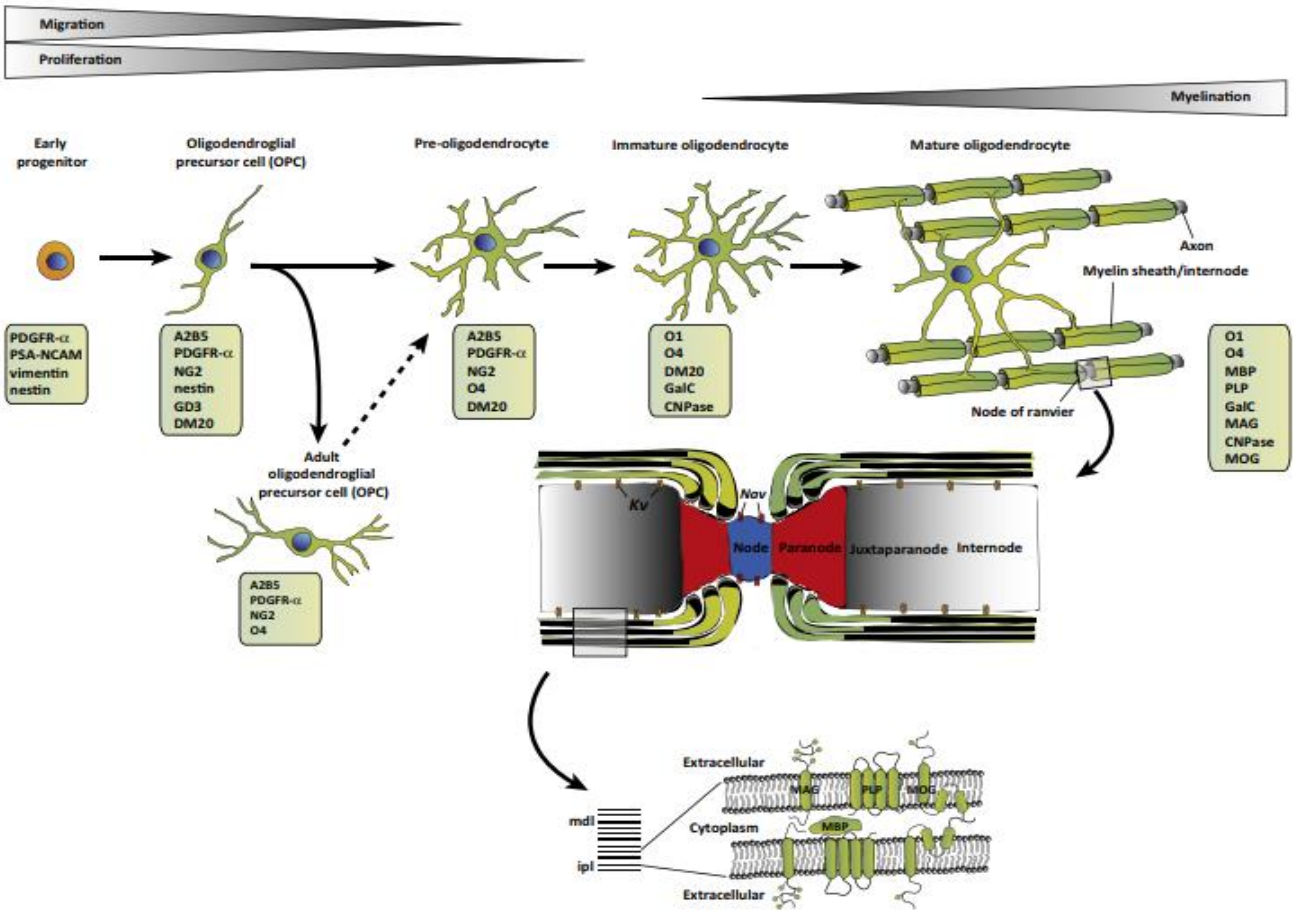


Figure 8. Oligodendrogenesis, Cellular Stages, and Markers.

During development, early (glial) progenitor cells give rise to oligodendroglial precursor cells (OPCs) that eventually transform into premature and immature oligodendrocytes. Cellular differentiation is accompanied by morphological conversion from bipolar precursor to mature cells with multiple processes forming a large network. Cells at different stages of maturation are characterized by specific combinations of marker proteins while their migratory activity and proliferation rates cease during the differentiation process. In the adult central nervous system (CNS), a fraction of immature oligodendroglial precursor cells (adult OPCs) persists. These cells are also referred to as adult NG2 cells.

In myelinated axons, voltage-gated sodium channels (Nav) are concentrated within central aspects of nodes, whereas voltage-gated potassium channels (Kv) are located in juxtaparanodes.

Insulating properties of internodes can be attributed to compact myelin that consists of approximately 30% proteins and 70% lipids. Structural proteins such as proteolipid protein (PLP) and myelin basic protein (MBP) are fundamental to the compaction process, resulting in the typical appearance of alliterating concentric major dense lines (mdl) and light layers (intraperiod lines, ipl).

(Kremer et al., 2016).

Autophagy and demyelination

As above mentioned, autophagy has been acknowledged as a significant component in a plethora of neurodegeneration diseases. Besides, the last years, autophagy seems to play a key role in various demyelinating disorders of the PNS in rodent models and postmortem human tissue as well as in the development of myelin-producing Schwann Cells (SCs).

Specifically, in humans, duplications of or point mutations in the gene encoding peripheral myelin protein 22 (PMP22) in Schwann cells cause the progressive demyelinating disease Charcot–Marie–Tooth disease type 1A (CMT1A). The levels of the aberrant PMP22 and Schwann cell health appear to be regulated by autophagy in mouse models of CMT1A, and autophagy upregulation may be a suitable strategy to consider for this class of diseases (Rangaraju et al., 2010).

One of the most common demyelination diseases is multiple sclerosis. Multiple sclerosis is an inflammatory disorder that is characterized by immune system reactivity against myelin in the central nervous system, resulting in varying degrees of either relapsing or progressive neurological degeneration (Nave, 2010).

Autophagy is tightly linked to the innate and adaptive immune systems during the autoimmune process, and several studies have shown that autophagy directly participates in the progress of MS or experimental autoimmune encephalomyelitis (EAE, a mouse model of MS). In detail, it is found that the mRNA and protein levels of autophagy-related (ATG)5, required for autophagosome formation, were increased in $CD4^+$ and $CD4^-$ T cells, but not B cells of MS patients compared to control subjects (Paunovic et al., 2018). Moreover, immune-related GTPase M (IRGM) 1 are increased, while ATG16L2 is decreased, in autoreactive T cells in EAE and actively relapsing-remitting MS brains (Igci et al., 2016). Inhibition of autophagy by loss of autophagy-related gene 7 (*Atg7*) in dendritic cells has shown a protective role in the EAE model (Bhattacharya et al., 2014). Finally, administration of rapamycin reduces relapsing–remitting EAE via inhibition of autophagy (Liang and We, 2015), indicating a pathological role of autophagy in MS.

Role of autophagy in oligodendrocytes

However, although a few studies implicate autophagy in CNS demyelinating pathologies, the role of autophagy in glia -especially in oligodendrocytes- remains poorly characterized.

In fact, Smith and colleagues were the first to study the relationship between autophagy and oligodendrocytes, by studies they performed in Long-Evans shaker rats (Smith et al., 2013). The Long–Evans shaker (les) rat has an abnormal insertion in the gene that encodes myelin basic protein (MBP). This mutation disrupts the splicing and subsequent translation of functional MBP (O'Connor et al., 1999). In detail, they demonstrated that les animals have increased levels of autophagy during dysmyelination and demyelination. However, they also showed that autophagy did not correlate with an increase in oligodendrocyte death in les and that an upregulation of autophagy is likely a mechanism for survival during myelin development and loss as les oligodendrocytes survive despite this cellular pathology. In addition, they proved that upregulating autophagy both in vitro in les oligodendrocytes and in les and control rats in vivo through intermittent fasting increased myelin production and the number of cells forming membrane extensions. Therefore, this study was the first evidence that autophagy can play a novel role in oligodendrocyte survival and myelin development that may have broader implications for therapies to treat myelin disease.

Finally, recently this year there was a publication published, indicating the role autophagy plays in oligodendrocytes after spinal cord injury (Saraswat- Ohri et al., 2018). More analytically, this research team demonstrated that specific deletion of autophagy in OLs is detrimental to OPC/OLs function after thoracic contusive spinal cord injury (SCI) and results in greater locomotor deficits and myelin loss. By using pharmacological gain and loss of function and genetic approaches -transgenic mice with conditional deletion of *Atg5* in OLs- they investigated the contribution of autophagy in OL survival and its role in the pathogenesis of thoracic contusive SCI in mice. Their study provides for the first time evidence that autophagy is an essential cytoprotective pathway operating in OLs and is required for hindlimb locomotor

recovery after thoracic SCI. However, the molecular pathways that link autophagy with myelin homeostasis remain unsearchable.

AIM OF THE STUDY

The goal of this study was to elucidate the role of autophagy in myelination in oligodendrocytes. Although, a few studies implicate autophagy in CNS demyelinating pathologies, knowledge about the cell-type specific effect of this mechanism in myelinating glia is extremely limited. In order to shed light on this role, we performed in vitro studies by using both wild type oligodendrocyte progenitor cells (OPCs), as well as wild type mature oligodendrocytes (OLs).

Thus, in this study we performed a series of experiments in order to:

- ✓ Characterize the requirement of autophagy in CNS myelination.
- ✓ Investigate whether autophagy is active in OPCs and OLs.
- ✓ Study the impact of inhibition of autophagy in OPCs.
- ✓ Analyze OL differentiation, maturation and membrane extension under normal conditions or pharmacological inhibition of autophagy.

MATERIALS AND METHODS

Animals

P0-2 pups from C57BL/6 mice were euthanized by decapitation as described in the International Animal Care and Use Committee, and then, they were used for the experiments. All research activities strictly adhered to the EU adopted Directive 2010/63/EU on the protection of animals used for scientific purposes. Animals were kept in the Animal House of the Institute of Molecular Biology and Biotechnology (IMBB-FoRTH, Heraklion, Greece), in a temperature-controlled facility on a 12 h light/dark cycle with food and water available *ad libitum*.

In vitro studies

Oligodendrocyte primary cultures

Primary mixed glial cell cultures were prepared from the brain of P0-2 (postnatal day 0-2) mouse pups. The whole brain was dissected out and the meninges were removed. Then, the brain hemispheres were chopped into small pieces and after enzymatically dissociation with trypsin, the brains were subsequently mechanically dissociated. Cells were plated onto poly-L-lysine (PLL, Sigma-Aldrich, 100 μg/ml)-coated T75 culture flasks and cultured in DMEM (+Glutamax, +4,5 g/L D-Glucose, -Pyruvate), supplemented with 10% FBS (Gibco) and 2% penicillin/streptomycin (P/S), until a clear layer of glial cells was formed. The culture medium was replenished twice a week. After about 2 weeks in culture, the flask contained astrocytes, OPC and some microglial cells. Once the mixed glial cell cultures achieved 90% confluence (after about 14 days), the microglia cells were isolated using an orbital shaker at 200 rpm for 1 h, 37°C. After removing the medium containing the microglial cells, fresh medium was added and the flasks were shaken overnight (16 h at 250 rpm, 37°C) to selectively detach the OPC from the underlying astrocytic cell layer on which they were growing. Contaminating astrocytes were removed by plating the OPC onto uncoated bacteriological Petri dishes for 30 min at 37°C. Astrocytes preferentially

attach to the flask, while the OPC remain floating. The cells were then replated on PLL-coated coverslips in 24 well plates at $3.5*10^4$ cells per well and incubated under 5% CO_2 at 37 °C and cultured in Proliferation Medium [DMEM (+Glutamax, +4,5 g/L D-Glucose, – Pyruvate) supplemented with 1% N2, 1 µM biotin, 1% BSA-FFa (Sigma-Aldrich), 60 µg/ml cysteine (Sigma-Aldrich), 1% P/S, 10 ng/ml FGF-2 (Peprotech) and 10 ng/ml PDGFα (Peprotech), the last two being essential growth factors for OPC proliferation]. After 2 days of expansion period, the cells were cultured for other 2-8 days in the same medium as before, without FGF-2 and PDGF-α, but with triiodothyronine (T3, Sigma-Aldrich, 40 ng/ml), an essential growth factor for OPC differentiation. This medium was called Differentiation medium and was replenished every other day. The cultures were named DIV0 the day the were passed to proliferation or differentiation medium.

Quantity		Stock Solut.	Final Conc.
50 ml	**PROLIFERATION MEDIUM FOR OPCs**		
47.45 ml	DMEM 1X GlutaMAX [+] 4.5 g/L D-Glucose, [-] Pyruvate (Gibco)		
0.5 ml	N2		1%
30 µl	Cysteine	100 mg/ml	60 µg/ml
25 µl	Biotin	200 µg/ml	100 ng/ml
0.5 ml	Penicillin/Streptomycin		1%
0.05 g	BSA fatty acid-free (Sigma, A6003)	100%	0.1%
0.5 ml	PDGFα	1 µg/ml	10 ng/ml
50 µl	hFGF	10 µg/ml	10 ng/ml

Quantity		Stock Solut.	Final Conc.
50 ml	**DIFFERENTIATION MEDIUM FOR OPCs**		
47.45 ml	DMEM 1X GlutaMAX [+] 4.5 g/L D-Glucose, [-] Pyruvate (Gibco)		
0.5 ml	N2	100x	1%
30 µl	Cysteine	100 mg/ml	60 µg/ml
25 µl	Biotin	200 ug/ml	100 ng/ml
0.5 ml	Penicillin/Streptomycin	100%	1%
0.05 g	BSA fatty acid-free (Sigma, A6003)	powder	0.1%
1 µl	T3	2 mg/ml	40 ng/ml

Immunocytochemistry

The cells were fixed in 4% paraformaldehyde (PFA) in 1xPBS for 10 minutes at 37°C. Then they were washed and incubated in Blocking Solution Solution of 5% bovine serum albumin (fraction V, BSA, Applichem) in 0.1% Triton X-100 in 1x PBS for 30 min, RT). Cells were incubated with primary antibodies diluted in Blocking Solution for 1 hour at room temperature. The primary antibodies were as follows: rabbit anti-Ki67 (1:200; Novocastra), rat anti-PDGFRα (1:100; Millipore), rat anti-MBP (1:200; Serotec), rabbit anti-p62 (1:500; MBL). After washing, the cells were labeled with secondary antibodies and DAPI for 30 minutes at room temperature. The secondary antibodies were as follows: anti-rat IgG-Alexa 488 (1:800; Molecular Probes, Thermo), anti-rabbit IgG-Cy3 (1:800; Jackson ImmunoResearch Laboratories) DAPI (0.1µg/mL in dH2O, Sigma-Aldrich). Finally, the samples were mounted with mounting medium containing mowiol mounting medium (see recipe below).

Mowiol mounting medium

2.6 g Mowiol 4-88 (Fluka, #81381, Sigma)

6 g Glycerol

12 ml Tris-Cl (0.2 M, pH 8.5)

Add 2.4 g of Mowiol 4-88 to 6 g of glycerol. Stir to mix. Add 6 mL of H_2O and leave for several hours at room temperature. Add 12 mL of 0.2 M Tris-Cl (pH 8.5) and heat to 50°C for 10 min with occasional mixing. After the Mowiol dissolves, clarify by centrifugation at 5000*g* for 15 min. Aliquot in tubes and store at −20°C. Stock is stable at room temperature for several weeks after thawing.

Measurement of OL cell viability

Cell viability was determined using the MTT (3-(4, 5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) assay protocol (Molecular Probes, Vybrant MTT Cell Proliferation Assay Kit). The MTT assay is based on the conversion of MTT into insoluble formazan by the metabolically active cells, which can then be solubilized and the concentration determined by attenuance (*D*) at 540 nm. The attenuance can be correlated to the number of cells present in the well. OPCs were plated at 8000 cells/well in poly-L-lysine-coated 96-well plates, allowed to attach and treated with AEF in basal DMEM without Phenol Red for 48 h. Following SBI treatment, 10 µl of MTT stock solution was added to each well and incubated at 37°C for 4 h. The absorbance of cell supernatants was measured at 540 nm using the Infinite® 200 PRO NanoQuant microplate reader (Tecan, Research Triangle Park, NC, USA). The analysis was conducted in triplicate. Cell viabilities were defined relative to control cells (considered to be 100%). Attenuance values from wells with no cells were subtracted from treatment wells to account for background signal. Mean attenuance values from triplicate runs were expressed as a percentage and untreated OPCs were

set to 100%. Significance of change in percent proliferation was compared between no treated and SBI-treated using a paired *t* test with significance at $p < 0.05$.

Analysis of OL morphology

OLs identified by the stage-specific marker MBP were analyzed and total number of 100 MBP+ cells were evaluated per coverslip and per condition (n = 3 coverslips). OL complexity was defined according to branching and to the degree of myelin membrane formation (see Figure 16). Briefly, type A were considered cells with MBP+ primary and secondary processes and with low ramification branching, type B were recognized as cells with MBP+ primary, secondary and tertiary processes with an increased branching level but lack of visible myelin membrane, whereas type C cells displayed an incremented level of complexity and myelin membrane. The quantification of MBP+ cells was done manually and blindly, while the quantification of total number of cells was performed by the high content screening system Operetta (Perkin Elmer), using the software Harmony 4.1.

Statistical analysis

Image processing, analysis and measurements were carried out using Photoshop 9.0-CS2 and NIH Image J software. Data are depicted as means ± standard deviation of the mean (SEM). Statistical analysis of the measured values for each group was performed using two-tailed, unpaired Student's t test or two-way analysis of variance (ANOVA) with Bonferroni or Tuckey post-hoc tests for multiple comparisons. Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (Prism, GraphPad). $P < 0.05$ was considered statistically significant.

Analysis of cell morphology and cell count

Confocal microscopy images were obtained using a Leica TCS-SP8 confocal laser scanning microscope (Leica Microsystems), and all confocal settings were set to the same parameters for each experiment. Light microscopy images were obtained using Zeiss Primo Vert inverted microscope.

Western Blot analysis

- **Sample preparation**

Cultured OLs (DIV2) were homogenized in ice-cold RIPA buffer (see recipe below) with an addition of protease inhibitor cocktail (Sigma), followed by a brief sonication on ice. The total protein concentration in each sample was quantified with the Bradford kit (Bio-Rad Laboratories). The samples were prepared with 2 µg of total protein with the addition of the appropriate amount of 4x Laemmli Sample Buffer (see recipe below) and DTT. After mixing, samples were incubated for 5 min at 95°C and subsequently cooled on ice.

RIPA Buffer	**4x Laemmli Sample Buffer**
150 mM NaCl	200mM Tris-Cl pH 6.8
50 mM Tris pH 8.0	8% sodium dodecyl sulfate (SDS)
1% w/v TritonX-100	40% glycerol
0.5% Sodium deoxycholate	0.2% bromophenol blue

- **SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

Samples (equal amount of total protein/lane) were resolved by a SDS-polyacrylamide gel of appropriate acrylamide percentage (15% in our case). The gel was placed in the wet electrophoresis tank (Bio-Rad Laboratories) according to manufacturer's instructions and covered with 1 L 1x SDS-PAGE Running Buffer (see recipe below). Samples were loaded in the buffer-submerged wells, next to a commercial protein

ladder (BlueStar Plus Prestained Protein Marker, Nippon). The electrophoresis device was set to 80 V during the packing of the samples in the stacking gel and then to 100-120 V until the bromophenol blue exits the gel.

- **Western Blotting**

The gel was removed from the device and proteins were transferred to a 0.45 μM Protran nitrocellulose transfer membrane (Schleicher & Schuel, Biosience) in the following way:

1. The stacking gel is removed and the rest of the gel is placed on top of a presoaked (in 1x Transfer Buffer) Protran nitrocellulose membrane of equal size between two pieces of presoaked Whatman paper (Whatman, GE Healthcare) on each side, flanked by two sponges, inside the plastic tray.

2. The whole plastic tray is closed carefully to avoid bubbles and placed in the transfer tank, with the black side towards the black side of the tank.

3. The tank is filled with freshly made cold 1x Transfer Buffer until the plastic tray is completely submerged in the buffer and the accompanying ice container is also placed in the tank (this helps maintain the temperature as low as possible).

4. The whole tank is placed in a container filled with ice and then the device is set to 310 mA for 1h to ensure the transfer of the proteins from the gel to the membrane.

- **Immunoblotting**

1. The membrane is carefully removed from the apparatus and briefly washed in 1xPBS-T.

2. Blocking with 5% BSA in 1xPBS-T for 1 h at RT.

3. Incubation with the appropriate antibodies follows [rabbit anti-LC3 (1:1000; Sigma) and rabbit anti-actin (1:1000) in 5% BSA in 1x PBS-T at 4°C overnight.

4. Washes (3x) with 1x PBS-T, 15 min each.

5. Incubation of the membrane with the appropriate horseradish-conjugated secondary antibodies [α-rabbit IgG horseradish-conjugated (1:5000, Jackson ImmunoResearch Laboratories)].

6. Washes (3x) with 1x PBS-T, 15 min each.

7. Visualization of signal with enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate (Merck).

- **Recipes**

Separating Gel (10ml)			**Staking Gel (5ml)**	
	15%			
1.5M Tris pH 8.8	2.5ml		1M Tris pH 6.8	1.5ml
30% Acrylamide	5ml		30% Acrylamide	900ul
dH_2O	2.5ml		dH_2O	3.6ml
10% APS	200ul		10% APS	240ul
TEMED	10ul		TEMED	12ul

10x RUNNING BUFFER (1 L)	**1x TRANSFER BUFFER (1 L)**
30 g Tris Base	100 ml 10x Running Buffer
144 g Glycine	200 ml MetOH
10 g SDS	700 ml dH_2O

RESULTS

In preliminary studies of our lab, we used a transgenic mouse line in which autophagy is ablated throughout the nervous system (*Nestin-Cre; Atg5 fl/fl*) (Hara et al., 2006). Our data have shown that both in early developmental stages (P16) and in adult mice, myelin basic protein (MBP) levels were significantly reduced in different CNS areas, including brain and the pure white matter tract of the optic nerve.

Our hypothesis is that autophagy is an essential mechanism for central myelin formation and maintenance. Thus, the present study aims to shed light on the significance of autophagy in myelination in the CNS, by *in vitro* analysis of oligodendrocytes.

Establishment of primary oligodendrocyte cultures

Our first step was to generate primary oligodendrocyte cultures from P0- P2 C57BL/6 mice, as previously described (McCarthy and de Vellis, 1980), with minor modifications. OPCs were cultured in medium supplemented with platelet-derived growth factor (PDGFα), the main mitogen for OPCs, in combination with basic fibroblast growth factor (FGF-2) that not only inhibit OPC differentiation, but also inhibit OPC maturation (Tang et al., 2000). Thus, most of the cells initially had a bipolar or unipolar morphology with primary unbranched processes (Figure 9A). After the plating on coverslips, the oligodendrocyte cultures were named as DIV0 to DIV6 OPCs with respect to the number of days cultured (DIV= days *in vitro*) under proliferating conditions. Differentiation of OPCs was induced by the elimination of the growth factors PDGFα and FGF-2 in the medium and the addition of thyroid hormone triiodothyronine (T3) (differentiation medium). In the absence of growth factors, OPCs stop dividing and prematurely differentiate into mature oligodendrocytes (OLs) with extended complex and highly branched processes (Figure 9B) (Noble and Murray, 1984).

OPCs cultured in differentiation medium were named OLs, with DIV0 defining the day they were passed in that medium. OLs were cultured in that medium for 4- 8 days (DIV4- DIV8) with replenishment every other day.

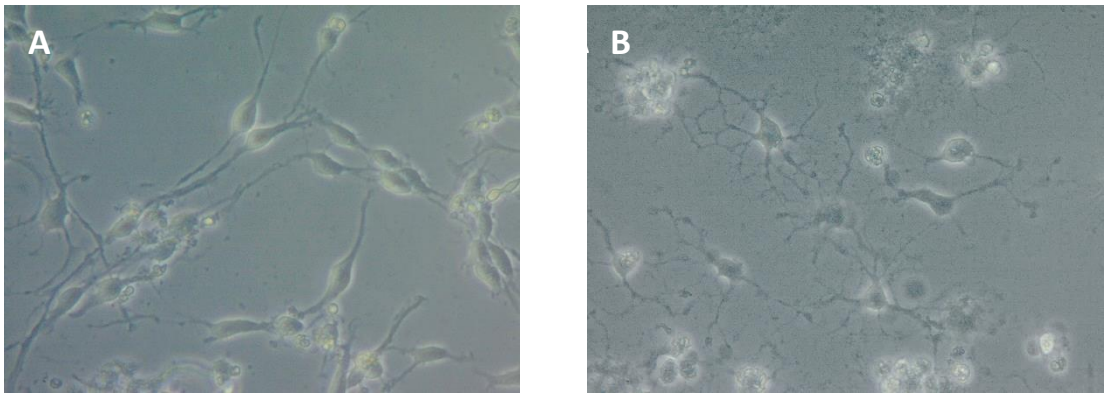


FIGURE 9. Oligodendrocyte primary cultures.

(A) OPCs (DIV2) cultured in proliferation medium supplemented with FGF-2 and PDGFα. OPCs initially displayed the typical morphology of immature cells, having a unipolar or bipolar morphology.

(B) In the absence of growth factors and presence of T3, OPCs differentiate to mature OLs (here DIV4), featured by the complicated reticulation of processes in the periphery.

Autophagy is active in OPCs

Recently it became known that autophagy is active in oligodendrocyte precursor cells (Saraswat- Ohri et al., 2018). Our first aim was to confirm this result and, additionally, to study which are the consequences of inhibition of autophagy in this cell population. Hence, we maintained our cultures in Proliferation medium that due to presence of FGF-2 and PDGFα helps OPCs maintain their ability to proliferate and prevents their differentiation in mature oligodendrocytes. OPCs were maintained for 6 DIV in normal Proliferation medium in order to synchronize, and then they were passed either in normal Proliferation medium or in Proliferation medium with the addition of SBI-0206965 for 4 more days. As already mentioned, SBI-0206965 is a potent and selective inhibitor of the serine/threonine autophagy-initiating kinases ULK1 and ULK2 with selectivity for ULK1. As shown in figure 10, the majority of cells were PDGFRα+, indicating they are still in the phase of the progenitors. SBI-0206965

administration led to an increase in the signal of p62 (Figure 10 C-D). Mammalian sequestosome 1 (p62/SQSTM1), hereinafter referred to as p62, is a multifunctional adapter protein that was identified as an ubiquitin-binding protein, transporting ubiquitinated proteins into autophagosomes through selective autophagy (Panvik et al., 2007). Since, p62 is an autophagic substrate, the increase in its signal when inhibiting autophagy was expected in case of an active autophagic machinery, verifying that autophagy is present in this type of glial cells.

Inhibition of autophagy leads to translocation of p62 to the nucleus of OPCs

In the course of these studies, we noticed to our surprise, that in case of autophagic inhibition, p62 seems to translocate to the nucleus (Figure 10 C-D). In the bibliography, it is established that the primary sequence of p62/SQSTM1 contains two nuclear localization signals (NLS) and one Nuclear Export Signal (NES), and the protein shuttles between the cytoplasm and the nucleus, where it seems to help in sequestering and degrading ubiquitin-conjugated nuclear proteins (Pankiv et al., 2010). However, it is not well known in which time points and why this translocation happens. Interestingly, there are also some publications that reveal the important role of p62 in cell proliferation. More specifically, it is demonstrated that autophagy deficiency promotes cell proliferation and migration through p62-dependent stabilization of an oncogenic transcription in mouse embryonic fibroblasts (MEFs) (Qiang et al., 2014) and it has been shown as well that p62-specific siRNA showed significantly decreased cell proliferation activity in cancer cell lines (Nakayama et al., 2017).

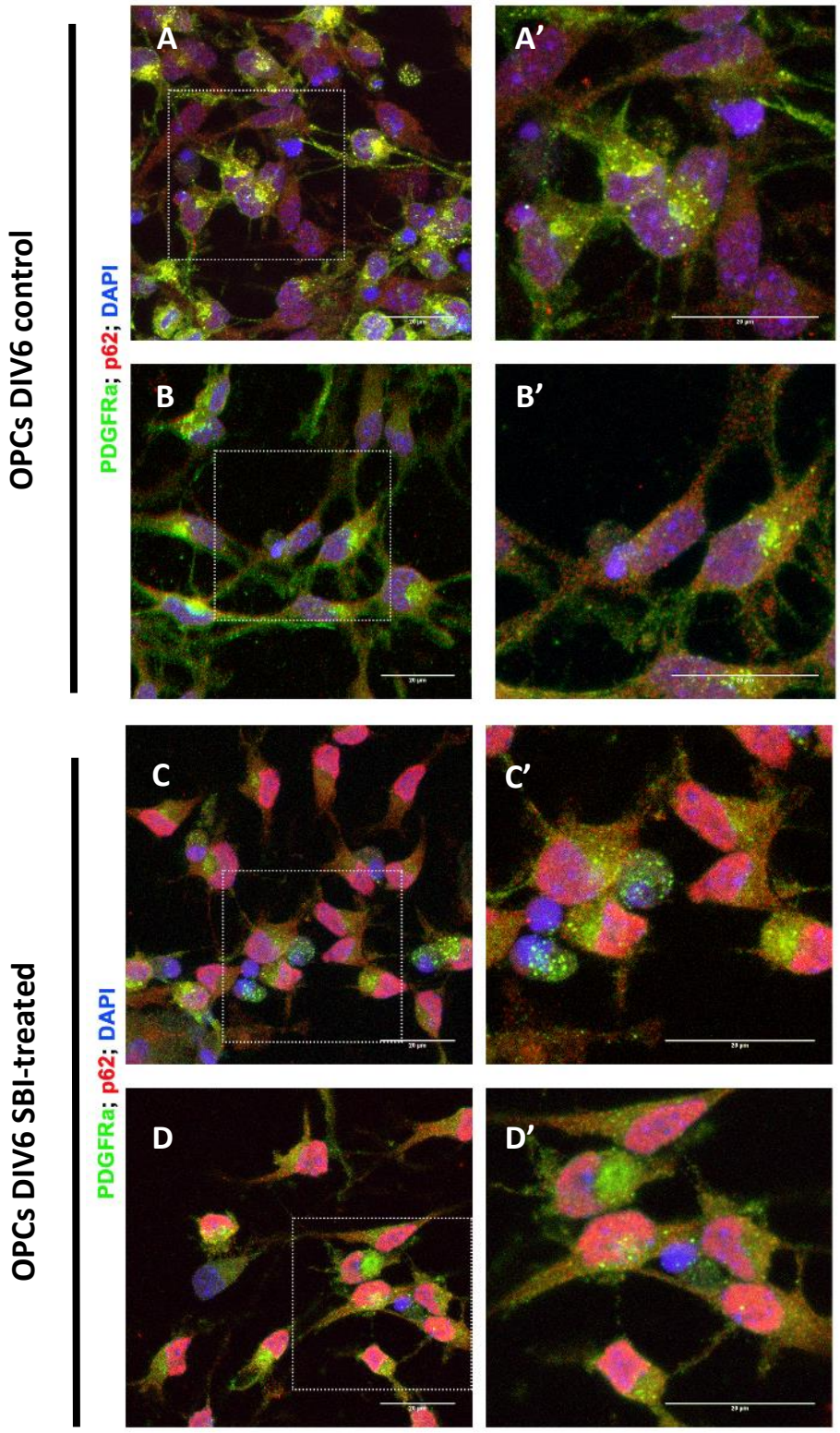


FIGURE 10. Treatment with SBI-0206965 leads to increased signal of p62 and its translocation to the nucleus in OPCs.

Representative images of immunocytochemical labeling for PDGFRα (green), p62 (red) and DAPI (blue).

(A-B) Untreated OPCs (PDGFRα+ cells) express low levels of p62 throughout the cell body.

(C-D) SBI- treated OPCs express higher levels of p62, a proof of active autophagy, and p62 signal seems to concentrate in the nuclei.

(A'-B', C'-D-) Higher magnification confocal view of the areas marked in white squares. Scale bars, 20 µm.

Inhibition of autophagy seems to increase proliferation of OPCs

Therefore, our next aim was to study whether this translocation of p62 to the nucleus could be associated somehow with increased proliferation in OPCs. To achieve that, we initially performed the MTT assay. This assay is based on the conversion of MTT into insoluble formazan, which can then be solubilized and the concentration determined by attenuance (*D*) at 540 nm. However, the ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity, which in turn may be interpreted as a measure of cell number/ proliferation/ viability/ survival/ toxicity. OPCs were plated at 8000 cells/well in poly-L-lysine-coated 96-well plates, allowed to attach and treated with SBI-0206965 in basal DMEM without Phenol Red for 24 h. The effect of SBI-0206965 was tested at two different concentrations, 0.5 µM and 5 µM. The protocol was followed as described above. Mean attenuance values from triplicate runs were expressed as a percentage and untreated OPCs were set to 100%. Significance of change in percent proliferation/ viability was compared between SBI-0206965-treated and control OPCs using an unpaired *t* test with significance at $P<0.05$. As shown in the diagram, there is an about 20% increase in cell proliferation/ viability in case of SBI-treatment with no statistical significant difference between the two concentrations (20.94% increase for 5 µM and 25.44% increase for 0.5 µM, Figure 11A). Therefore, we decided to continue our experiments using SBI-0206965 at 0.5 µM concentration.

Since the increase in the number of OPCs detected using the MTT assay could be due to both increased OPC proliferation and/or survival after SBI-0206965 treatment, and in order to elucidate which cellular mechanism was responsible for the increased number of OPCs, we performed immunocytochemistry experiments in OPCs using anti-Ki67 antibody (Figure 11C-D). The Ki67 protein is a cellular marker for proliferation (Scholzen and Gerdes, 2000). Ki67 protein is present during all

active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent in quiescent cells (G_0) (Bruno et al., 1992).

In the case of SBI treatment, there is an increase in Ki67+ cells (Figure 11B), confirming our findings with the MTT assay and in fact revealing that this increase is due to an increase in cell proliferation. Thus, to our surprise, we found that inhibition of autophagy leads to increased proliferation in OPCs.

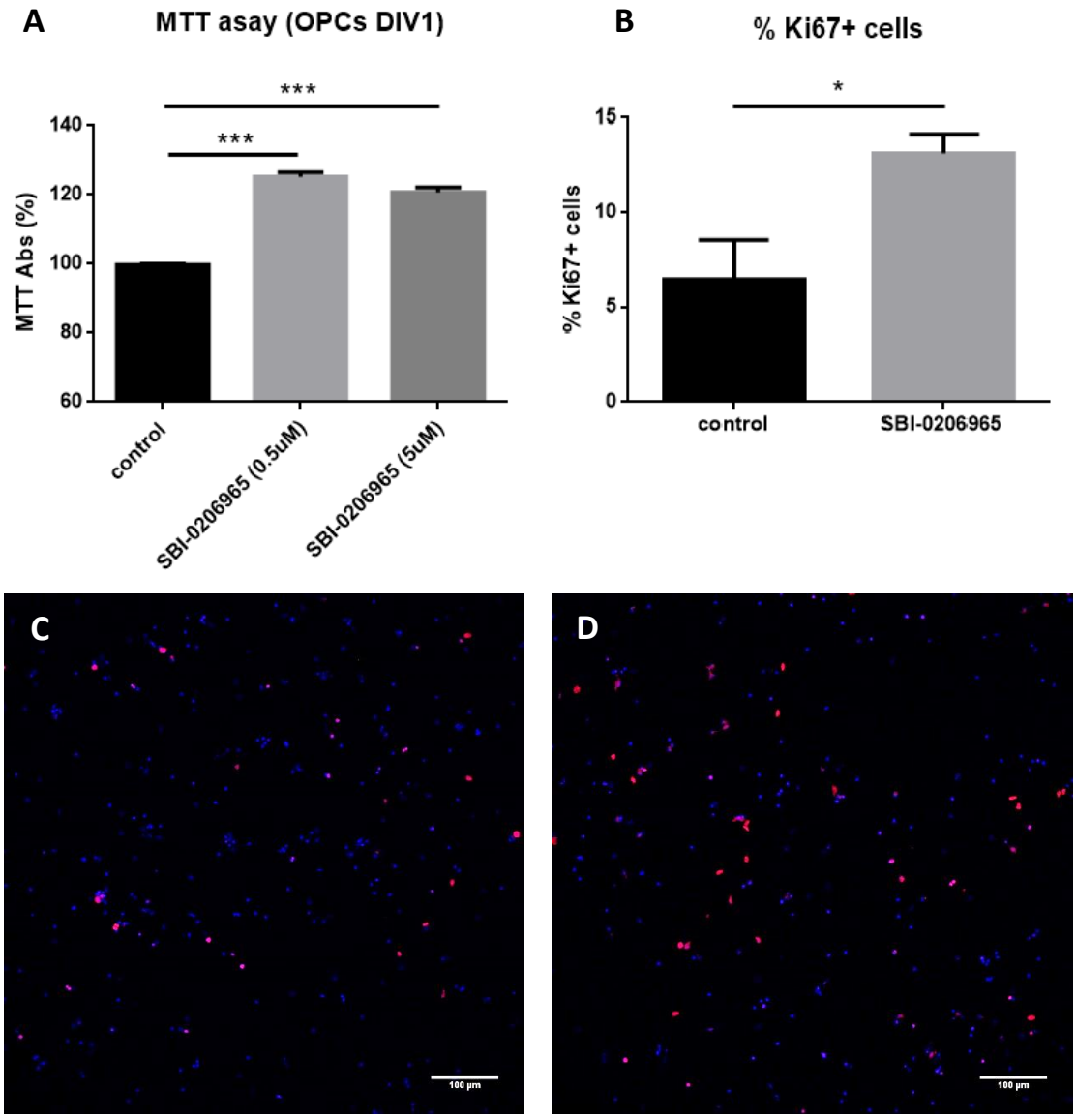


FIGURE 11. SBI-0206965 treatment increases proliferation of OPC.

(A) Quantification of cell viability using the MTT assay. Data expressed as an absorbance ratio to the control (untreated) OPC cultures. OPC incubated with SBI-0206965 for 24 h show an increase in the amount of formazan production, which correlates with the number of OPCs present in each well using the MTT assay. This increase of cell viability is independent

of the dose of SBI-0206965 (0.5 μM & 5 μM). Data are means ± SEM. Unpaired t-test *p< 0.05, **p< 0.01, n =3.
(B) Quantification of the number of cells found positive for Ki67. Data are means ± SEM. Unpaired t-test *p< 0.05, n =3.

(C - D) Representative immunocytochemistry of oligodendrocyte primary cultures showing proliferating OPCs (nuclear staining of Ki67+ cells, in red), in control cultures and in cultures after SBI-0206965 addition (0.5 μM). Nuclei are stained with DAPI (blue). Scale bars, 100 μm.

Autophagy is active in OLs

Subsequently, we asked whether autophagy is active in mature OLs as well. In order to examine this hypothesis we performed western blot analysis from oligodendrocytes DIV1 protein lysate for LC3 protein. As above mentioned, LC3 is conjugated to PE to form LC3-II, which is localized to isolation membranes and autophagosomes on both outer and inner membranes (Kabeya et al., 2000). LC3 is currently the most widely used autophagosome marker because the amount of LC3-II reflects the number of autophagosomes and autophagy-related structures (Zhang et al., 2013). In order to determine the lysosome-dependent degradation, a potent V-ATPase inhibitor, e.g. bafilomycin A_1, is commonly used. The difference in the amount of LC3-II between samples with and without this inhibitor represents the level of autophagic flux. Autophagic flux in normal cells is estimated by an increase in the LC3-II amount under bafilomycin A_1 treatment (Yoshii and Mizushima, 2017). Therefore, according to the elevated levels in LCE-II signal in our cells after the administration of bafilomycin A_1 (Figure 12), we can accept that autophagy is active in mature oligodendrocytes, under normal conditions.

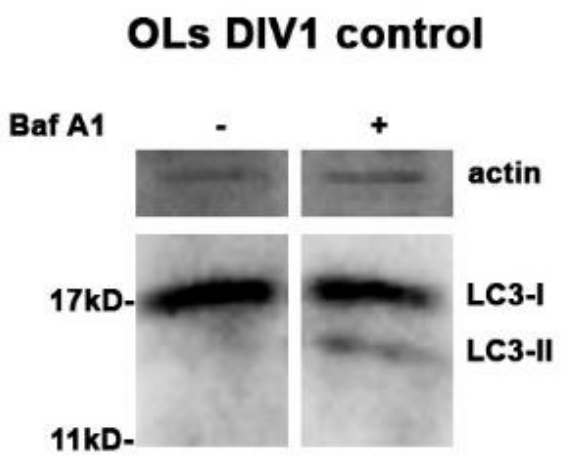


FIGURE 12. Autophagy is active in OLs.

Lysates of control OLs DIV1 treated with Bafilomycin A_1 for 6 h, show an increase in LC3-II levels in the presence of Bafilomycin A_1 on a representative Western blot, proving the existence of an active autophagic machinery in these cells.

Inhibition of autophagy in OLs results in altered morphology

To further investigate the impact of inhibition of autophagy in OLs, we tried to analyze the morphology of mature oligodendrocytes in case of active or inhibited autophagy. Therefore, we performed immunocytochemical analysis for MBP in mature DIV4 OLs and we investigated if there is a different morphology among four different conditions we established:

1. Condition 1: control (normal proliferation & differentiation medium)
2. Condition 2: Administration of SBI-0206965 in the proliferation medium
3. Condition 3: Administration of SBI-0206965 in the differentiation medium
4. Condition 4: Administration of SBI-0206965 in both the proliferation & differentiation medium.

Firstly, we analyzed the morphology of control cells, which were cultured in normal proliferation and differentiation medium. In this case, we noticed a beautiful myelin

sheath, spreading like a veil around the cell body of the oligodendrocyte. There are not many MBP+ fragments and the myelin sheath seems to cover an extensive area around the cell body.

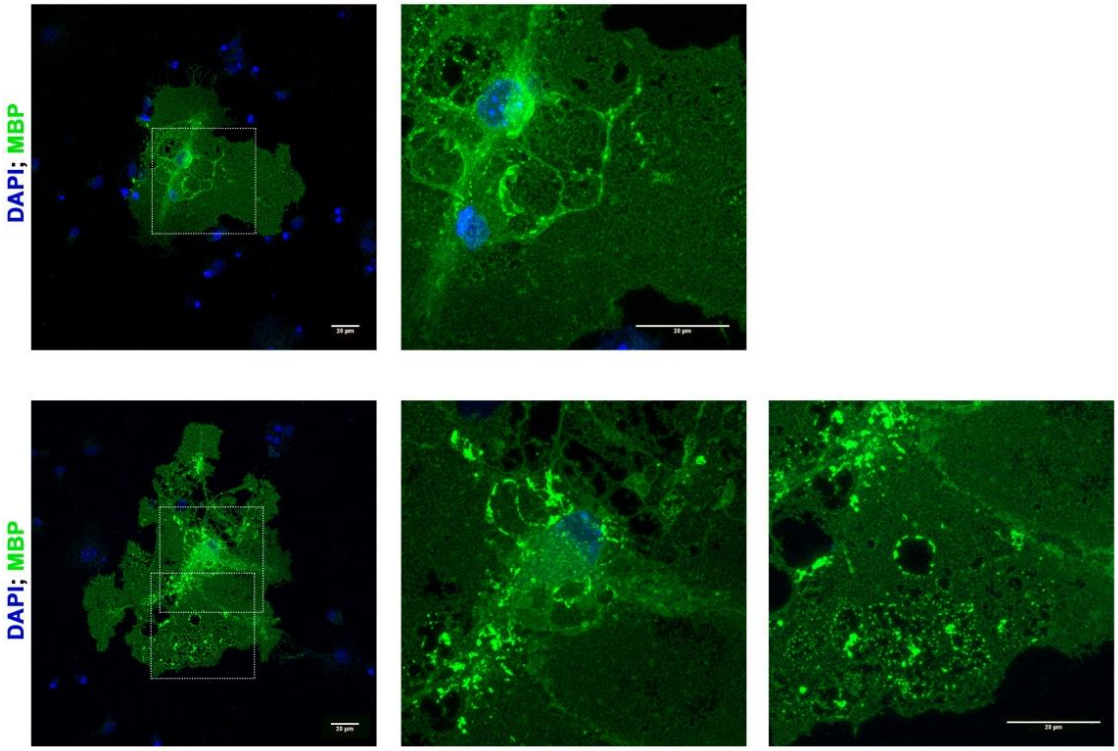


FIGURE 13. Immunocytochemical analysis of control OLs (DIV4), cultured in normal proliferation and differentiation medium.

MBP (green) and DAPI (blue), double immunolabeling. OLs exhibit the "classic" morphology of a mature oligodendrocyte, with a myelin sheath surrounding its cell body. Higher magnification confocal view of the areas marked in white squares. Scale bars, 20 μm.

Next, in case of administration of SBI-0206965 only in the Proliferation medium (2nd condition), we observed morphological alterations. More specifically, we noticed many myelin fragments, especially over and around the cellular body, as well as many long thin myelin processes that do not form a myelin sheath around them (Figure 14A).

When we tried to block autophagy only in the differentiation phase (3rd condition, Figure 14B), we saw a similar morphology compared to the 2nd condition, with the only difference that the MBP+ fragments seem to expand also in the periphery.

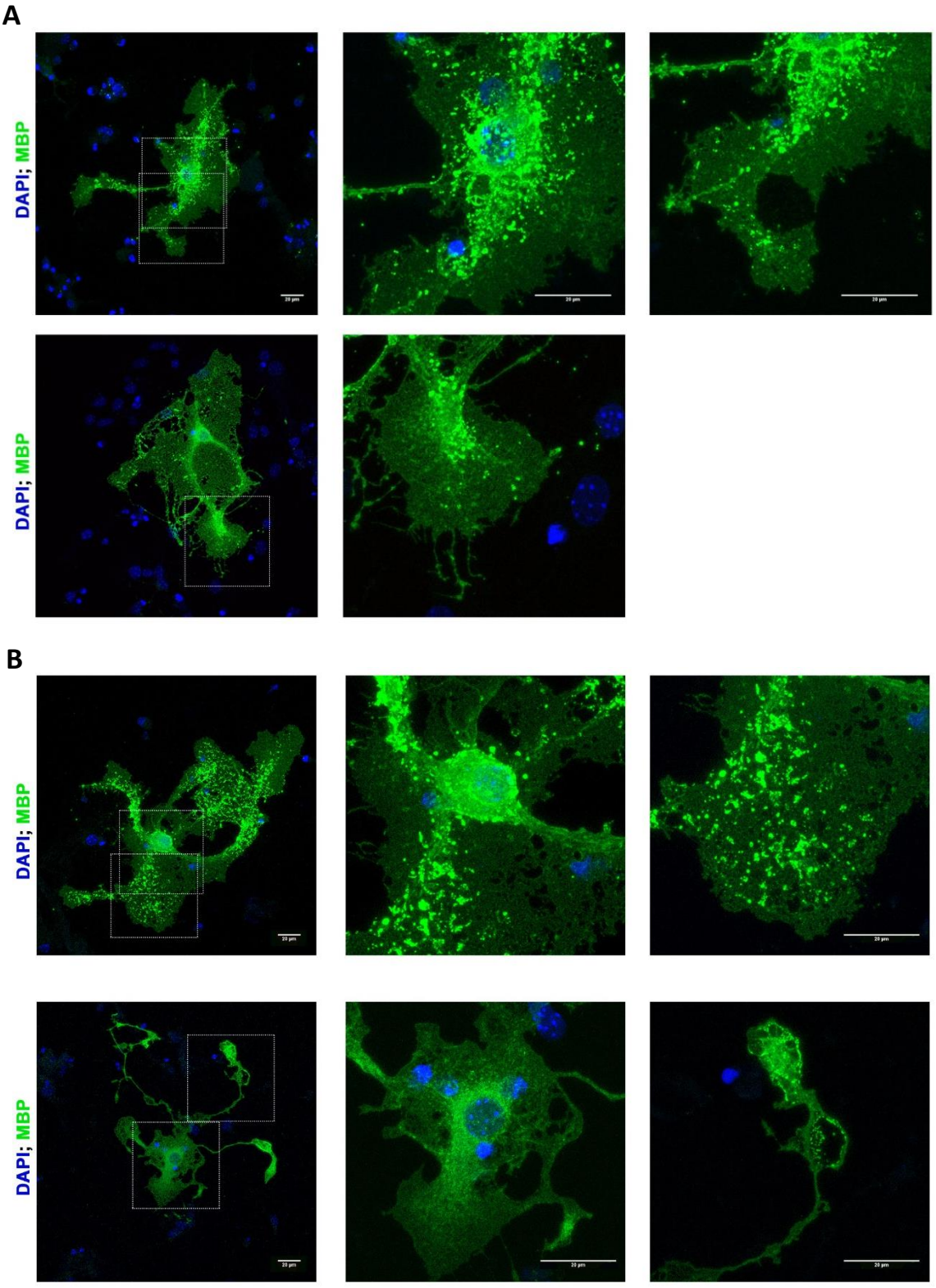


FIGURE 14. SBI-0206965 administration in either the proliferation or the differentiation phase of a developing OL leads to morphological alterations.

MBP (green) and DAPI (blue), double immunolabeling. SBI-0206965 is added in either the proliferation **(A)** or the differentiation medium **(B)**. We notice, many myelin fragments, especially over and around the cellular body, but also in the area of myelin sheath. Also, there are many long thin myelin processes that do not form a myelin sheath around them.

Higher magnification confocal view of the areas marked in white squares. Scale bars, 20 μm.

Finally, when we added SBI-0206965 throughout the culture (both in proliferation and differentiation medium) we observed that there are severe morphological defects, compared to the control. More specifically, we can see myelin fragments, and a plethora of very thin processes that do not form myelin sheaths. Moreover, the surface that the myelin sheath should cover, seems extremely limited in the area that it extents (Figure 15).

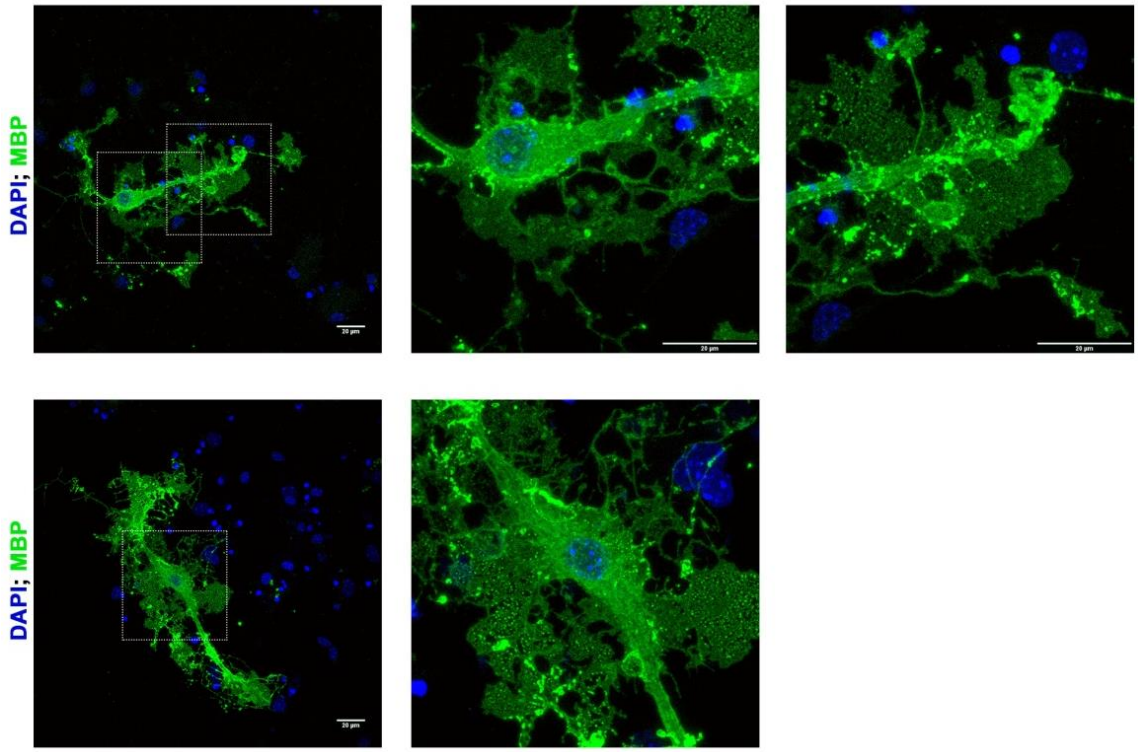


FIGURE 15. SBI-0206965 administration throughout the culture leads to severe morphological defects.

Administration of SBI-0206965 from the beginning to the end of the culture leads to the formation of a tiny myelin sheath, MBP+ fragments and a plethora of thin processes that seem unable to form a myelin veil around them.

Higher magnification confocal view of the areas marked in white squares. Scale bars, 20 μm.

Collectively, these data prove that inhibition of autophagy in proliferating and mature oligodendrocytes leads to severe morphological defects, with most important alterations noticed when SBI-0206965 is administered throughout the culture, in both proliferating and differentiating phases.

Inhibition of autophagy seems to delay the maturation of OLs and severely block their terminal differentiation

In order to explain the morphological differences detected, we wondered whether the inhibition of autophagy could also lead to changes in the maturation of oligodendrocytes. Towards testing this hypothesis, we studied mature oligodendrocytes at three different time points, and more specifically at DIV2, DIV4 and DIV8. Mature oligodendrocytes are segregated in three morphological categories/ types according to their differentiation stage (category A, B, C) of that can be identified according to previously published work (Lourenço et al., 2016). More analytically: category A includes cells with low ramification branching (Figure 16A), category B displays increasing levels of process outgrowth and branching without myelin membranes (Figure 16B), whereas category C (fully myelinated) cells exhibit incremented levels of complexity and myelin membranes (Figure 16C). Thus, in order to investigate the role of SBI in the later stages of oligodendrocyte development and maturation, we classified MBP+ OLs in one of the three categories that reflected increasing branching complexity and myelin membrane sheet formation.

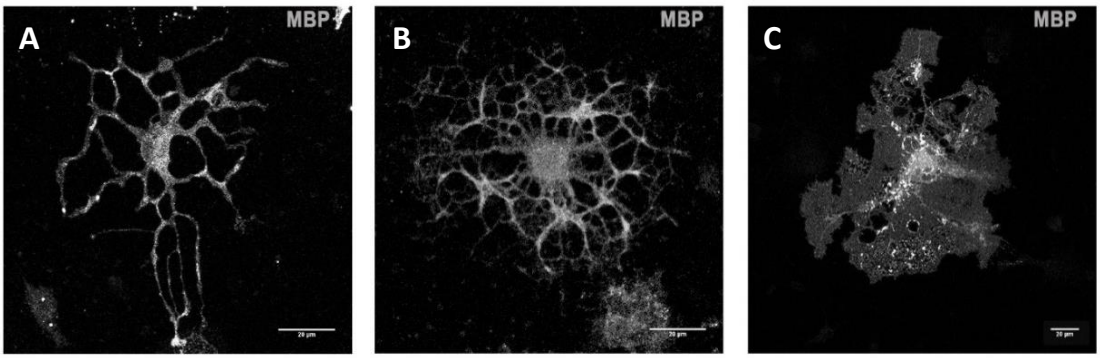


FIGURE 16. Representative examples of MBP-expressing OLs.

(A) category A shows low ramification branching,

(B) category B displays increasing levels of process outgrowth and branching without myelin membranes,

(C) whereas type C exhibits incremented levels of complexity and myelin membranes. Scale bars, 20 µm.

In this experiment, OPCs were cultured for two days in normal proliferation medium and SBI was administered only in differentiation medium. Firstly, we studied DIV2 mature oligodendrocytes, which showed no differences in the number of cells per category between the control and the SBI-treated group (Figure 17).

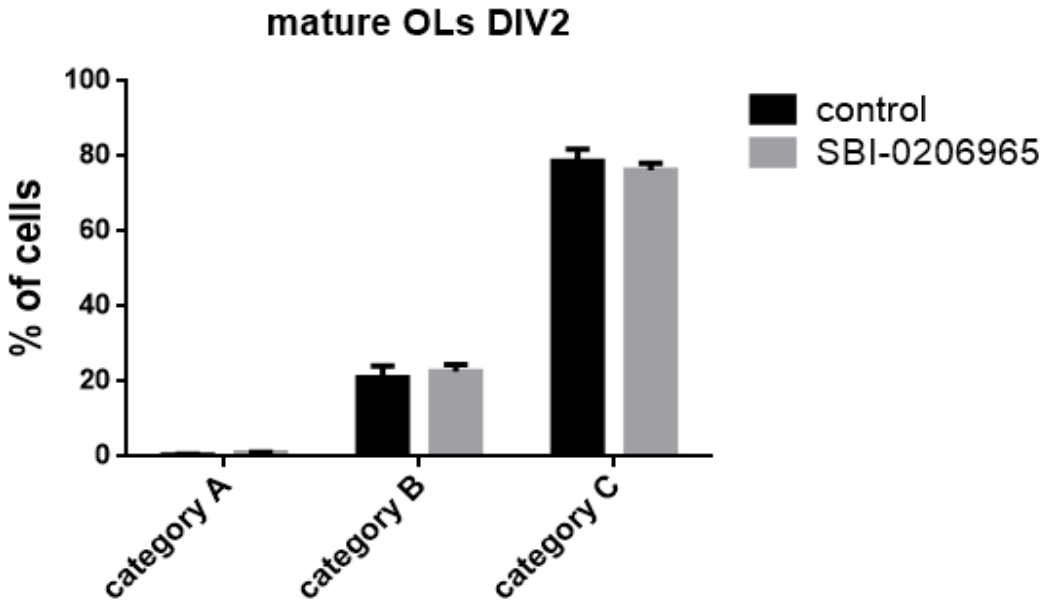


FIGURE 17. Effect of SBI-0206965 in development of OL primary cultures.

Quantification of OL complexity in OL cultures, after the addition of SBI-0206965: the percentage of cells within each category is shown for control DIV2. No statistically significant difference was detected (Two-way ANOVA, n=3).

However, when we examined DIV4 oligodendrocytes we noticed that SBI administration increased the number of category A OLs and decreased the number of category C OLs, compared to control (Figure 18). Thus, we can presume that inhibition of autophagy causes a delay in the maturation of OLs.

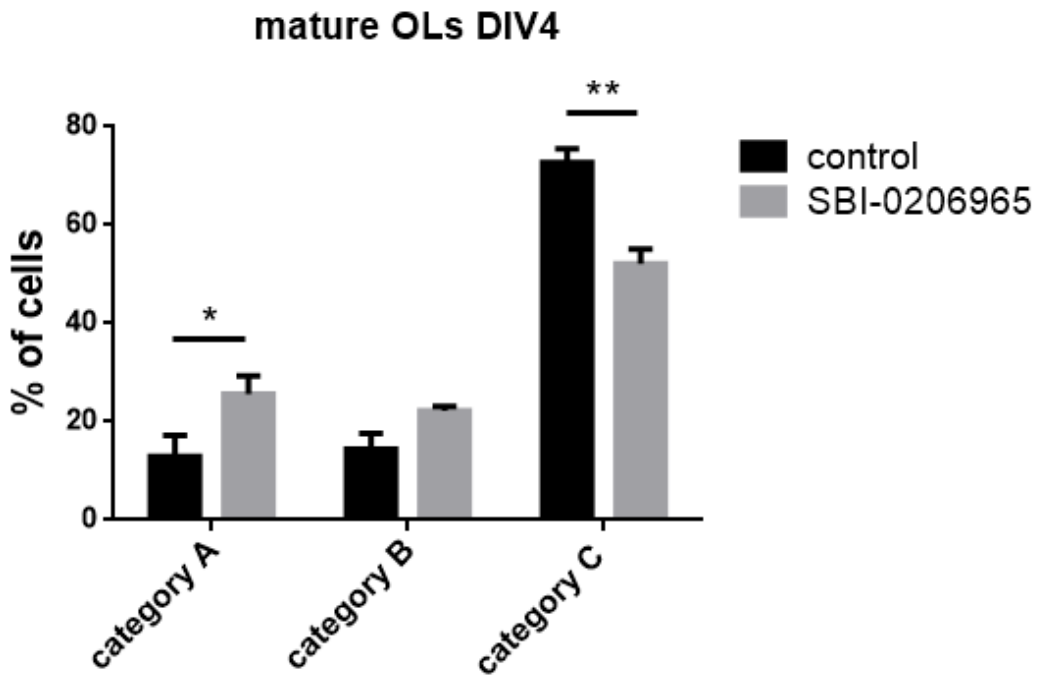


FIGURE 18. SBI-0206965 seems to cause delay in maturation of OLs.

SBI administration increased the number of category A OLs and decreased the number of category C OLs, compared to control.

For the quantification of OLs complexity, the Two-way ANOVA was used. Comparison with control group: * $p < 0,05$; ** $p < 0,01$; n=3.

Finally, when studying DIV8 oligodendrocytes, no numerical differences among MBP+ OLs in the three categories between control and SBI-treated groups were observed (Figure 19).

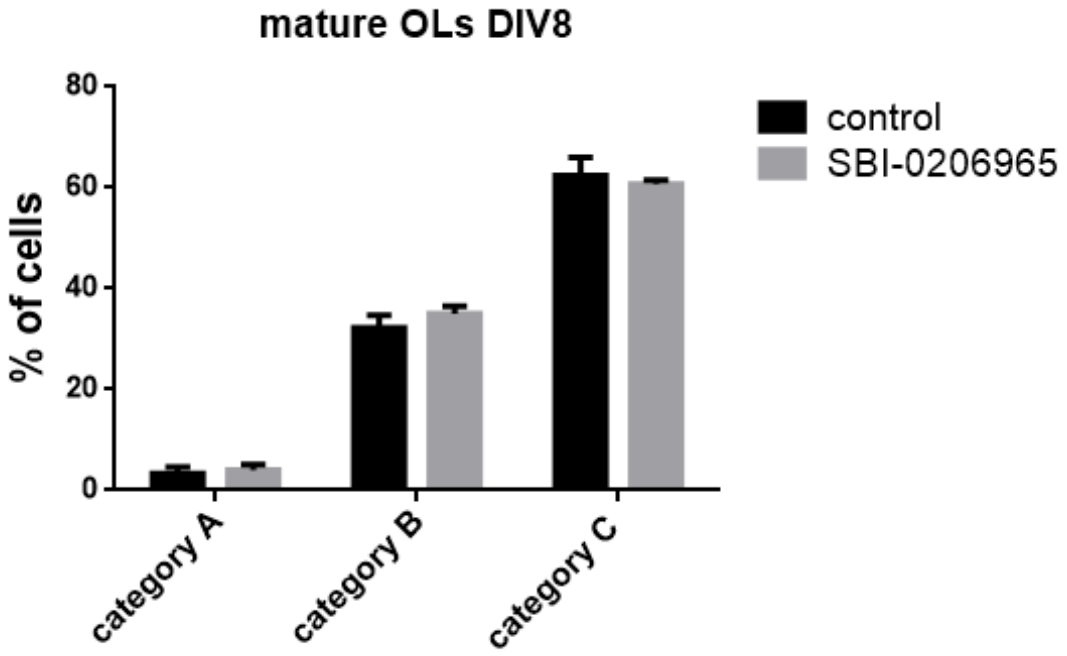


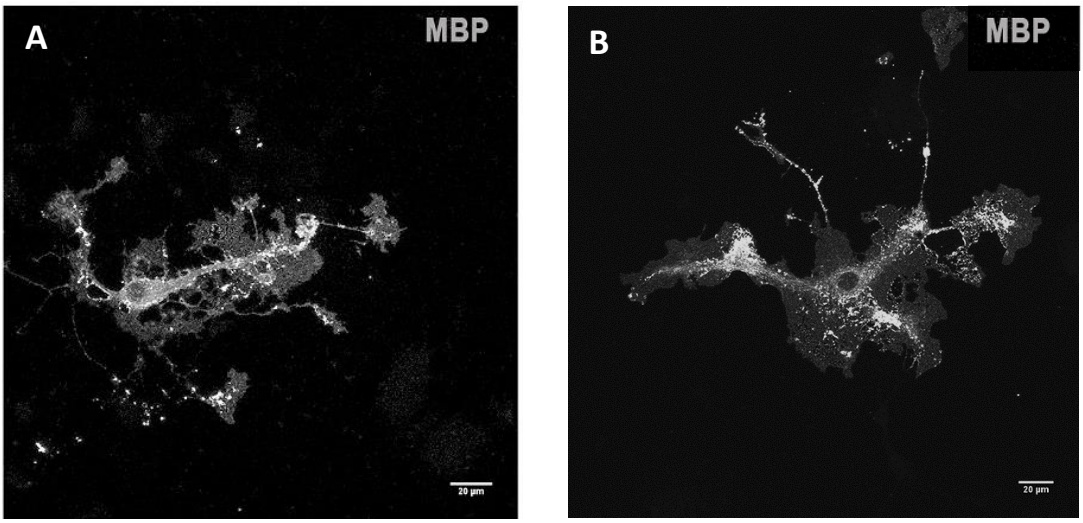
FIGURE 19. Effect of SBI-0206965 in maturation of DIV8 OLs.

Quantification of OL complexity in OL cultures, after the addition of SBI-0206965: the percentage of cells within each category is shown for control DIV8. No statistically significant difference was detected (Two-way ANOVA, n=3).

However, we noticed that although there is no difference in the number of cells at DIV8 cultures, SBI- treated category C cells showed a defective morphology, as described also in the previous section. In the literature, there are no specific criteria established in order to characterize an oligodendrocyte as morphologically defective. Therefore, we decided to establish a list of criteria to be met in order to characterize an OL as morphologically defective, according to the common morphological traits the majority of SBI-treated cells exhibited. Thus, in order to characterize an OL as "abnormal" it should:

1. have a ratio of MBP signal in myelin sheath/ MBP signal in the cell body ≤ 1, meaning that the myelin sheath should occupy less surface than the cell body,
2. and also to show increased number of long and thin myelin processes (more than two).

If an OL met these two criteria, it was considered defective (Figure 20A-B). Extensively fragmented myelin sheath or very intense MBP fragments in cell body were considered as additional confirmatory data for the categorization. Thus, after careful observation of the DIV8 OLs we concluded that, although the total number of category C cells between control and SBI-treated was indeed the same, the number of category C cells that were morphological defective was more than tripled in the case of SBI treated OLs (Figure 20C).



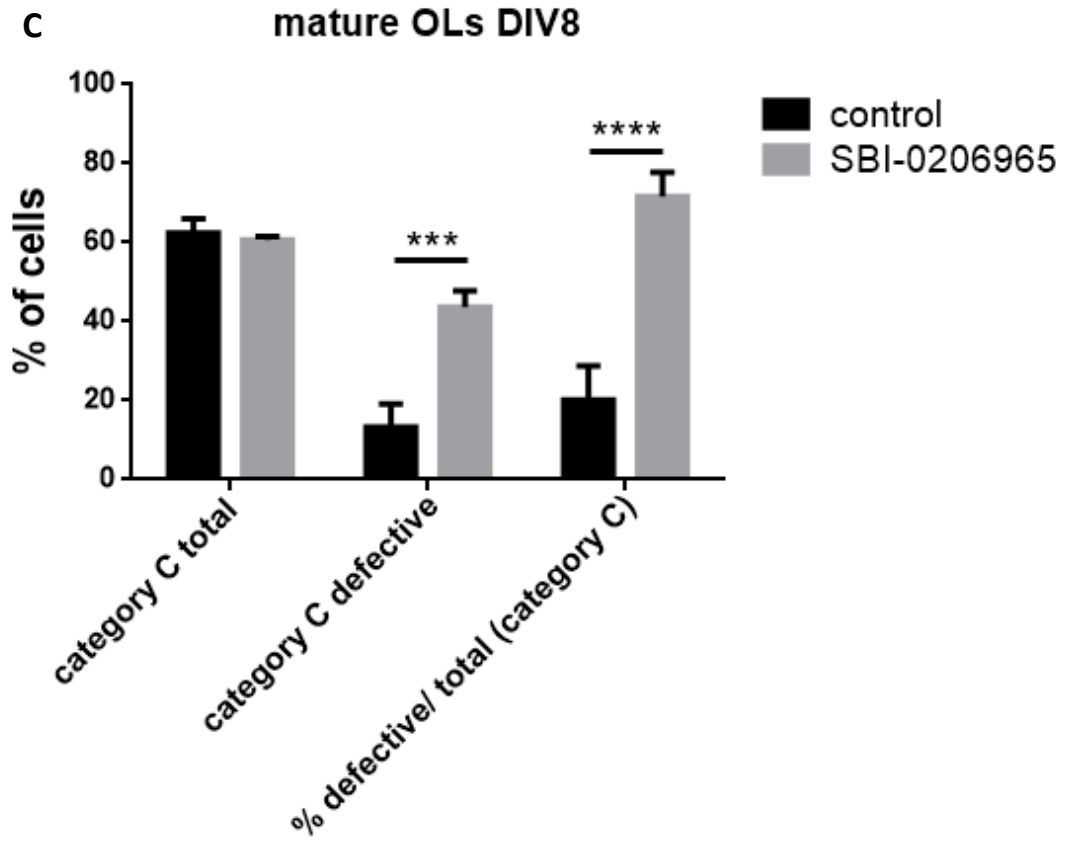


FIGURE 20. SBI-0206965 administration causes severe morphological defects in category C DIV8 OLs, without altering their total number.

(A - B) Representative examples of cells defined as morphological defective. Development of a tiny myelin sheath with long thin processes at its ends and usually with appearance of intense MBP fragments. Scale bars, 20 μm.

(C) although the total number of category C cells between control and SBI-treated is the same, the number of category C cells that were morphological defective was about treble in the case of SBI treated OLs.

Two-way ANOVA test was used. Comparison of SBI-treated with control group: ***$p \leq 0.001$; ****$p \leq 0.0001$; n=3.

Inhibition of autophagy in OLs does not affect their differentiation index

Subsequently, we tested whether there was any change in the differentiation index (% of MBP+ cells/ total no of cells) between control and SBI-treated OLs, meaning whether there were more cells differentiated to mature oligodendrocytes (MBP+

cells) in any of the two groups. A higher value of the differentiation index indicates more advanced differentiation of OPCs to mature oligodendrocytes. We manually and blindly counted the number of MBP+ cells in 3 coverslips per condition as well as the total number of cells plated per coverslip with the help of the Operetta High-Content Imaging System in each group. We found no statistically significant difference between control and SBI-treated groups, suggesting that the inhibition of autophagy does not alter the differentiation index of OLs. The total number of cells did not significantly differ between groups (Figure 21B).

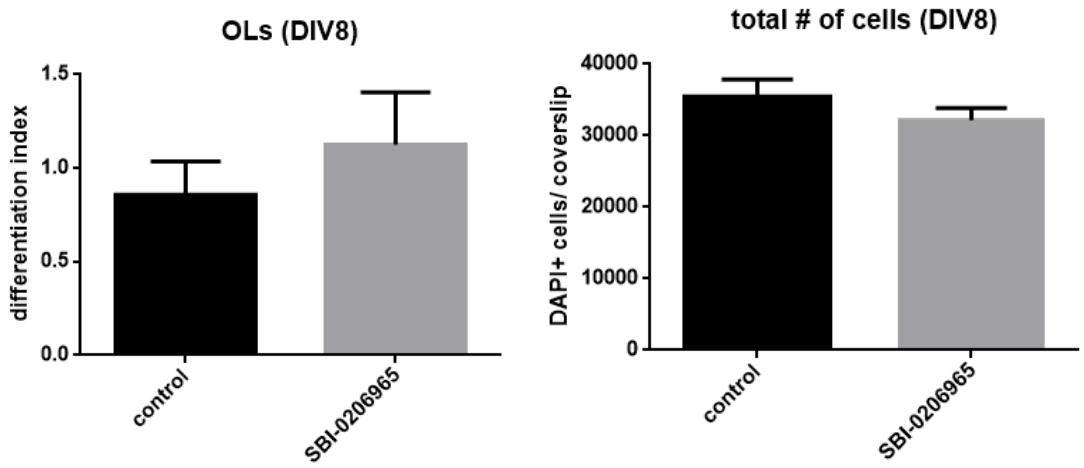


FIGURE 21. SBI-0206965 does not alter differentiation index or the total number of cells in OL cultures.

(A) To detect whether there was a variation in the number of OLs upon SBI-0206965 treatment, we analyzed the differentiation index between control and SBI-treated groups. There was no statistically significant difference.
(B) The total number of cells plated was also the same between the two groups.
(Unpaired t-test).

Conclusions

The main findings of this study are:

- ✓ Autophagy is active in both OPCs and OLs.
- ✓ Pharmacological inhibition of autophagy with SBI-0206965 leads to translocation of p62 to the nucleus of OPCs.
- ✓ SBI-0206965 seems to increase proliferation of OPCs.
- ✓ Inhibition of autophagy in OLs results in morphological defects without affecting the differentiation index.
- ✓ Inhibition of autophagy seems to delay the maturation of OLs and severely block their terminal differentiation.
- ✓ It is suggested that there is a different mechanism of autophagy regulation between OPCs and OLs.

DISCUSSION

Autophagy comprises a major degradation mechanism, which engulfs, removes, and recycles unwanted cytoplasmic material including damaged organelles and toxic protein aggregates. Through its role in energy production and cellular homeostasis, autophagy is crucial during development in many tissues and organisms, and its dysregulation has been linked to several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and most recently in multiple sclerosis as well. Although many studies the last years have documented the mechanisms of autophagy in neurons, the role of autophagy in glia - and especially in oligodendrocytes- remains poorly characterized. Thus, aim of the present study was to shed light on the significance of autophagy in myelin homeostasis in the CNS, focusing on both oligodendrocyte precursor cells and mature oligodendrocytes.

Autophagy is active in OPCs and its inhibition appears to lead in increased proliferation

Oligodendroglial precursor cells (OPCs) are considered the main source for generating mature oligodendrocytes and myelin during development. It is- also- of high interest that there exists a sizeable population of these cells that remain undifferentiated after completion of myelination. In recent years, adult OPCs have generated much interest as a reservoir of cells with the potential to self-renew, differentiate, and remyelinate the CNS (Rivers et al., 2008). In the literature, references as to the role of autophagy in these cells are scarce. Therefore, our first aim was to investigate the role that this catabolic process plays in OPCs.

Immunocytochemical studies that we performed revealed not only that autophagy is indeed active in these progenitor cells, but also that inhibition of autophagy leads to translocation of p62 to their nucleus. Finally, using proliferation assays, we showed that administration of the autophagy inhibitor SBI-0206965 increases the proliferation of OPCs. This increased proliferation may be correlated to the

translocation of p62 to the nucleus, but a series of additional experiments in order to substantiate this connection confidently are needed. Moreover, cell death of these cells should be determined by TUNEL assay or propidium iodine staining, in order to elucidate whether inhibition of autophagy plays a role in cell death as well.

Autophagy is active in OLs and its inhibition results in morphological defects

Our next question was whether autophagy is also active in mature oligodendrocytes. To this end, we performed immunoblot analysis for LC3 in wild type mature OLs under normal medium or treatment with Bafilomycin A_1, a selective inhibitor for autophagosome-lysosome fusion. As mentioned above, LC3-II reflects the number of autophagosomes and autophagy-related structures (Zhang et al., 2013). Therefore, the elevated levels in LCE-II signal in our cells after the administration of Bafilomycin A_1, propose that autophagy is active in mature oligodendrocytes, under normal conditions.

Subsequently, to further investigate the role of autophagy in OLs *in vitro*, we tried to analyze the morphology of mature oligodendrocytes in case of active or inhibited autophagy. For this reason, we performed immunocytochemical analysis of myelin basic protein (MBP). As already mentioned, the myelin membrane has a highly specialized molecular composition, consisting of a high content of lipids and a small number of proteins, with MBP being one of the most abundant (Jahn et al., 2009). MBP is essential for normal myelination and its multiple functions suggest that a tight temporal and spatial regulation of expression is required for normal myelination. Our results propose that administration of SBI-0206965 at different maturation stages causes morphological defects in OLs. More specifically, we can see MBP+ fragments, especially over and around the cellular body, as well as many long thin myelin processes that do not form a myelin sheath around them in case of inhibition of autophagy only during the proliferation phase. When inhibiting autophagy only during differentiation phase we see a similar phenotype with the difference that MBP+ fragments seem to be scattered throughout the cell body and

the periphery. Finally, in case of inhibition of autophagy from the beginning to the end of the culture, the phenotype seems even worse, with the existence of many MBP+ fragments, and a plethora of very thin processes that do not form myelin sheath around them (Figure 22).

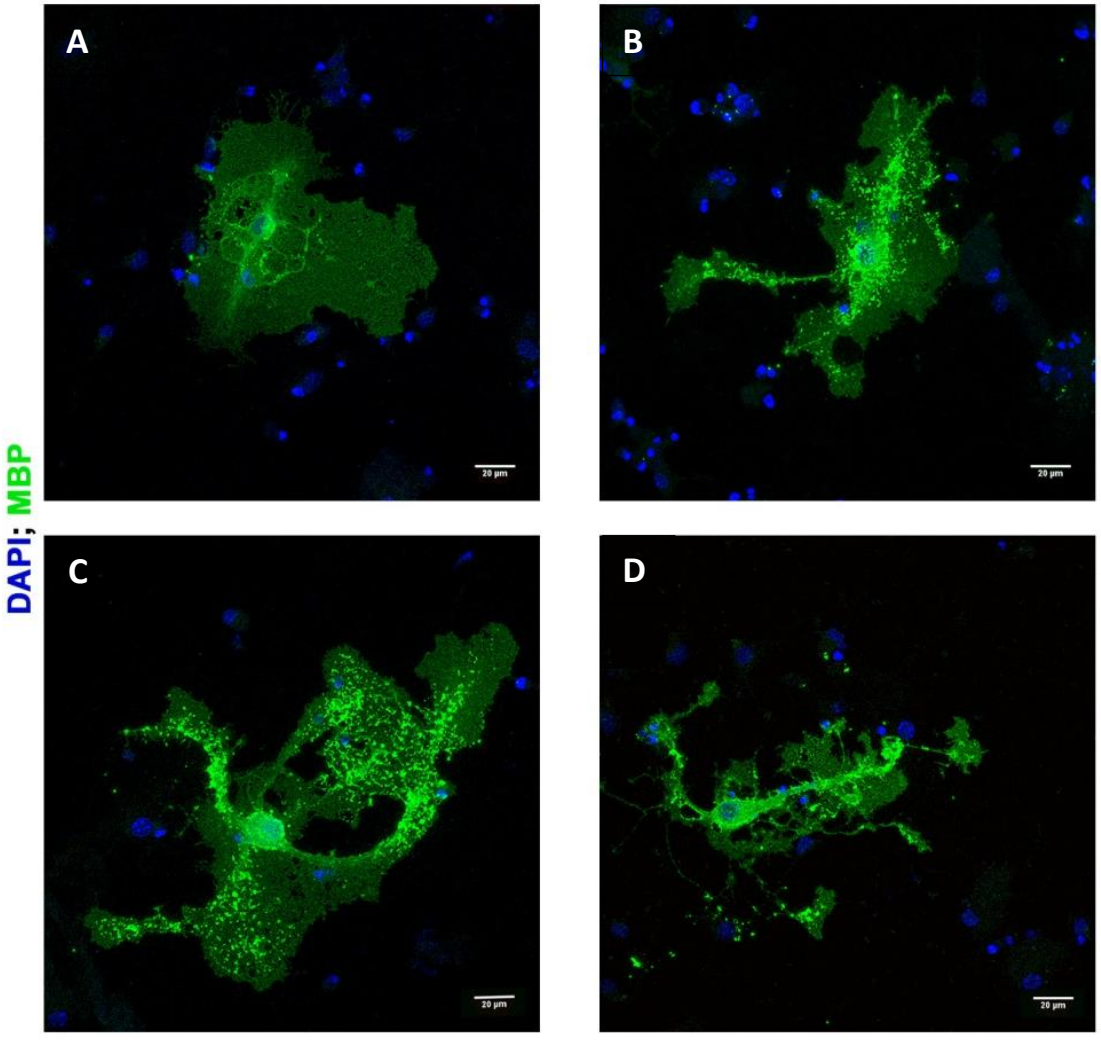


FIGURE 22. Representative examples of the morphological alterations in OLs among the four different conditions we tested.

- **(A)** Condition 1: control (normal proliferation & differentiation medium)
- **(B)** Condition 2: Administration of SBI-0206965 in proliferation medium
- **(C)** Condition 3: Administration of SBI-0206965 in differentiation medium
- **(D)** Condition 4: Administration of SBI-0206965 throughout the culture.

MBP (green) and DAPI (blue), double immunolabeling. Scale bars, 20 μm.

As already mentioned, and as shown in the summary figure (Figure 22), we can notice the morphological deficits in OLs in all cases of pharmacological inhibition of autophagy. This notice leads us to the conclusion that autophagy plays a vital role throughout the oligodendrocyte development, not only during the differentiation stage. Furthermore, it would be very useful to proceed with co-cultures of oligodendrocytes with neurons to figure out whether there is also a functional effect on the neurons when autophagy is ablated in oligodendrocytes.

Moreover, focusing on the MBP signal we observe that there are many MBP+ fragments in cases of inhibition of autophagy. MBP is targeted to the membrane sheath by mRNA transport (Ainger et al., 1993, 1997) and local translation (Colman et al., 1982), which ensures that its expression is temporally and spatially restricted. It is suggested that a tight temporal and spatial regulation of expression is required for normal myelination. Moreover, *in vitro* studies have started to delineate the molecular mechanisms involved in controlling the different steps of mRNA transport and local translation (Müller et al., 2013). The fact that we show is that MBP is expressed, but seems to be "trapped" around the cell body when blocking autophagy. We do not know yet whether less MBP protein is produced. Therefore, we have two hypotheses: 1. that autophagy plays a role in the trafficking of MBP or 2. that autophagy is essential for the production of MBP of proper quality and quantity.

For the investigation of the first hypothesis, we will recruit neuron-OL coculture system and block autophagy at the various stages, including the fusion with the lysosome. If blocking the last step of the fusion in OL single or co-cultures do not affect MBP trafficking, it means that autophagy does not play –or at least does not only play- a role in oligodendrocytes as a catabolic procedure responsible for degradation, but mainly as a mechanism indispensable for trafficking. For the investigation of the 2nd hypothesis we realized that we should look for a connecting link between macroautophagy and MBP. Interestingly, we recognized one transcription factor, called myelin gene expression factor-2 (Myef-2) that belongs to the LIR motif-containing proteins (iLIR autophagy database https://ilir.warwick.ac.uk/) and is known to repress the expression of MBP (Haas et

al., 1995). Myef-2 is up-regulated in mouse brains before the onset of myelination and regulates MBP expression by OLs, while the underlying mechanism is unknown (Haas et al., 1995). We hypothesize that Myef-2 might be targeted by the autophagy-lysosome pathway via its LIR motif, allowing for the expression of myelin proteins and the progression of myelination. As a result, it is possible that Myef-2 is the missing key that connects autophagy with myelin homeostasis.

Therefore, future plans of the project include the study of the role of autophagy in Myef-2. To accomplish this, we could perform both biochemical and immunocytochemical experiments in OPC cultures, in order to investigate whether there is an alteration in the expression of the specific protein in case of pharmacological inhibition of autophagy.

Inhibition of autophagy seems to delay the maturation of OLs without affecting their differentiation index.

In addition to the above and in an effort to find out whether autophagy plays a role in the maturation of OLs, we placed mature OLs expressing MBP in three different categories (categories A, B, C) that reflect increasing branching complexity and myelin membrane sheet formation. This categorization was done at three different time points (DIV2, DIV4, DIV8) and data between control and SBI-treated groups were compared. In DIV2 OLs there was no statistically significant difference detected between control and SBI-treated cells. This could possibly be because cells of both groups were initially cultured in normal proliferation medium, in which they could be synchronized. However, in DIV4 OLs we noticed that there is an increase in the number of category A cells and a decrease in the number of category C. Together these data support that autophagy impacts maturation of OLs, and more specifically delays their maturation since we see more cells of category A and fewer cells of category C in SBI-treated OLs compared to the control. However, what seems surprising to us is the fact that this delay in maturation does not seem to exist in DIV8 as well, possibly showing once more that autophagy is a really dynamic process

that may play distinct roles during development and maturation of oligodendrocytes. Nevertheless, the DIV8 OLs treated with SBI-0206965 did not exhibit a delay in their maturation, but as might be expected from the morphological studies that preceded, they exhibited morphologic defects.

Finally, there was no difference detected in the value of the differentiation index between control and SBI-treated OLs, meaning the total number of OPCs that differentiated to mature oligodendrocytes (MBP+ cells) was statistically the same between the two groups at DIV 8 OLs.

There might be a different mechanism of autophagic regulation between OPCs and OLs.

From the data that we have, we can say with confidence that autophagy is active in both OPCs and mature OLS. However, the role that this catabolic procedure plays in these cellular categories seems complicated. Inhibition of autophagy leads to increased proliferation, which initially led us to believe that autophagy could play a dual role during myelin development and that downregulation of autophagy could be "beneficial" for OPCs. However, the morphological defects that we observe in mature oligodendrocytes, even when autophagy is inhibited exclusively during the proliferation phase, shows that autophagy plays a crucial and probably much more complex role, than we first assumed, in this cell population.

Our second hypothesis is that the role of autophagy is indeed dynamic during oligodendrocyte development. In other words, we assume there could exist a developmental variation in autophagy, meaning it could be less "active" in progenitors and more in mature oligodendrocytes. If the role of autophagy is indeed dynamic during oligodendrocyte development, the regulation of this switch from low levels to elevated levels has not been elucidated and needs to be explored for a complete understanding of this process. In order to determine if assumption is true, we should initially measure and compare the autophagic flux of these cells.

However, it will also be necessary to further refine our mouse models. In particular, we will need to genetically modulate autophagy, not only in a spatial and cell type-specific fashion (meaning only to oligodendrocytes), but also temporally (at particular stages of development). Such models are possible with currently available tools; conditional mutants where autophagy is ablated specifically in OLs will be generated by the crossing of *plp*-CreERT (The Jackson Laboratory, stock No: 005975) with *Atg5 fl/fl* mice and tamoxifen administration in lactating mice (*plp-CreERT; Atg5 fl/fl*). Initially, *in vitro* analysis of OPCs and OLs should be performed in order to study differentiation, maturation, and morphology of these cells.

Thinking more longer term, myelination can be analyzed with morphological, biochemical, transcriptional and immunohistochemical experiments. Thanks to our genetic tools, autophagy can be ablated in the oligodendrocyte lineage in different developmental stages. Thus, three different developmental stages will be evaluated: a. the initiation of myelination (2 weeks of age, 2wo), b. the peak of myelination (3 weeks of age, 3wo) and c. a stage when myelination is complete (8 weeks of age, 8wo). Lastly, the delineation of the autophagic cargo in oligodendrocytes will allow for a greater understanding of how this process is integrated into the metabolism of the cell.

REFERENCES

Ainger, K., Avossa, D., Diana, A. S., Barry, C., Barbarese, E., and Carson, J. H. (1997). Transport and localization elements in myelin basic protein mRNA. J. Cell Biol. 138, 1077–1087.

Ainger, K., Avossa, D., Morgan, F., Hill, S. J., Barry, C., Barbarese, E., et al. (1993). Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. J. Cell Biol. 123, 431–441.

Axe, E. L., Walker, S. A., Manifava, M. & Chandra, P. (2008). Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J. Cell Biol. 182, 685–701.

Baba, M., Takeshige, K., Baba, N., Ohsumi, Y. (1994). Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization. J. Cell. Biol. 124(6), 903- 913.

Bauer, N.G., Richter-Landsberg, C., Ffrench-Constant, C. (2009). Role of the oligodendroglial cytoskeleton in differentiation and myelination. Glia 57, 1691-1705.

Bhattacharya, A., Parillon, X., Zeng, S., Han, S., Eissa, N.T. (2014). Deficiency of autophagy in dendritic cells protects against experimental autoimmune encephalomyelitis. J. Biol. Chem. 289(38), 26525-32.

Bruno, S., Darzynkiewicz, Z. (1992). Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. Cell Proliferation. 25 (1), 31–40.

Chang, A. et al. (2000). NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. J. Neurosci. 20, 6404–6412.

Colman, D. R., Kreibich, G., Frey, A. B., and Sabatini, D. D. (1982). Synthesis and incorporation of myelin polypeptides into CNS myelin. J. Cell Biol. 95, 598–608.

Crawford, A.H. et al. (2014). Oligodendrocyte progenitors: adult stem cells of the central nervous system? Exp. Neurol. 260, 50–55.

Cuervo, A. M. & Wong, E. (2014). Chaperone-mediated autophagy: roles in disease and aging. Cell Res. 24, 92–104.

Dikic, I., Elazar, Z. (2018). Mechanism and medical implications of mammalian autophagy. Nat. Rev. Mol. Cell Biol. 19(6), 349-364.

Fewster, M.E., Hirono, H., Mead, J.F. (1976). Lipid composition of myelin in multiple sclerosis. J. Neurol. 213, 119–131.

Gomez-Sanchez, J.A., Carty, L., Iruarrizaga-Lejarreta, M., Palomo-Irigoyen, M., Varela-Rey, M., Griffith, M., Hantke, J., Macias-Camara, N., Azkargorta, M., Aurrekoetxea, I., et al. (2015). Schwann cell autophagy, myelinophagy, initiates myelin clearance from injured nerves. J. Cell Biol. 210(1), 153–168.

Haas, S., Steplewski, A., Siracusa, L.D., Amini, S., Khalili, K. (1995). Identification of a sequence-specific single-stranded DNA binding protein that suppresses transcription of the mouse myelin basic protein gene. J. Biol. Chem. 270, 12503–12510.

Hara, T. et al. (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441, 885-889.

Hirata, K., Kawabuchi, M. (2002). Myelin phagocytosis by macrophages and nonmacrophages during wallerian degeneration. Microsc. Res. Tech. 57, 541–547.

Igci, M., Baysan, M., Yigiter, R., Ulasli, M., Geyik, S., Bayraktar, R., et al. (2016). Gene expression profiles of autophagy-related genes in multiple sclerosis. Gene 588(1), 38–46.

Inouye, H., Kirschner, D.A. (1988). Membrane interactions in nerve myelin. 1. Determination of surface-charge from effects of pH and ionic-strength on period. Biophys. J. 53, 235–245.

Inouye, H., Kirschner, D.A. (1988). Membrane interactions in nerve myelin. 2. Determination of surface-charge from biochemical data. Biophys. J. 53, 247–260.

Jahn, O., Tenzer, S., and Werner, H. B. (2009). Myelin proteomics: molecular anatomy of an insulating sheath. Mol. Neurobiol. 40, 55–72.

Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., Ohsumi, Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J. Cell Biol. 150(6), 1507-1513.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J., 19, 5720–5728.

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M., Richardson, W.D. (2006). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. Nat. Neurosci. 9, 173–179.

Kremer, D., Gottle, P., Hartung, H. P., Kury, P. (2016). Pushing forward: remyelination as the new frontier in CNS diseases. Trends Neurosci. 39, 246–263.

Komatsu, M., Kageyama, S., Ichimura, Y. (2012). p62/SQSTM1/A170: physiology and pathology. Pharmacol. Res. 66, 457–62.

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E., and Tanaka, K. (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature. 441, 880–884.

Komatsu, M., Waguri, S., Koike, M., Sou, Y.S., Ueno, T., Hara, T., Mizushima, N., Iwata, J., Ezaki, J., Murata, S., et al. (2007). Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 131, 1149–1163.

Lee, J.A. (2012). Neuronal autophagy: a housekeeper or a fighter in neuronal cell survival? Exp Neurobiol. 21, 1–8.

Li, W.-W., Li, J. & Bao, J.-K. (2012). Microautophagy: lesserknown self-eating. Cell. Mol. Life Sci. 69, 1125–1136.

Liang, P., Le, W. (2015). Role of autophagy in the pathogenesis of multiple sclerosis. Neurosci. Bull 31(4), 435–44.

McCarthy, K. D., de Vellis, J. (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. The Journal of Cell Biology 85, 890–902.

Menzies, F.M., Fleming, A., and Rubinsztein, D.C. (2015). Compromised autophagy and neurodegenerative diseases. Nat. Rev. Neurosci. 16, 345–357.

Menzies, F.M., Fleming, A., Caricasole, A., Bento, C.F., Andrews, S.P., Ashkenazi, A., Fullgrabe, J., Jackson, A., Jimenez Sanchez, M., Karabiyik, C., et al. (2017). Autophagy and neurodegeneration: pathogenic mechanisms and therapeutic opportunities. Neuron. 93, 1015–1034.

Mizushima, N., Levine, B., Cuervo, A. M. & Klionsky, D. J. (2008). Autophagy fights disease through cellular self-digestion. Nature 451, 1069–1075.

Mizushima, N., Komatsu, M. (2011). Autophagy: Renovation of cells and tissues. Cell 147, 728–741.

Müller, C., Bauer, N. M., Schafer, I., and White, R. (2013). Making myelin basic protein—from mRNA transport to localized translation. Front. Cell. Neurosci. 7, 169.

Lourenço, T., Paes de Faria, J., Bippes, C. A., Maia, J., Lopes-da-Silva, J. A., Relvas, J. B., & Graos, M. (2016). Modulation of oligodendrocyte differentiation and maturation by combined biochemical and mechanical cues. Science Reports, 6, 21563.

Nakayama, S., Karasawa, H., Suzuki, T., Yabuuchi, S., Takagi, K., Aizawa, T., et al. (2017). p62/sequestosome 1 in human colorectal carcinoma as a potent prognostic predictor associated with cell proliferation. Cancer Med. 6(6), 1264–74.

Nave, K.A. (2010). Myelination and support of axonal integrity by glia. Nature 468, 244–252.

Nave, K.A. and Werner, H.B. (2014). Myelination of the nervous system: mechanisms and functions. Annu. Rev. Cell Dev. Biol. 30, 503–533.

Nikoletopoulou, V., Papandreou, M.E., Tavernarakis, N. (2015). Autophagy in the physiology and pathology of the central nervous system. Cell Death Differ. 22, 398–407.

Nishiyama, A. et al. (2009.) Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. Nat. Rev. Neurosci. 10, 9–22.

Norton, W.T., Autilio, L.A. (1966). The lipid composition of purified bovine brain myelin. J. Neurochem. 13, 213–222.

O'Connor, L.T., Goetz, B.D., Kwiecien, J.M., Delaney, K.H., Fletch, A.L., Duncan, I.D. (1999). Insertion of a retrotransposon in Mbp disrupts mRNA splicing and myelination in a new mutant rat. J Neurosci. 19, 3404 –3413.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., et al. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J. Biol. Chem. 282, 24131–24145.

Paunovic, V., Petrovic, I.V., Milenkovic, M., Janjetovic, K., Pravica, V., Dujmovic, I., et al. (2018). Autophagy-independent increase of ATG5 expression in T cells of multiple sclerosis patients. J Neuroimmunol. 319, 100–5.

Podbielska, M., Banik, N.L., Kurowska, E., Hogan, E.L. (2013). Myelin recovery in multiple sclerosis: the challenge of remyelination. Brain Sci. 3, 1282–1324.

Poliak, S. and E. Peles (2003). The local differentiation of myelinated axons at nodes of Ranvier. Nat. Rev. Neurosci. 4(12), 968-980.

Qiang, L., Zhao, B., Ming, M., Wang, N., He, T.C., Hwang, S., Thorburn, A., He, Y.Y. (2014). Regulation of cell proliferation and migration by p62 through stabilization of Twist1. Proc. Natl. Acad. Sci. U S A. 111, 9241–9246.

Rangaraju, S. (2010). Rapamycin activates autophagy and improves myelination in explant cultures from neuropathic mice. J. Neurosci. 30, 11388–11397.

Rivers, L.E., Young, K.M., Rizzi, M., Jamen, F., Psachoulia, K., Wade, A., Kessaris, N., Richardson, W.D. (2008). PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice. Nat. Neurosci. 11, 1392–1401

Rogov, V., Dotsch, V., Johansen, T. & Kirkin, V. (2014). Interactions between Autophagy Receptors and Ubiquitin-like Proteins Form the Molecular Basis for Selective Autophagy. Molecular Cell. 53, 167-178.

Saraswat- Ohri, S., Bankston, A.N., Mullins, S.A., Liu, Y., Andres, K.R, Beare, J.E., Howard, R.M., Burke, D.A., Riegler, A.S., Smith, A.E., Hetman, M., Whittemore, S.R. (2018). Blocking Autophagy in Oligodendrocytes Limits Functional Recovery after Spinal Cord Injury. J. Neurosci. 38(26), 5900-5912.

Scholzen, T., Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. Journal of Cellular Physiology. 182 (3), 311–22.

Simons, M., Misgeld T., and Kerschensteiner M. (2014). A unified cell biological perspective on axon-myelin injury. J. Cell Biol. 206, 335–345.

Smith, C.M., Mayer, J.A., Duncan, I.D. (2013). Autophagy promotes oligodendrocyte survival and function following dysmyelination in a long-lived myelin mutant. J Neurosci. 33, 8088–100.

Suzuki, K., Kubota, Y., Sekito, T., Ohsumi, Y. (2007). Hierarchy of Atg proteins in pre autophagosomal structure organization. Genes Cells 12(2), 209-218.

Tang, D.G., Tokumoto, Y.M., Raff, M.C. (2000). Long-term culture of purified postnatal oligodendrocyte precursor cells. Evidence for an intrinsic maturation program that plays out over months. J. Cell Biol. 148, 971–984.

Tanida, I., Nishitani, T., Nemoto, T., Ueno, T., Kominami, E. (2002). Mammalian Apg12p, but not the Apg12p.Apg5p conjugate, facilitates LC3 processing. Biochem. Biophys. Res. Commun. 296, 1164–1170.

Teter, S.A., Eggerton, K.P., Scott, S.V., Kim, J., Fischer, A.M., Klionsky, D.J. (2001). Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase. J. Biol. Chem. 276(3), 2083-2087.

Williams, K.A., Deber, C.M. (1993). The structure and function of central nervous system myelin. Crit. Rev. Clin. Lab. Sci. 30, 29–64.

Yang, Z., Huang, J., Geng, J., Nair, U., Klionsky, D.J. (2006). Atg22 recycles amino acids to link the degradative and recycling functions of autophagy. Mol. Biol. Cell 17(12), 5094-5104.

Yoshii, S.R., Mizushima, N. (2017). Monitoring and measuring autophagy. Int. J. Mol. Sci. 18(9) pii: E1865.

Zhang, X., Chen, S., Huang, K., Le, W. (2013). Why should autophagic flux be assessed? Acta Pharmacol. Sin. 34, 595-599.