Μεταπτυχιακή Εργασία

Ο ρόλος του φυλετικού διμορφισμού στη μυελίνωση του ΚΝΣ σε διαγονιδιακούς μύες με έλλειψη αυτοφαγίας

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Η εργασία κατατέθηκε στην Ιατρική σχολή
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Περίληψη

Η εκκαθάριση κυτταροπλασματικών συστατικών επιτυγάνεται μέσω μιας καταβολικής διαδικασίας, γνωστή και ως αυτοφαγία. Το μονοπάτι της μάκρο-αυτοφαγίας (ΜΑ) προσφάτως κέντρισε το ενδιαφέρον της ερευνητικής κοινότητας λόγω της πιθανής συνεισφοράς της στην φυσιολογική λειτουργία των Ολιγοδενδροκυττάρων (ΟΔ). Τα προαναφερθέντα κύτταρα εντοπίζονται στο Κεντρικό Νευρικό Σύστημα (ΚΝΣ) και είναι υπεύθυνα τόσο για την δημιουργία νέας μυελίνης όσο και για την συντήρηση της ήδη υπάρχουσας. Ιδιαίτερα ενδιαφέρον καθίσταται το γεγονός ότι τα κύτταρα Ολιγοδενδροκυτταρικής γενεάς, και κατ' επέκταση η μυελίνωση του ΚΝΣ, διαφέρουν μεταξύ ενήλικων αρσενικών και θηλυκών μυών. Δεδομένου αυτού, στόχος της εξής μελέτης ήταν να διευκρινιστεί κατά πόσο η ομαλή λειτουργία της ΜΑ είναι απαραίτητη για την συντήρηση της ομοιόστασης της μυελίνης στον εγκέφαλο ενήλικων μυών, καθώς και αν υπάρχει κάποια φυλετικά διμορφική επίδρασης της ΜΑ στη μυελίνη κατά την ενήλικη ζωή. Για τον σκοπό αυτό, μπλοκάραμε το μονοπατι της ΜΑ (μέσω της διαγραφής του γονιδίου ATG5) στα ΟΔ ενηλίκων αρσενικών και θηλυκών μυών και εξετάσαμε τις συνέπειες της αντίστοιχης επίδρασης τρεις μήνες αργότερα. Σύμφωνα με τα αποτελέσματα, οι αρσενικοί μύες με έλλειψη ΜΑ (cKO) εμφάνισαν υψηλότερα επίπεδα της πρωτενίνης PLP, άξονες με μικρότερα g-ratios, υψηλότερη πυκνότητα αξόνων με δομικά αποδιοργανωμένη μυελίνη, υψηλά επίπεδα αξονικής εκφύλισης και μειωμένη ικανότητα μάθησης κινητικών μοτίβων. Στους θηλυκούς cKO μύες, από την άλλη πλευρά, εντοπίστηκε μόνο αύξημένος αριθμός αξόνων με δομικά αποδιοργανωμένη μυελίνη. Συνοψίζοντας, ενώ η ομαλή λειτουργία της ΜΑ παίζει σημαντικό ρόλο στην διατήρηση της φυσιολογικής μυελίνωσης του ΚΝΣ τόσο των αρσενικών όσο και των θηλυκών μυών, υπάρχει μια φυλετικά διμορφική επίδραση της ΜΑ στην αντίστοιχη διαδικασία.

Master Thesis

Sexual dimorphic effects in the absence of autophagy in CNS myelin

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Summary

Autophagy is a catabolic process that mediates the clearance of cytoplasmic constituents through a variety of pathways (i.e., chaperone-mediated autophagy, micro-autophagy, macro-autophagy). Macro-autophagy has recently drawn the attention of researchers due to its potential contribution to the normal function of Oligodendrocytes (OLs), the myelinating glia of the Central Nervous System (CNS). On a functional level, OLs are responsible for both the formation of new myelin and the maintenance of existing myelin in the CNS. Of particular interest is the fact that OL-lineage cells and by extension myelination display sexual dimorphism during adulthood. Taking into account that the relationship between macro-autophagy and CNS myelination is an intriguing research concept that has yet to be fully elucidated, the aim of this study was to examine the significance of a well-regulated macro-autophagic mechanism for the maintenance of myelin homeostasis in the adult mouse brain and to identify whether there is a sexually dimorphic effect of basal autophagy on CNS myelin during the respective period. For this purpose, we ablated the macro-autophagic pathway (through deletion of ATG5), specifically in OLs of adult male and female mice, and examined the results of that blockade three months later on a cellular, structural and behavioral level. According to the respective results, cKO males displayed higher levels of PLP, axons with smaller g-ratios, increased density of axons with myelin decompaction, high levels of axonal degeneration and motor-learning deficiencies. Female mice, on the other hand, were characterized only by an increase in the number of axons with decompacted myelin. Overall, our data confirmed not only the contribution of the macro-autophagic pathway in the maintenance of normal myelination in the adult CNS of both males and females, but also a sexually dimorphic effect in the respective process.

I. Introduction

1.1 The Myelinating Glia of the Central Nervous System

The central nervous system (CNS) is occupied by a vast variety of cells. In this study we focused on Oligodendrocytes (OLs), the myelinating glia of the CNS, and their progenitors known as oligodendrocyte progenitor cells (OPCs) (Kuhn et al. 2019).

OPCs are migratory, proliferating adult progenitor cells whose generation, in the mouse brain (e.g., telenchephalon), can be distinguished into three temporal waves after the closure of the neural tube (Kuhn et al. 2019; Timsit et al. 1995). More specifically, the first wave originates from the ventral ventricular zone (VVZ) of the medial ganglionic eminence (MGE), takes place at embryonic day 12.5 (E12.5), and is immediately followed by a second one originating from the lateral ganglionic eminence (LGE). By E16 the respective cells have already invaded the developing cortex in a lateral to medial direction. As far as the last wave is concerned, it takes place postnatally and it originates from the cortex itself (Kessaris et al. 2006). The aforementioned overproduction of OPCs is counter-balanced by an increase in apoptosis triggered by the lack of available space and growth/survival factors (e.g., PDGF-A, FGF-2, IGF-1, NT-3, CNTF) (Barres and Raff 1993; Miller 2002; Raff et al. 1993; Trapp et al. 1997; Kessaris et al. 2006; Barres and Raff et al. 1992; Barres and Raff et al. 1999). With the completion of the migration process, OPCs can be detected in both the grey and the white matter. The latter, however, displays a higher abundance of the aforementioned cells (Dawson et al. 2003). To add to this, white matter OPCs appear to have greater capacity for differentiation, when compared to those of the grey matter (Dimou et al. 2008; Kang et al. 2010; Kuhn et al. 2019; Simons et al. 2016; Bergles et al. 2016).

OPCs give rise to mature oligodendrocytes through a multi-stage process of differentiation. The aforementioned maturation process has been thoroughly investigated thanks to the fact that each one of respective stages (i.e., from OPCs to myelinating OLs) is characterized by the expression of specific markers. To be more precise, apart from expressing DM-20 mRNA (proteolipid protein isoform), OPCs can be identified by the expression of the receptor for the mitogen PDGF-a (PDGFR-a) (Pringle and Richardson 1993; Timsit et al. 1995; Noble et al. 1988; Yeh et al. 1991; Calver et al. 1998). Pre-myelinating OLs, with the capacity to build filamentous myelin outgrowths (transition from progenitor to differentiated OLs), specifically express CNPase (20-, 30 -cyclic-nucleotide 30 -phosphodiesterase) and the cell-surface markers O4, O1 (Sommer et al. 1981; Braun et al. 1988; Jakovcevski et al. 2009). Last but not least, fully differentiated OLs are characterized by the production of myelin and the expression of myelin-related proteins: MBP (myelin basic protein), PLP (proteolipid protein), MAG (myelin associated glycoprotein), MOG (myelin-oligodendrocyte protein) and the membrane marker GalC (galactocerebroside) (Brunner et al. 1989; Linnington et al. 1984; Barbarese et al. 1988; Trapp 1990; Raff et al. 1978; Timsit et al. 1995). To add to this, irrespective of the developmental stage, cells of OL-lineage express Olig1, Olig2, SOX10 and Nkx2.2 (Zhou et al 2000; Zhou et al 2002; Kuhn et al. 2019).

Although the production of myelinating OLs reaches its peak between the 2nd and 4th postnatal week, myelinating OLs continue to be generated for a at least 7 more months in a steadily declining rate (i.e., well into adulthood). This can be attributed to the fact that a percentage of OPCs stays in the precursor state, thus providing the ability to the respective organism of generating OLs throughout most of its life (Rivers et al. 2008; Kuhn et al. 2019; Simons et al. 2016; Bergles et al. 2016).

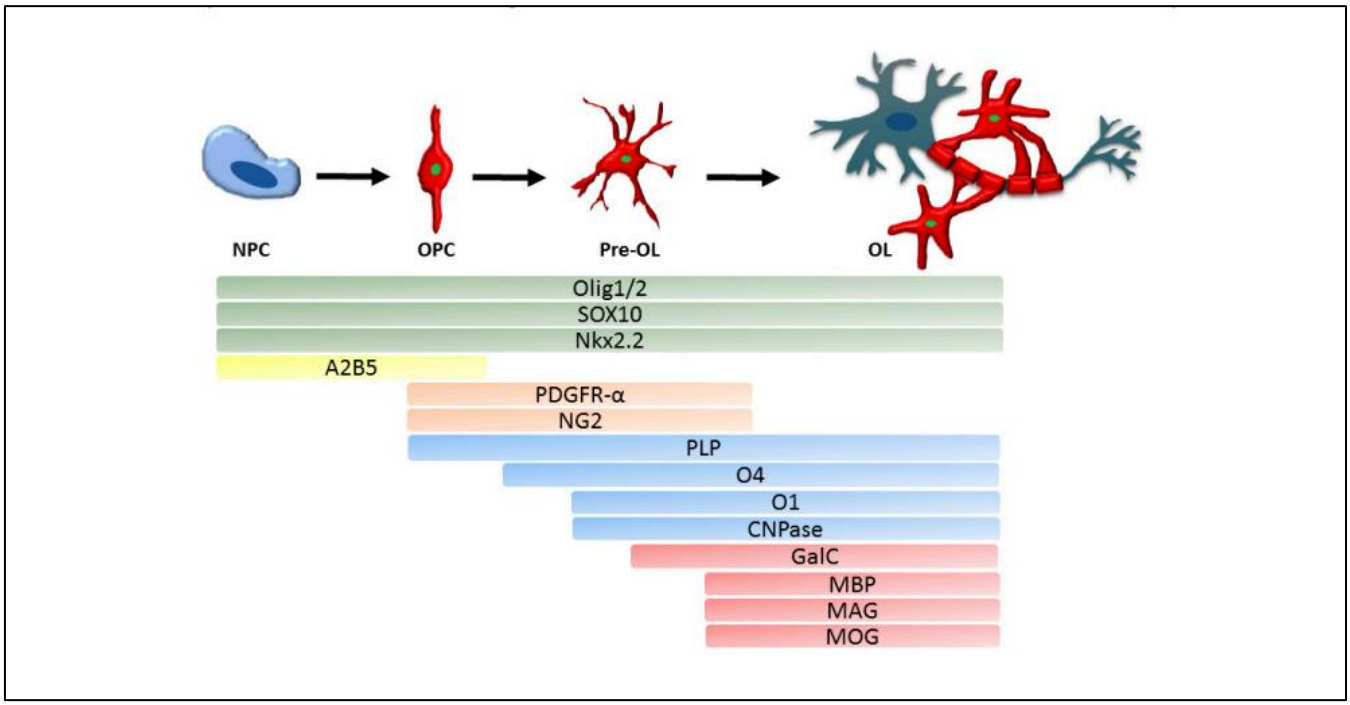


Figure 1: Schematic depiction of oligodendroglial lineage markers specific for different developmental stages from neuronal progenitors to myelinating OLs (Kuhn et al. 2019).

1.2 Myelination of the Central Nervous System

1.2.1 The Myelin Sheath

Mature oligodendrocytes have the ability to extent their plasma membrane and wrap it around neighboring axons in a concentric fashion (Lee et al. 2012; Kuhn et al. 2019). The end result is the formation of a structure called myelin that consists mainly of lipids and proteins. Due to the nature of its composition (e.g., high levels of saturated, long chain fatty acids, glycosphingolipids and cholesterol), myelin displays tremendous stability (O'Brien 1965; Coetzee et al. 1996; Simons et al. 2016).

On a structural level, myelin is described as a multilayered stack of uniformly thick membranes in which electron dense- and light- layers (i.e., major dense and the intra-period line, respectively) periodically alternate. The aforementioned adhesion zones actually represent the apposed cytoplasmic and extracellular myelin membrane bilayers. Tight junctions, consisting of claudin-11, run radially through

myelin holding together the respective lamellae (Gow et al. 1999; Morita et al. 1999). Despite the high compaction level of myelin, non-compacted cytoplasmic spaces (e.g., outer and inner periaxonal tongues, paranodal loops) are also present. As far as the paranodal loops are concerned, they constitute the ends of a myelin internode where septate junctions are formed with the axonal surface (adhesion proteins Caspr, contactin and Neurofascin-155 hold together the two surfaces) (Salzer et al. 2008; Simons et al. 2016). Of particular significance is the fact that the myelin structure allows the communication of oligodendrocytes with the respective neurons. This is possible due to the low compaction of the lamellae positioned directly above the periaxonal space (i.e., adaxonal part of the myelinic channel system) (Snaidero et al., 2014; Simons et al. 2016).

On a functional level, compacted myelin is responsible for the facilitation of rapid transmission of action potentials that, in turn, are necessary for efficient sensory, motor and cognitive function. This form of transmission, which is significantly faster than that in non-myelinated axons, is possible due to the fact that myelin provides high electrical resistance, along with low capacitance, and due to the existence of sodium channels only at the nodes of Ranvier. As a result, axon potentials are allowed to practically jump from one node of Ranvier to the next, a phenomenon known as saltatory conduction (Kuhn et al. 2019).

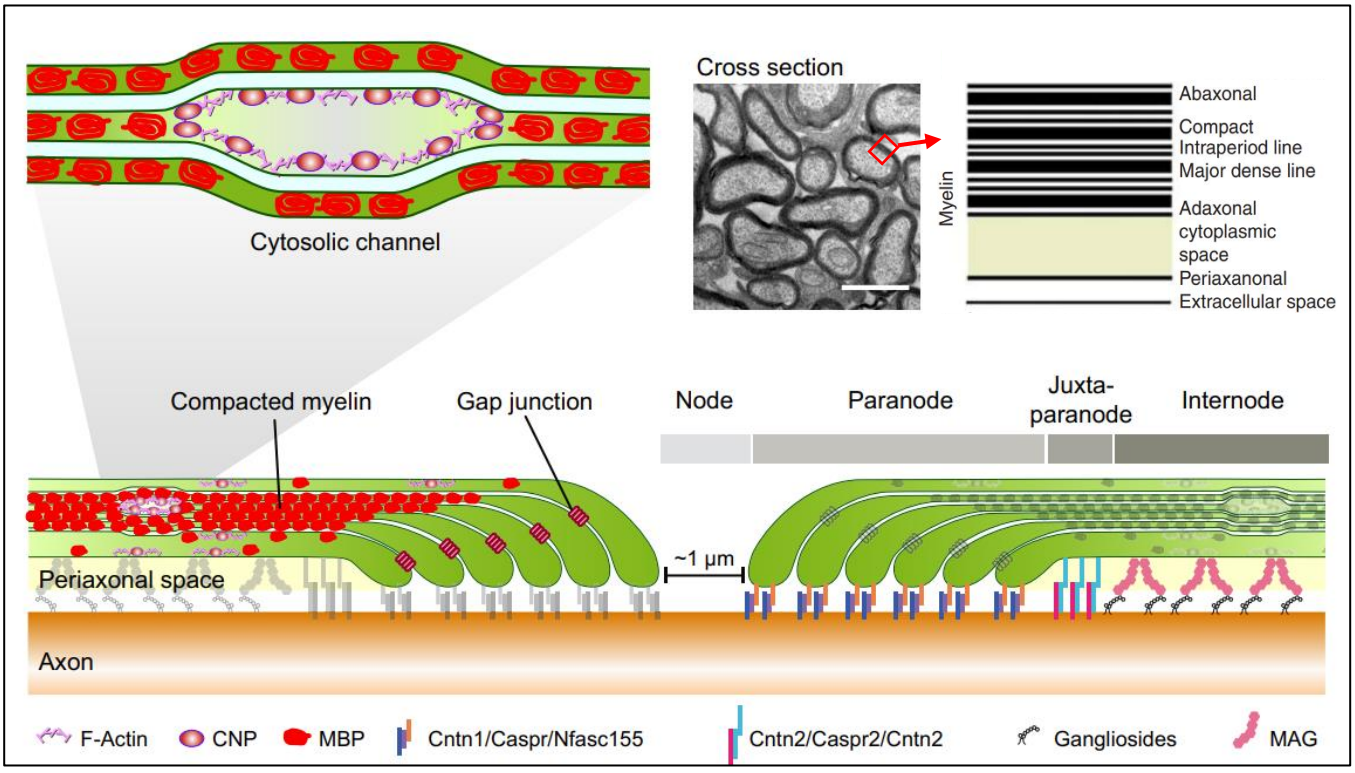


Figure 2: Schematic illustration of CNS myelin structure including different domains of myelinated axons and cross-section view of myelin (Adapted from Saab and Nave 2017; Simons et al. 2016).

1.2.2 The process of Myelination

Oligodendrocytes are particularly selective as to which axons to myelinate. They identify the target axons by continuously extending/ retracting their processes, ensuring at the same time the maintenance of discrete territories between cells of OL lineage. Following the identification of an axon, the formation of myelin begins, a process highly dependent on the PI(3,4,5)P3/ Akt/ mTOR and ERK1/2 signaling pathways (Flores et al. 2008; Tyler et al. 2009; Goebbels et al. 2010; Ishii et al. 2013; Simons et al. 2016; Narayanan et al., 2009; Bercury and Macklin 2015).

The latest model of myelination in the CNS proposes that, once a process from an OL contacts the axon of interest, two coordinated motions take place. More specifically, while the inner tongue moves in coiled turns, the membrane spreads sidewards on the axon towards the nodal regions (Sobottka et al., 2011, Snaidero et al., 2014). This results in the lateral cytoplasmic-rich edges of each myelin layer to align at the future node, thus forming the paranodal loops. The compaction of the myelin sheath is controlled by several factors including CNP1 (compaction-delaying factor) and the previously mentioned MBP (compaction-promoting factor). As far as the latter is concerned, it is believed to zipper two adjacent cytoplasmic membrane surfaces by polymerizing with previously deposited monomers (Aggarwal et al. 2013). To add to this, since MBP is synthesized at the tip of the glial process, myelin compaction progresses from the outside to the inside of the sheath (Snaidero et al. 2014). A direct consequence of the aforementioned process is the generation of a diffusion barrier which, in turn, limits the entry of proteins with large cytoplasmic domains, thus turning compact myelin into a protein poor membrane (Aggarwal et al. 2011). Higher levels of compaction are presumably achieved after the resolving of a system of cytoplasmic channels that, up until then, served the transportation of myelin components to the growth zone (communication of inner-/outer- zone) (Snaidero et al. 2014; Bercury and Macklin 2015).

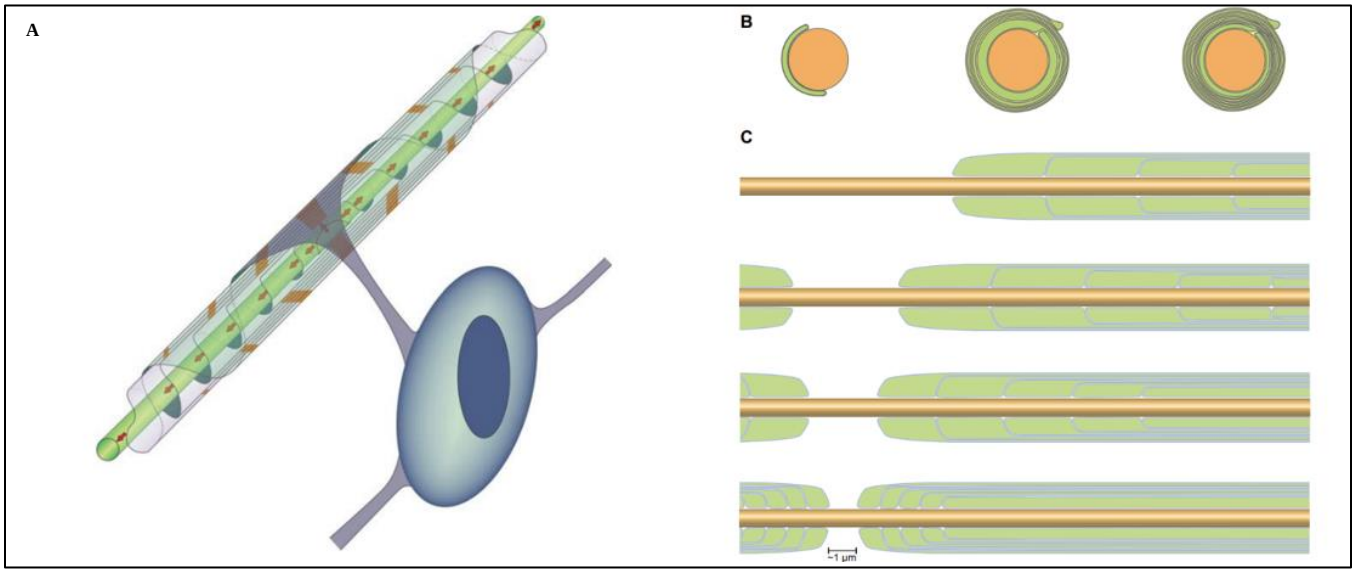


Figure 3: Model of myelination in the CNS: (A) Motions of wrapping (Simons et al. 2016). (B, C) Cross-section and longitudinal view of myelinated axon at different stages, respectively (Adapted from Chang et al., Snaidero et al.)

1.3 The role of Oligodendrocytes in the Central Nervous System

1.3.1 Metabolic Support of Axons

Recent evidence suggests that myelin, apart from playing a role in the rapid propagation of signals, is essential for the metabolic support of axons which, as we mentioned previously, are relatively isolated from the extracellular milieu (Funfschilling et al., 2012; Bercury and Macklin 2015).

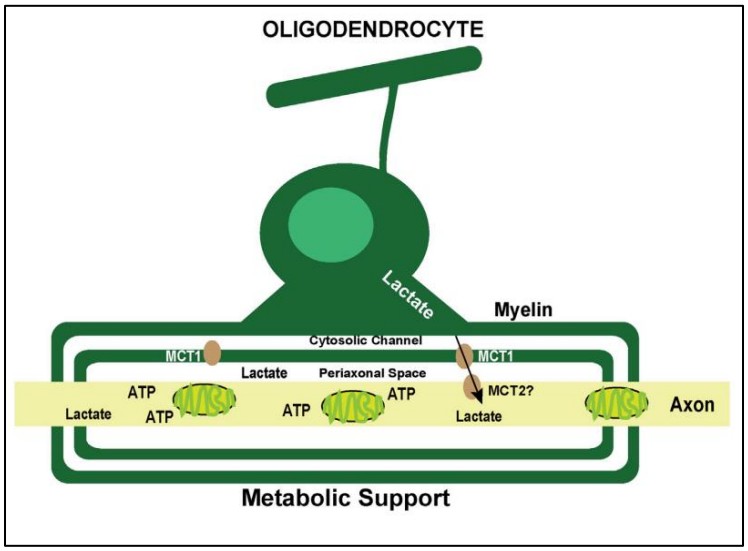


Figure 4: Model of oligodendrocyte-derived lactate delivered by the MCT1 transporter to the axons (Adapted from Funfschilling et al 2012).

Under conditions of a dysfunctional electron transport mechanism, mature OLs take advantage of the aerobic glycolysis process to generate both lactate and pyruvate. The aforementioned products are used by OLs for the maintenance of the respective myelin sheaths which, in turn, contribute significantly to the survival and normal function of axons. To add to this, several glycolytic and Krebs cycle enzymes (i.e., succinate dehyfrogenase, furmarase) appear to be expressed in the myelin sheath, something which hints the axonal use of OL-derived lactate for the generation of ATP. In support of that notion is the fact that reduced expression of MCT1 (monocarboxylate transporter 1), a lactate transporter highly localized to OLs (adaxonal membrane), severely affects the long-term maintenance of myelinated axons (i.e., axonal degeneration). To add to this, Kang et al. (2013) showed that impaired OL function enhanced the vulnerability of motor neurons to ALS-linked genetic insults, ultimately leading to axonal degeneration (Funfschilling et al., 2012; Lee et al., 2012; Ravera et al. 2013; Kang et al., 2013; Philips et al. 2021; Bercury and Macklin 2015; Kuhn et al. 2019). Last but not least, lack of CNP1 expression appears to lead to a pathological phenotype characterized by wide-spread axonal loss, swollen inner tongue and premature death of the respective mice. Since CNP1 counteracts membrane compaction by MBP, the aforementioned results can be attributed to a lack of metabolic support that could, in turn, stem from the closure of myelinic channels that mediate the metabolic coupling of OLs and axons (Snaidero et al. 2017; Lappe-Siefke et al. 2003; Edgar and Nave 2009; Bercury and Macklin 2015; Kuhn et al. 2019).

1.3.2 Adaptive Myelination

The ability of oligodendrocytes to myelinate axons has also been associated with several types of skill learning in humans (e.g., playing a musical instrument, learning a new language) (Bergles et al. 2016).

The process of learning involves the repetitive firing of specific neural circuits. The respective pattern of neuronal activity has the capacity to trigger OL-mediated changes in myelination which, in turn, lead to alterations in the structure of white matter (Bergles et al. 2016). The aforementioned activity-dependent generation of new myelin, also known as adaptive myelination, is only possible due to the fact that the adult CNS has a high abundance of OPCs capable of producing new mature OLs (Young et al. 2013). More specifically, through the expression of neurotransmitter receptors (e.g., NMDA-R, AMPA, GABA-R, Ach-R), voltage-gated ion channels (i.e., Na^+, K^+, Ca^{2+}) and the formation of transient synapses with glutamatergic neurons (sampling of respective axonal activity), OPCs can alter their proliferation/ differentiation rates accordingly to the neuron-emitted signals. To add to this, the generated OLs, apart from forming synapses with unmyelinated axons, also respond to electrical stimuli (Barres et al. 1990, Bergles et al. 2016).

In vivo studies examining activity-dependent alterations in the myelin pattern in the CNS of rodents confirm adequately the link between myelination and learning capacity (e.g., motor skill learning). More specifically, McKenzie et al. (2014) showed that OL production was briefly increased in the brain of adult mice that underwent motor skill learning using the complex wheel apparatus (i.e., wheel with irregular spaced bars). To add to this, mice unable to produce new OLs, due to conditional deletion of MyRF (myelin regulatory factor, a transcription factor necessary in OLs to maintain the myelination program) in OPCs during adulthood, were prevented from mastering the aforementioned skill (McKenzie et al. 2014; Long and Corfas 2014). Light-induced electrical stimulation of motor cortex V neurons was found to increase oligodendrogenesis which, in turn, increased both MBP expression and myelin sheath thickness. As a result, a marked difference in performance on the Catwalk apparatus was detected (Gibson et al. 2014). Finally, activity-dependent alterations in OL-lineage cells and myelination can also be triggered by environmental stimuli such as physical exercise and social interactions (Simon et al. 2011; Liu et al. 2012; Makinodan et al. 2012; Bercury and Macklin 2015; Bergles et al. 2016).

To sum up, the formation of new myelin patterns (triggered by neuronal excitability) and learning during adulthood are two intertwined processes for which normal function of OL-lineage cells is undoubtedly required.

1.3.3 Remyelination

Perturbations of myelin sheath integrity, due to injury or pathological conditions, are far from uncommon in the adult CNS. Mature oligodendrocytes have the ability to restore, at least to some degree, the proper myelination of the CNS through the process of remyelination.

OPCs, apart from assessing the surrounding microenvironment using their filopodia extensions, can detect inflammatory stimuli (e.g., TNFa) thanks to the expression of cytokine receptors (Arnett et al. 2001; Hughes et al. 2013). In response to demyelination, OPCs migrate to the respective sites of myelin lesions (Kuhn et al. 2019). An increase of SOX2 and TCF7L2 (i.e., Wnt signaling) expression is also triggered by demyelination which, in turn, causes OPCs to remain in their proliferative state. As a result, large amounts of myelinating OLs are generated in order to prevent extensive damage and neurodegeneration (Levine et al. 1999; Fancy et al. 2004; Franklin et al. 2017). A few studies have suggested that the remyelinated internodes are both thinner and shorter than normal (Blakemore and Murray, 1981; Gledhill and McDonald, Blakemore 1974). Recent data, however, have shown that a substantial recovery time (e.g., 6 months) can result in remyelinated fibers with comparable internode length and thickness to developmentally myelinated axons (Powers et al., 2013; Kuhn et al. 2019; Bercury and Macklin 2015).

Of particular interest is the fact that the capacity of remyelination appears to decline with age (Franklin et al. 2017). More specifically, apart from a diminished remyelination levels, aged organisms are also characterized by an increased level of white matter atrophy and a decline in motor learning (Kuhn et al. 2019). These can be attributed to both an inadequate recruitment of differentiation-capable OPCs and a high level of mature OL apoptosis (Sim et al. 2002; Zhu et al. 2011, Shields et al. 1999). As far as the latter is concerned, OLs are exposed to high metabolic stress (i.e., myelination, myelin maintenance, metabolic support of axons) for prolonged periods of time, whilst not having the appropriate levels of antioxidants to prevent their damage (Lee et al. 2012, Juurlink et al. 1998; Thorburne et al 1996; Liu et al. 2000). As a result, aging OLs gradually become more and more susceptible to DNA damage which, in turn, leads to their apoptosis (Kuhn et al. 2019). The rejuvenation, however, of the myelination capacity is feasible once the accumulated myelin debris are successfully removed (Ruckh et al. 2012; Kuhn et al. 2019).

1.4 Autophagy in Myelinating Glia of the Central Nervous System

1.4.1 Autophagic Machinery and Pathways

Autophagy is a process of intracellular clearance through which cytoplasmic contents, membranes and pathogens are degraded. Of particular significance to the respective process are autophagy-related (ATG) proteins which, in turn, are responsible for the control of both the core autophagic machinery and the regulatory complexes that mediate the initiation of the mechanism (Dikic and Elazar, 2018).

There are at least three types of autophagy, namely chaperone-mediated autophagy (targets unfolded cytosolic proteins), micro-autophagy (lysosomal-mediated engulfment of cytoplasmic portions) and macro-autophagy (induced by hypoxia, nutrient depletion, cellular damage, ROS production, ageing). As far as the latter is concerned, it begins with the formation of a double membraned autophagic precursor (i.e., phagophore) from ω-shaped nucleation sites (i.e., omegasomes). More specifically, the ULK1

complex (unc-51 like autophagy-activating kinase 1) activates the PI3KC3 complex (Class III phosphoinositide 3-kinase) which leads to the production of PI3P (phosphatidylinositol 3-phosphate) and the catalysis of the nucleation and elongation processes. During the expansion phase of the pre-autophagosomal structure, the ATG12–ATG5–ATG16L1 protein complex (E3-ligase function) is recruited for the conjugation of LC3-II to PE (phosphatidyl-ethanolamine), located on the membrane of the phagophore. The autophagosome is formed only after a substrate is engulfed by the phagophore. The formation process, which is mediated by a variety of signaling pathways, requires mTORC1 (mammalian target of rapamycin complex 1) to be inhibited and AMPK (AMP-activated protein kinase) to be activated. Subsequently, the autophagosome is trafficked along microtubules to the perinuclear region where the lysosomes are located. The degradation of the respective substrate is achieved through the fusion of the autophagosome with a lysosome (Belgrad et al. 2020; Fleming and Rubinsztein 2020).

Degradation of both organelles (e.g., dysfunctional mitochondria, peroxisomes, endoplasmic reticulum) and aggregate-prone proteins is possible through selective autophagy. In such cases, autophagy receptors and adaptors (e.g., SQSTM1/p62 and NBR1) bind the target cargoes to LC3 (ATG8), via ubiquitinated residues, thus sequestering them within the respective autophagosomes. The following fusion of the autophagosomes with lysosomes is mediated by both the STX17-VAMP8-SNAP29 trans-SNARE complex and an ARL8B-dependent recruitment of the homotypic fusion and protein sorting complex to lysosomes. Finally, the hydrolyzation of the aforementioned cargo allows the reuse of both lipids and amino acids (Belgrad et al. 2020; Fleming and Rubinsztein 2020).

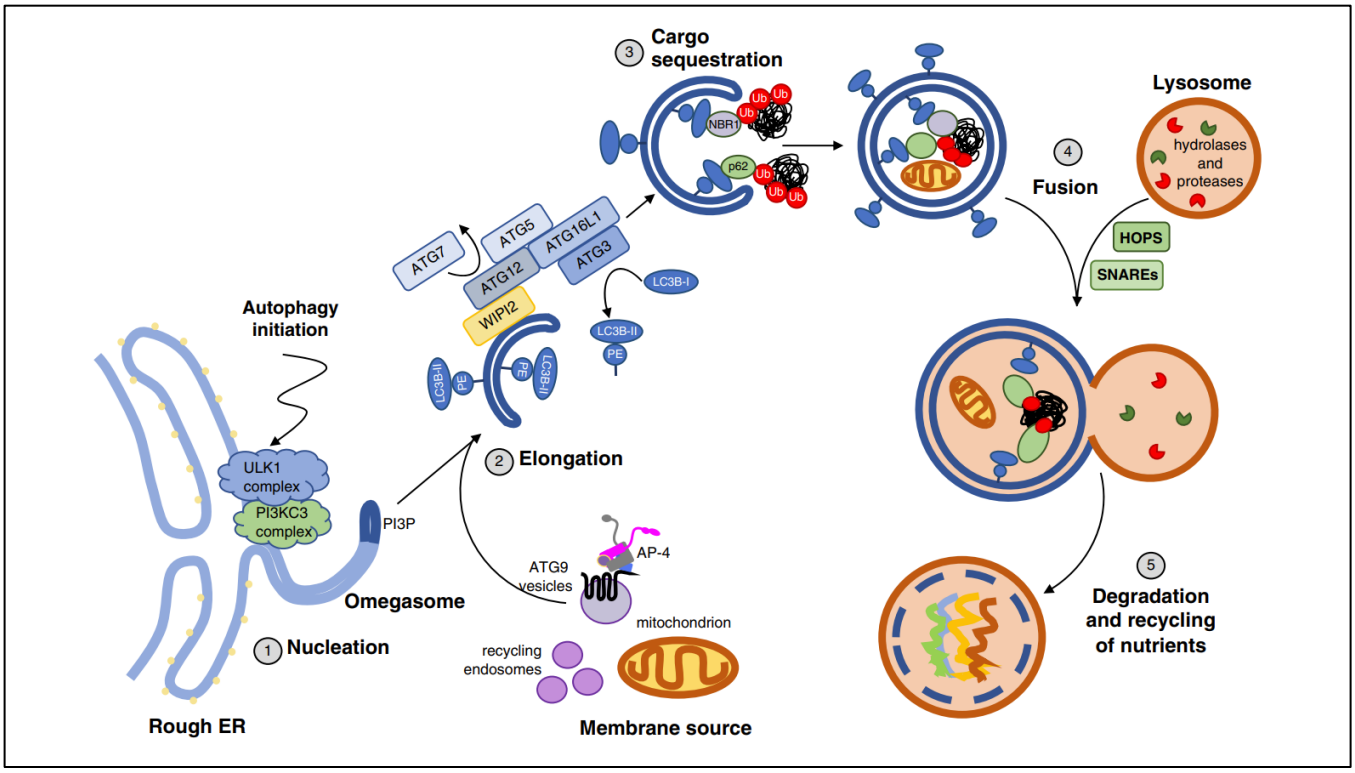


Figure 5: Schematic representation of autophagic pathway (Belgrad et al. 2020).

1.4.1 The role of Autophagy in Oligodendrocyte Maturation and Function

Recent evidence suggests that autophagy plays a significant role in both the development of OL-lineage cells and their function as myelinating glia of the central nervous sytem.

The contribution of autophagy in the normal differentiation of OPCs was proposed by Bankston et al. (2019) due to the presence of increased autophagic flux and autophagic puncta in the distal processes of the respective cells (Bankston et al., 2019). This notion was actually confirmed by the work in our lab that examined the effect of pharmacological inhibition (using SBI-0206965) and genetic blockade of macro-autophagy in primary OL cultures. According to these results, fewer mature OLs (i.e., stage 4) were detected alongside an increase in the number of early-stage OLs (i.e., stage 0-3) (Ktena et al. 2022). Interestingly, the respective *in vitro* data did not indicate an effect on cell viability, something which contradicted existing *in vivo* data (Ktena et al. 2022; Bankston et al. 2019). It is important to point out, however, that the transgenic line (i.e., PDGFRα-Cre^{ERT2}; ATG5$^{-/-}$ mice) used in the study by Bankston et al (2019) did not inhibit autophagy exclusively in OL-lineage cells, as PDGRFα is also expressed in pericytes (cells responsible for both capillary blood flow and microcirculation). Since the dysfunction of these cells has been related to white matter changes (e.g., loss of myelin, axons and OLs), there is a possibility that the inhibition of ATG5 in pericytes played a role in the reduced survival of OLs (Montagne et al. 2018; Bankston et al. 2019).

Apart from a lower density of OPCs/OLs, Bankston et al. (2019) also observed low myelination levels (e.g., MBP protein levels) in the CC, increased events of abnormal myelin (i.e., expanded inner tongue, poor compaction, myelin outfoldings) less myelinated axons and premature lethality in mice lacking ATG5 in their OL-lineage cells. *In vitro* experiments, using OL-neuro cocultures, further supported the previously mentioned role of autophagy in normal myelin development. More specifically, while autophagy induction (using Tat-Beclin1) increased myelin segment length, autophagy inhibition (using KU559533 or verteporfin) dramatically reduced both the number and the length of the myelin segments on the respective neurons. The promotion of myelin compaction seems to also be regulated by autophagy considering the thicker myelin sheaths (containing pockets of cytoplasm) that ATG5$^{-/-}$ mice had when compared to their counterparts (Bankston et al., 2019). In relation to this, taking into account that in adults the number of myelin wraps can change, it is possible that autophagy affects myelin plasticity through its role in cytoplasm decompaction (Dutta et al., 2018).

Another fact that supports the association of autophagy with myelination is that both processes depend on the normal activity of mTOR (mammalian target of rapamycin) (Dello Russo et al., 2013; Figlia et al., 2018). Of particular interest, however, is the fact that CNS myelination appears to be greatly dependent on the balanced activity of mTORC1, with perturbations in its activity (i.e., OL-specific overactivation) causing hypomethylation (Lebrun-Julien et al., 2014).

1.4.3 Oligodendrocyte-related Autophagy and CNS Homeostasis

The existence of a well-regulated oligodendrocyte-associated autophagy is crucial for the maintenance of CNS homeostasis. This is evident considering the vast variety of pathological conditions characterized by both dysfuction of the autophagic machinery and myelin abnormalities.

Amyotrophic lateral sclerosis (ALS), an adult-onset motor neuron disease in which upper/ lower motor neurons degenerate and proteins aggregate, is a prominent example of such a case. More specifically, the contribution of defective autophagy in the emergence of the respective disease is hinted by the fact that several ALS-causing genes have been linked with the regulation and initiation of the autophagic process (e.g., TBK1, C9ORF72, CCNF, ALS2 and B/C VAPB), with the maturation of autophagosomes (i.e., TDP-43, VCP) and with the fusion of autophagosomes-lysosomes (e.g., CHMP2B, FIG4, PIKFYVE). The contribution of autophagy-defective OLs in the onset/progression of ALS is further supported by the fact that TDP-43- and FUS-positive inclusions have been detected in the respective cells. To add to this, ALS mouse models appear to have low levels of OL-related MCT1 (transporter associated with the metabolic support of axons) which, in turn, have been correlated with events of neuronal death in those animals (Strohm and Behrends 2020; Belgrad et al. 2020).

Another example is Multiple system atrophy (MSA), a progressive adult-onset sporadic neuro-degenerative disorder accompanied by parkinsonism, ataxia and autonomic dysfunction symptoms. A hallmark of the respective pathology is the presence of α-synuclein-containing glial cytoplasmic inclusions originating in OLs. According to *in vitro* experiments using cultured rat brain derived OLs, this is the result of defects in both the proteasomal and the autophagic machinery. Of particular interest is the fact that although the latter seems to be upregulated (increase of LC3-II) during pathogenesis, presumably due to impairment of the proteasome, the aggregated proteins are still not efficiently cleared from the cytoplasm of the respective cells (Schwarz et al. 2012; Belgrad et al. 2020).

The significance of a well-regulated oligodendrocyte-associated autophagy can also be acknowledged in events of injury. More specifically, mice lacking ATG5 (i.e., defective macro-autphagy) display significantly reduced functional recovery after spinal cord injury (SCI). The active role of the autophagic mechanism in both the maintenance and the protection of myelin is further supported by the decrease in spared WM, detected 6 weeks later in the respective subjects (Saraswat Ohri et al., 2018). On the other side of the spectrum lies the case of traumatic brain injury (TBI), in which an immediate increase of OL-related autophagy appears to be responsible for the extensive damage in both white and gray matter. This is further supported by the fact that administration of an autophagy-inhibitor limits the extent of injury (Belgrad et al. 2020).

1.5 Sex Hormones in the Central Nervous System

1.5.1 Expression and Distribution of Sex Hormones and Receptors

Although the shaping of the CNS is influenced by a vast variety of factors, sex hormones appear to have a particularly significant role in the respective process. These neuroactive steroids, which are generated through cholesterol conversion mainly in the CNS (i.e., neural and glial cells) and the peripheral glands (i.e., testis, ovary, adrenal glands), can be categorized into three distinct families: androgens, estrogens and progestogens (Melcangi et al. 2016).

On a functional level, androgens (e.g., testosterone) and estrogens (e.g., 17-beta-estradiol) are responsible for the masculinization of brain and behavior that persists into adulthood (Gegenhuber et al. 2019). This is attributed to the fact that they can affect several developmental processes such as neurogenesis, neuronal survival, synaptogenesis and glial cell differentiation. To add to this, progesterone is an important regulator of both cerebellar Purkinje cells and OL development (Melcangi et al. 2016). As far as estrogens are concerned, they modulate masculinization and defeminization of mice by binding to specific receptors, namely ERα and ERβ. Of particular interest is testosterone's mechanism of action as, apart from influencing male-typical behavior associated neural circuits (via AR activation), it can also affect estrogen-mediated neural circuits. This is possible due to the fact that testosterone is a precursor of estrogen *in vivo*, something which also explains the existence of the latter in the male brain (Neigh et al. 2016). It is of great importance to point out that all sex-steroid-related actions are the result of the regulation of gene transcription by the respective receptors (i.e., AR, ERa, Erb, PR). This is achieved either by (sex steroid receptors) binding directly to hormone response elements on DNA or by interacting with co-factors (e.g., c-fos) that bind on gene promoters. To add to this, interaction with kinases (e.g., MAP, PI3) or membrane receptors (e.g., mGluR) also leads to transcriptional changes (Neigh et al. 2016).

Sex hormone receptors are abundantly expressed in areas such as the bed nucleus of stria terminalis (BNST), the medial preoptic hypothalamus (MPOA), the medial amygdala (MeA) and the ventrolateral nucleus of ventromedial hypothalamus (VHM). This comes as no surprise considering that sex hormones play a significant role in the regulation of the innate reproductive behavior. To add to this, since all four receptors are present in the suprachiasmatic nucleus of the hypothalamus, gonadal hormones can directly influence fluctuations in the adrenal output, sleep and mood. The arcuate nucleus, which is responsible for the regulation of homeostasis (e.g., feeding, energy balance), also displays prominent levels of AR, ERα and PR expression. ERα, ERβ and PRs are additionally detected in several cortex and midbrain regions, such as the ventral tegmental area (VTA), the substantia nigra (SNc), the periaqueductal gray (PAG) and raphe. Lastly, functional ARs are detected in the primary visual cortex and the prefrontal cortex (Manoli et al. 2018).

Evidently, adult rodents display brain-region specific differences in the expression levels of the aforementioned sex steroids. More specifically, while testosterone and DHT (dihydrotestosterone) levels

are higher in the CNS (i.e., hippocampus, cerebral cortex, cerebellum, spinal cord) and the cerebrospinal fluid (CSF) of males, diestrus females have higher levels of DHET (dehydroepiandrosterone) in their CSF, HIP and the CC. To add to this, females display higher levels of 17 beta-estradiol throughout their brain, when compared to their counterparts. As far as progesterone is concerned, it displays a complex expression pattern with diestrus females having higher levels in the CSF, males having higher levels in the CC and the CB (no differences in either the HIP or the SC). Interestingly, higher levels of the progesterone metabolites (i.e., dihydroprogesterone, allopregnanolone, isopregnanolone) were detected in most of the CNS regions of diestrus females, when compared to males (Melcangi et al. 2016).

Sexual dimorphism is also detected in the expression of the respective receptors. To be more precise, while females seem to express higher levels of ERs in regions such as the trigeminal ganglia, the MeA, the posterior BNST (BNSTp), the MPOA and ventrolateral VMH (VMHvl), males possess more AR positive neurons in the BNSTp and MPOA (Gegenhuber et al. 2019). In addition, PR levels are higher in the VMHvl and lower in the BNSTp of females, in comparison with that of males. Surprisingly, the sex-dependent difference in hypothalamic PR expression becomes even more prominent during the phases of the estrus cycle with peaking estrogen levels (Warfvinge et al. 2020; Gegenhuber et al. 2019). This indicates that, although progesterone can downregulate its own receptors, the expression of PRs is particularly sensitive to estrogen levels (Neigh et al. 2016).

Overall, the sexual dimorphism that appears to characterize the expression of hormones and steroid receptors could ultimately be responsible for sex-dependent differences in gene regulation and expression in the respective cells of the CNS (Gegenhuber et al. 2019).

1.5.2 Effect of Sex Hormones on Oligodendrocyte-Lineage cells

Sexual dimorphism exists at all levels of the nervous system of rodents (i.e., rats and mice). An extensive amount of literature focusing on the region of the corpus callosum (CC), being the largest brain commissure responsible for the interhemispheric connectivity, has shown that OL-lineage cells are no exception of the previously stated notion.

OPCs display sex-specific transcriptional profiles and cellular characteristics. To be more precise, while adult male-derived OPCs appear to have greater capacity for differentiation into myelinating OLs (higher density of newly-generated OLs), female-derived OPCs demonstrate higher migration and proliferation rates, with the latter being attributed to a higher expression level of the proliferation-promoting factors Olig1, Olig2, NF1 and PDGFRa (Cerghet et al., 2006; Patel et al., 2013; Yasuda et al., 2020). According to Cerghet et al, the seeming greater differentiation capacity of male-derived OPCs into OLs is a result of a shorter lifespan of female-derived OLs. More specifically, a higher generation rate is counter-balanced by a higher cell death rate (higher active caspase 3 levels). This ultimately results in adult male rodents having a greater density of myelinating OLs, than females, in regions such as the CC, the fornix and the ventral funiculus of the spinal cord (Cerghet et al., 2006; Patel et al., 2013).

It is no secret that males and females differ, under both physiological and pathological conditions, as far as their CNS hormonal environment is concerned (Roberto C. et al., 2016). Since OLs express a vast variety of steroid receptors (e.g., estrogen receptors α and β, androgen receptors, progesterone receptors), they are amenable to the effects of both sex-hormones and their metabolites (Nunez et al., 2000; Jung-Testas et al., 1998; Santagati et al., 1994; Zhang et al., 2004; Hirahara et al., 2009; Finley et al., 1999; Jung-Testas et al., 1994; Takao et al., 2004). *In vitro* studies examining the effect of progesterone, estrogen 17 beta-estradiol and dihydrotestosterone (DHT) on OPC differentiation and OL survival have shown that these processes are greatly dependent on the concentration of the respective hormones (Marin-Husstege et al, 2004; Swamydas et al., 2008). To add to this, specific combinations of hormones had different effects on male- and female-derived cells, thus proving the fact that OL-lineage cells have indeed a sexually-dependent sensitivity to the concentrations of neuro-steroids (Swamydas et al., 2008; Marin-Husstege et al, 2004). The aforementioned notion of sex hormones being responsible for the sexual dimorphism in OL density of adult rodents is further supported by *in vivo* studies examining the effect of gonadal hormones on OLs. More specifically, removal of endogenous testosterone (via castration) in male mice resulted in a female-like OL phenotype (i.e., increased cell death rate, decreased density in CC and fornix) (Cerghet et al., 2006; Pesaresi et al, 2015), whereas estradiol supplementation in gonadectomized groups (male and female) increased the total number of OLs (Patel et al. 2013). According to Abi Ghanem et al. (2007), the respective difference in OL density can be ultimately attributed to a higher transient expression of androgens (e.g., testosterone and the more potent 5a-DHT), ARs and type 2 5α-reductase (converts testosterone to 5α-DHT) that takes place in males postnatally. This was confirmed by the fact that the administration of an AR antagonist in males and 5α-DHT in females, during that time period, resulted in decreased and increased OL density during adulthood, respectively (Abi Ghanem et al., 2007).

To sum up, although the interaction between OLs and hormones *in vivo* is undoubtedly influenced by a myriad of factors (e.g., neuros, glia, growth factors, neuro-steroid synthesis, estrus and pregnancy), the hormonal environment definitely contributes to the OL-associated differences in the adult rodent brain.

1.5.3 Effect of Sex Hormones on Myelination and Axon Morphology

As mentioned previously, the generation of new myelin and the maintenance of pre-existing myelin are some of the core functions of mature OLs. Evidently, the sexual dimorphism that characterizes OL-lineage cells also affects their capacity for myelination. Differences can be spotted even at the transcriptional level, with male-derived OLs displaying higher expression mRNA levels of myelinating-promoting factors (e.g., MBP, MAG, CNP, and Myrf) (Cerghet et al., 2006; Yasuda et al., 2020).

On a morphological level, several studies investigating the CC in adult rodents suggest the sexual dimorphism of the respective region. More specifically, Yates and Juraska (2007) reported that in adult male rats the genu and splenium are bigger and a larger area is occupied by myelin (Yates and Juraska, 2007). Previous examinations had already revealed that males, apart from having a higher percentage of

myelinated axons, they also had higher percentage of large caliber axons when compared to age-matched females (Nunez et al., 2000; Mack et al., 1995; Kim et al., 1996; Kim et al., 1997; Pesaresi et al, 2015). According to Shatarat et al (2020), however, male rats have higher mean axon diameter of myelinated axons in the splenium and the anterior trunk of the CC, whereas females have higher mean diameter in the posterior trunk and the genu. Nevertheless, of particular significance is the fact that sex-dependent differences were also observed in the myelin sheath thickness (i.e., males > females in anterior trunk and females > males in posterior trunk and genu) (Shatarat et al., 2020).

In vitro studies examining the effect of sex hormones on the myelination process have shown that there is indeed a correlation between the two. To be more precise, the presence of progesterone can apparently lead to increased synthesis of myelin proteins, such as MBP and CNPase, in vitro (Jung-Testas et al., 1994, Ghoumari et al., 2003). To add to this, both progesterone and 17β-estradiol were able to restore myelination under pathological conditions (i.e., demyelination models, SCI) by modulating myelin protein expression (e.g., PLP, MBP) (Acs et al., 2009; De Nicola AF et al., 2006; El-Etr et al., 2015). On the same side of the spectrum lie evidence from *in vivo* studies examining the effect of gonadal hormones on the level and pattern of CNS myelination. More specifically, it appears that testosterone (activates both ARs and ERs if aromatized) plays a significant role in the maintenance of normal myelination in the adult brain of rodents as surgical gonadectomy of adult males resulted in decreased female-like levels of myelination, smaller number of myelinated axons and lower g-ratios (Patel et al., 2013; Cerghet et al., 2006; Pesaresi et al, 2015). The same applies for females as they were affected in a similar way by ovariectomy, but not to such an extent. To add to this, all gonadectomized groups displayed more severe demyelination and inefficient remyelination when compared to the intact groups, something which in turn confirms the positive role of sex hormones in axon myelination (Patel et al., 2013).

All these differences in myelination levels (i.e., more myelinated axons in males) and pattern (i.e., thicker myelin sheaths in males, lower g-ratio) can be attributed once more to a higher postnatal expression of androgens and AR receptors. Apart from an effect on OL density, Abi Ghanem et al. (2017) also detected reduction in the expression of MBP, in the percentage of myelinated axons and in the thickness of myelin sheaths (reflected by an increased g-ratio) in the region of the CC of adult mice lacking functional ARs (in neurons astrocytes and OLs). Furthermore, a sex difference that was detected in the length of myelinated axons segments was completely abolished after treatment of males with an AR-antagonist postnatally (sex hormones affect myelin organization) (Abi Ghanem et al., 2017). Finally, the fact that estradiol-, testosterone- and DHT-supplementation in gonadectomized animals restored to a different degree normal myelination/callosal conduction and remyelination, further supports the differential effect of sex hormones in myelination regulation and the maintenance of myelin sheath integrity (Patel et al., 2013).

According to Moore et al (2013), the emergence of the aforementioned differences between males and females could be partially attributed to the effect of sex chromosomes (e.g., asymmetry in expression of

myelination-related genes residing in the X chromosome). In support of that notion is the fact that functional remyelination (after cuprizone demyelination) is higher in XX animals (of both gonadal sexes), when compared to that of XY animals. However, due to the fact that this difference arises only the absence of circulating hormones (i.e., gonadectomized animals), the chromosomal contribution to both myelination and remyelination is suspected to be relatively subtle (Moore et al., 2013).

To sum up, sex hormones play a crucial role in the regulation of myelin-related processes including the shaping of the myelination pattern, the maintenance of normal myelination, the regeneration of myelin sheaths and the protection under pathological conditions (i.e., immune attacks) (Abi Ghanem et al., 2017).

1.5.4 Sex hormone-mediated Autophagy Regulation

A particularly intriguing theory has recently emerged that sex hormones can differentially regulate autophagy in the brain. The aforementioned notion was based primarily on results from CNS disease models and the fact that hormone receptors are abundantly expressed in the CNS (Noh et al., 2022).

Evidently, estradiol can either stimulate of inhibit the respective process by acting on multiple signaling autophagy-related pathways (Azcoitia et al., 2019; Xiang et al., 2019). Stimulation may be achieved in a few different ways: by inhibiting GSK3β (serine/ threonine kinase glycogen synthase kinase 3β), by inhibiting mTORC1 (through class III PI3K/AKT), by activating APMK (under conditions of ATP depletion) or by inhibiting NFKb (associated with reduced autophagy during neural cell differentiation) (Saraceno et al., 2018; Wang et al., 2019; Perez-Alvarez et al., 2018; Guo et al., 2017a; Cook et al., 2018; Yun et al., 2018; FitzPatrick et al., 2018). On the other hand, the estradiol-mediated inhibition of the autophagic machinery was detected in the hippocampus after stroke and it involved downregulating LC3-II, ATG5 and Beclin1 (mediate phagophore formation) (Li et al., 2017). Another female hormone that has been associated with the regulation of autophagy is progesterone. Both *in vitro* and *in vivo* experiments have shown progesterone to activate autophagy in astrocytes and microglia thus preventing the accumulation of neurotoxic aggregates and NLRP3 inflammasome activation, respectively (Kim et al.2012; Espinosa-Garcia et al., 2020). Contrary to female hormones, the effect of testosterone on autophagy in the brain has not been thoroughly investigated. However, judging by the fact that it can be aromatized into estradiol and that it interrupts the initiation of autophagy in rat cardiac tissue (by upregulating Bcl2 which in turn binds to Beclin1), it is highly possible that it exerts similar effects in CNS cells (Fu et al., 2017; Shay et al., 2018). Finally, an *in vitro* model of Parkinson's disease (i.e., SH-SY5Y cells) has shown that ERα contributes to the elevation of LC3-II levels and to the enhancement of the lysosomal enzyme activities (Li et al., 2015). Therefore, it is clear that under specific circumstances steroid hormones have the ability to significantly affect not only the formation of autophagy vesicles, but also the efficiency of cargo degradation (by maintaining the integrity of lysosomes).

In relation to the previous data, Noh et al. (2022) proposed that sex chromosomes have the potential to amplify an already existing autophagy-related sexual dimorphism. This is attributed to the fact that several

genes associated with the regulation of the autophagic machinery in the brain localize to the X chromosome. Wdr45 and Rab39b, which have been known to mediate the formation and maturation of autophagosomes, are two of those genes. Hdac6, which is directly involved in the recruitment of autophagy cargo, is another example. Last but not least, Atp6ap2 and Lamp2 are the necessary for the maintenance of lysosomal integrity. Deficiency in any of these genes can severely affect the autophagic process in the brain, thus leading to neurodegeneration and brain dysfunction. Although the X-chromosome inactivation that occurs in females ensures the similar expression levels of the aforementioned genes in males and females, a possibility of skewing cannot be ignored (Noh et al., 2022).

To sum up, based on the fact that the expression level of sex hormones/ sex hormone receptors is both sexually dimorphic and brain region-specific, one could not easily dispute a sex-dependent regulation of autophagy in the CNS (Noh et al., 2022).

1.5.5 Neuroprotection

There is a vast variety of CNS-associated pathological conditions that display sexual dimorphism in aspects such as prevalence, disease progression and mortality. This comes as no surprise considering the differences in genome constitution and gene expression regulation between males and females. Sex hormones are believed to also contribute to the aforementioned differences since they can act as neuro-protective agents.

Several animal studies are in agreement with human data suggesting the existence of sexual dimorphism in ALS pathology. More specifically, males display earlier age of onset, quicker disease progression, more aggressive disease phenotypes and earlier mortality. According to the existing literature, the hormonal environment may play a significant role in the emergence of the aforementioned sex-dependent vulnerability. Direct evidence about the neuroprotective effect of estrogens is provided by a study by Trieu and Uckun (1999), in which the administration of phytoestrogens (e.g., genistein) in murine models of familial ALS resulted in the elimination of the observed dimorphism (Trieu and Uckun 1999). The anti-apoptotic effect of estrogens is also supported by the fact that 17-beta-estradiol treatment delayed disease progression in female mice (hSOD1 or mSOD1 G39A), whereas ovariectomy had the opposite effect (Groeneveld et al. 2004; Choi et al. 2007). Furthermore, 17-beta-estradiol administration on pre-symptomatic or symptomatic male SOD1 mice enhanced motor performance and increased survival of lumbar spinal cord motor-neurons by reducing expression of inflammasome proteins NLRP3 and the levels of caspase 1 and mature IL1-beta (Heitzer et al. 2017). Progesterone seems to function in a similar way since it managed to slow down the progression of the disease, extend the life span of male mice (tgSOD1 ALS) and reduce motor-neuron death by inducing the autophagic degradation of mutant SOD1 without, however, delaying the onset of symptoms (Kim et al. 2013).

Experimental models of stroke (i.e., middle cerebral artery occlusion), a neurodegenerative disease with higher incidence and worse prognosis in males (at least until menopause in females), are another

source of evidence supporting the original notion. More specifically, female rats display less serious brain injury than age-matched males, while gonadectomized females display a phenotype even more severe than that of males, after induction of cerebral ischemia (Alkayed et al. 1998). Since the administration of 17-beta-estradiol protects both male and female estrogen-deficient brains, there is a clear neuroprotective effect of estrogens in the aforementioned conditions (Toung et al. 1998; Rusa et al. 1999). Surprisingly the combined use of estradiol and progesterone does not always lead to synergistic action and the manner of its effect is brain-region specific (Rubio et al. 2018). Traumatic brain injury (TBI) models have also demonstrated sex-dependent neuroprotection, since female rats have less severe edema phenotype than males and pseudo-pregnant females (maximal levels of progesterone and progesterone metabolites) have virtually no edema at all (Roof et al. 1993). This proves, at least to some degree, that the higher levels of circulating female hormones in the respective animals are responsible for the higher level of neuroprotection in case of TBI.

Studies examining the effect of hormones on remyelination after injury (i.e., SCI) and demyelination (i.e., in cuprizone, LPC, EAE animal models, mentioned below) further support the role of sex hormones as potent neuroprotectants with rehabilitative properties for OLs. More specifically, in a case of SCI, 17-beta-estradiol administration to adult rats attenuated not only OL apoptosis (via inhibition of RhoA and c-Jun-N-terminal kinase activation), but also axonal degeneration (via inhibition of caspase 3/9 activation), thus improving functional recovery (i.e., hindlimb motor function) (Lee et al. 2012). In murine models of multiple sclerosis (MS), 17-beta-estradiol partially reduced OL loss and demyelination in the CC of male mice, following cuprizone-induced demyelination (Taylor et al. 2010). The aforementioned neuroprotective role of estrogen is also apparent in middle aged ovariectomized rats. To be more precise, receival of one month of replacement therapy retained the volume of white matter myelin sheaths and increased myelin fiber length and diameter (improved spatial learning), compared to placebo treated mice (He et al. 2017; Luo et al. 2016). Progesterone has similar effects on the efficiency of remyelination in the adult CNS of injury models. Starting with SCI, significant enhancement of OPC proliferation/differentiation (via increase of Nkx2.2 and Olig 2 expression) and remyelination (increase in Olig1, PLP expression and OL maturation) were observed in male rats alongside anti-inflammatory effects (e.g., prevention of microglial activation/proliferation) (Labombarda et al. 2011; Labombarda et al.2008). To add to this, the binding of progesterone to the respective receptor contributes to OPC survival after injury by reducing the levels of pro-inflammatory cytokines (Labombarda et al. 2015). In an Lysophosphatidycholine (LPC)-induced demyelination model, progesterone pre-treatment antagonized the injury (spinal cord) in adult males, something which was evident by the increased myelination levels, the increased OPC/OL numbers and the reduced microglial/macrophage response, compared to their age-matched counterparts (Garay et al. 2011). Similar effects were observed in a cuprizone-demyelination model focusing on the CC and cortex of female mice (El-Etr et al. 2014). Lastly, improved clinical outcomes have been detected in female EAE (experimental autoimmune encephalomyelitis) mice due to

progesterone treatment (caused less inflammatory cell infiltration and recovery of MBP, PLP). Evidently, the administration of both progesterone and estradiol seems to produce a synergistic effect on the restoration of myelination and the reduction of inflammation in female EAE mice models (Garay et al. 2008; Acs et al. 2008). As far as the former is concerned, 17-beta-estradiol and progesterone induced the production of IGF-1 by astrocytes in a cuprizone-induced demyelination model which, in turn, promoted OL proliferation and differentiation (Acs et al. 2008). Data on androgen-mediated neuroprotection, on the other hand, hint a modulatory effect on inflammation rather than a direct effect on OL-lineage cells and myelin. More specifically, the protective aspect of testosterone is indicated by the fact that castration of relapsing-EAE mice induces relapses with greater influx of activated-T cells in the CNS and the fact that testosterone treatment reduces clinical scores in both males and females (Bebo et al. 1998; Dalal et al. 1995). DHT administration has similar results in the spinal cord of rats (i.e., reduced gliosis, inflammation, oxidative stress, tissue damage) (Giatti et al. 2015). As far as cuprizone models are concerned, androgens seem to enhance remyelination, amongst others, as testosterone action through ARs rescues the number of OPCs/ OLs and the expression of MBP in gonadectomized males and females (Hussain et al. 2013). Similar results were obtained in a LPC mouse model of spinal cord demyelination using male mice (Bielecki et al. 2016; Breton et al. 2021).

To sum up, although the neuroprotective properties of sex steroids have been well documented, it appears that they are sex-dependent, brain region-dependent and pathology-dependent. As a result, males and females display different vulnerability to specific pathologies.

1.6 Thesis Hypothesis and Aims

According to the aforementioned literature, the existence of a well-regulated autophagic machinery appears to be critical for the normal function of the CNS in both males and females. However, its newly revealed role in the normal development and function of OLs has caused further questions to arise (Bankston et al. 2019; Ktena et al. 2022). In relation to this, the significance of macro-autophagy for the maintenance of myelin homeostasis and the contribution of the sex-factor to the aforementioned process are questions that our laboratory team has chosen to address (Ktena et al. 2022).

Our preliminary (unpublished) data hinted the existence of a sexually dimorphic effect of macro-autophagy on myelination. Taking the aforementioned into consideration, we sought to identify whether the contribution of autophagy in the normal function of OPCs/ OLs, and by extension myelination, during adulthood is sexually dimorphic. The relationship between autophagy and oligodendrocytes has only recently attracted the attention of researchers, as opposed to the relationship between autophagy and the myelinating glia of the Peripheral Nervous System (Schwann cells), so it has yet to be well documented. To add to this, the relationship between autophagy and CNS myelination is examined mainly in male-only groups. Therefore, the work was divided into the following specific aims:

1. Examine whether and how the ablation of macro-autophagy affects the level of myelin proteins in the CNS of adult male and female mice.
2. Examine whether and how the ablation of macro-autophagy affects myelin and axonal integrity in adult male and female mice.
3. Examine whether and how the ablation of macro-autophagy affects motor skill learning and coordination in male and female mice.
4. Examine whether and how the ablation of macro-autophagy affects OPC/ OL density and survival in the adult CNS of male mice

II. Materials and Methods

2.1 Mouse lines and Tamoxifen Treatment

The animals used in this study were adult (> 3 months old) and of C57BL/6 genetic background. More specifically, apart from male mT/mG; plpCre^{ERT2} mice, both male and female plpCre^{ERT2}; atg5 mice were used. All subjects were kept at temperature-controlled conditions under a 12h light/dark cycle. They were housed in groups (2-4 per cage) and were provided with standard mouse chow and water ad libitum by the animal facility of the Institute of Molecular Biology and Biotechnology (IMBB) – Foundation for Research and Technology Hellas (FORTH). In order to ensure the welfare of laboratory animals, all experiments complied with the NC3Rs guidelines.

All mice were administered 1 dose (100 μg) of 4-hydroxytamoxifen intraperitoneally for 10 consecutive days (with a two-day break in between) at 2.5 months (i.e., P75 – P86). The aforementioned treatment plan resulted in the formation of two experimental groups for the plpCre^{ERT2}; atg5 mouse line. More specifically, mice of the Control (CTR) group were either of following genotypes: plpCre$^{ERT2\ (-)}$; atg5 $^{(fl/fl)}$, plpCre$^{ERT2\ (-)}$; atg5 $^{(+/fl)}$, plpCre$^{ERT2\ (-)}$; atg5 $^{(+/+)}$, plpCre$^{ERT2\ (+)}$; atg5 $^{(+/+)}$. The conditional Knockout (cKO) group on the other hand, consisted exclusively of plpCre$^{ERT2\ (+)}$; atg5 $^{(fl/fl)}$ mice. All tests were performed after the mice had reached the age of 6 months (i.e., P180).

2.2. Estrous Cycle Staging Identification

The reproductive cycle of female mice is divided in four stages (i.e., proestrus, estrus, metestrus, diestrus). Each is defined by unique ovarian steroid levels (i.e., 17-beta-estradiol, progesterone, luteinizing hormone, follicle stimulating hormone, prolactin) and cell typology in the murine vaginal canal.

In order to keep track of the aforementioned fluctuation of hormones, during the behavioral testing period (i.e., Handling, Open-field, Rotarod, Clasping), vaginal cell samples were collected. The sampling was performed at approximately 11 pm each day, prior to the conduction of the respective experiment, using a non-invasive protocol known as Vaginal Lavage. More specifically, a drop of ddH2O was placed at the opening of the vaginal canal using a 200μl tip (latex bulb attached at the end). By consecutively

increasing/decreasing the pressure on the bulb a sufficient number of cells was acquired. The respective fluid was subsequently placed on a glass slide and left to air-dry at room temperature (RT). The process was then repeated for each of the remaining subjects. All smears were stored at RT for staining (using Crystal violet) and microscopic examination at a later date (McLean et al. 2012).

2.3. Behavioral Experiments

Prior to conducting any behavioral experiments, a 4-day period of handling (i.e., 180 – 183) of the mice (number of animals used is indicated in the results section) took place so as to familiarize them with human contact. The process was performed after a 1-hour acclimation session in the room that future experiments would be conducted under low illumination conditions.

2.3.1. Open Field Test

Following the completion of the handling, an assessment of the locomotor activity and of the anxiety levels of the animals took place.

The subjects were moved to the experimentation room one hour prior to the test in order to acclimate. Each animal was then placed in the middle of an open field arena (45 x 45 x 45) and was left to move freely for 10 min. Its activity was video recorded during the whole task with the experimenter being outside the room so as to ensure minimal noise and distractions (not within the animal's sight). The arena was cleaned with 70% ethanol between test subjects.

Since rodents display a natural aversion to brightly lit open areas, the anxiety levels of each mouse were evaluated based on its desire to stay close to the walls and edges of the field (Thigmotaxis). In order to calculate the degree of thigmotaxis, three individual zones were established: the central zone, the peripheral zone, the outer zone (i.e., close to walls and edges). The respective index [(Time spent in the outer zone) / (Total time of test)] is representative of the anxiety levels during the task. Locomotion, in turn, was evaluated by keeping track of each animal's travel path and then calculating the total distance travelled. The videos were analyzed using JWatcher, EthoVision XT and ToxTrac and the respective results were based on each subject's activity during the first 5 min of the test.

2.3.2. Rotarod Test

The motor behavior of mice (i.e., motor learning and motor coordination) was assessed using a rotarod apparatus with falling sensors (MK-660D, Muromachi-Kikai, Tokyo, Japan).

The Rotarod test lasted 5 consecutive days with a total of four trials per day. A 15 min interval would take place in between trials during which mice were returned to their home cage, away from the device. A day prior to the onset of the experiment (i.e., the same day of the open field testing), all mice were familiarized with the device by being placed on the rotating rod (speed: 4rpm) for 1 min. Each of the following testing days began with the animals being acclimated to the experimentation room for 1 hour.

The first three days of the experiment (motor learning testing phase) the subjects were placed on the rod rotating at a speed of 4 rpm. The rod would then accelerate gradually for 5 min until it reached a speed of 40 rpm. On the fourth and the fifth day (motor coordination testing phase), however, mice were placed on a still rod which then began to rotate at a constant speed of 20 rpm and 32 rpm respectively, for 5 min. The test was conducted under low illumination conditions and mice of only one cage were put on the rotarod each time. The device was wiped clean with 70% ethanol before placing subjects of a different cage on it. Every trial was video recorded and three specific time points were noted for each subject: latency to one rotation (1R), latency to two rotations (2R), latency to fall. A trial was considered over when all mice had performed 2 rotations, when all mice had fallen off or when the cutoff time was reached (i.e., 5 min). An average of the four trials per day for each mouse was calculated.

2.3.3. Hindlimb Clasping Test

The motor coordination of male and female mice that participated in the rotarod test was also assessed through the hindlimb clasping test (marker of disease progression in mouse models of neurodegeneration and ataxias). The task took place on Day 5 of the rotarod test, under low illumination conditions.

As far as the experimental protocol is concerned, it involved grasping each subject by the tail, lifting it clear of all surrounding objects and keeping in the air for 10 seconds. Following the end of the session each animal was returned to its home cage. Every session was video recorded and mice were scored based on the motor behavior of their hindlimbs. More specifically, animals with hindlimbs consistently splayed outwards (away from their abdomen) and normal escape tension were awarded a score of 0. A score of 1 was assigned to subjects who had one hindlimb retracted towards the abdomen, for more than 5 sec. In the case of both hindlimbs being partially retracted, for more than 50% of the time, a score of 2 was assigned. Lastly, mice with fully retracted hindlimbs (for more than 5 sec) received a score of 3.

2.4. Western Blot analysis

Tissues from 6 months old male and female mice (number of animals used is indicated in the results section) were collected and stored at -80^{o}C until their homogenization. The optic nerves (ONs) were sonicated (50 Hz) in RIPA buffer (500mM Tris-HCL pH 7.2, 1MNaCL, EDTA, Triton 100-X, Na-deoxycholate, 10% SDS) supplemented with protease inhibitors (Sigma) so as to achieve their lysis. The samples were subsequently centrifugated at 12,000 rpm at 4^{o}C for 30 min and their supernatant was then centrifuged a second time for 20 min. The acquired protein extracts were then aliquoted and the appropriate amounts of Laemmli buffer (500mM Tris-HCL pH 6.8, 100% glycerol, 10% SDS, bromophenol blue) and dithiothreitol (DTT) were added to each aliquot (in accordance to the results of the Bradford Protein Assay).

The samples were separated on a 15% polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore). Before being incubated in the respective primary antibody solutions overnight at 4^{o}C, the

membranes were firstly incubated in blocking solution (3% BSA, Phosphate Buffer Saline – 0.1% Tween 20) for 1 hour at room temperature (RT). The following are the primary antibodies that were used: anti-PLP (1:1000, rabbit), anti-MBP (1:3000, rat), anti-CNPase (1:1000, rabbit), anti-tubulin (1:5000, mouse).

The next day the membranes were washed in PBS-T (10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween-20) for 10 min three times before being incubated for 1 hour at RT in the corresponding secondary horseradish peroxidase-conjugated antibodies (i.e., goat anti-rabbit HRP 1:5000, goat anti-mouse HRP 1:5000, donkey anti-rat HRP 1:5000). The last step involved the development of the blots by chemiluminescence which was achieved by using the Immobilon Classico Western HRP substrate (Merck). Images were acquired through the ImageLab software.

After acquiring the images, the membrane carrying the female samples was incubated in 0.025% azide-containing solution for 20 min. As a result, the respective secondary antibodies were stripped (i.e., goat anti-rabbit HRP 1:5000). The membrane was then washed thoroughly in PBS-T and incubated in primary antibody solution containing anti-tubulin (1:5000, mouse), overnight at 4ºC. The next day the membrane was washed in PBS-T and incubated in goat anti-mouse HRP (1:5000) solution, for 1 hour at RT. After developing the blots, using the aforementioned manner, new images were collected. The intensity of the respective bands was quantified using the Fiji/ImageJ Gel Analyzer plugin.

2.5. Transmission Electron Microscopy

Both male and female mice (number of animals used is indicated in the results section) were firstly anesthetized with an average of 70 mg of a ketamine/xylazine mix (4/3 ratio) before being subjected to transcardial perfusion with 2.5% glutaraldehyde (GDA) in 0.1 M phosphate buffer (PB), pH 7.25. The harvested optic nerves were placed in the aforementioned fixative overnight at 4ºC. After numerous washes in 0.1 M PB, the ONs were then incubated in 2% (wt/vol) osmium tetroxide and 1.5% (wt/vol) $K_4[Fe(CN)_6]$ in 100 mM PB buffer for 1 hour on ice. The subsequent washes of the samples with water were followed by an incubation for 1 hour in 1% (wt/vol) tannic acid in 100 mM PB buffer. Once that was over a third incubation took place in 1% (wt/vol) uranyl acetate for 2 hours at RT. The next steps of the tissue preparation procedure involved the dehydration of the samples at RT, through gradual ethanol cycles, and their infiltration with an ethanol/Epon-Araldite (EMS) mix. Finally, the samples were flat embedded and polymerized for 24 hours at 60ºC (Kolotuev, 2014, Pasquettaz et al, 2021).

Using a 90º diamond trim tool, polymerized blocks were acquired which, in turn, were used to obtain 70 nm sections (use of 35º diamond knife mounted on a Leica UC6 microtome). As far as the sectioning is concerned, the samples were oriented in such a way so as to obtain coronal sections of the optic nerve starting roughly 1 mm from the optic nerve head (Kolotuev, 2014, Burel et al, 2018). Polyetherimide-coated carbon slot grids were used to collect the respective sections.

The analysis of the TEM samples was conducted using an FEI CM100 electron microscope at 80kV. Apart from being equipped with a TVIPS camera, the microscope was also piloted by the EMTVIPS

program. Two types of images were collected, single frames and stitched mosaic panels covering a larger sample region. Images of the latter type (generated using the IMOD software package) were used for the assessment of myelin sheath thickness [using g-ratio = (radius of axon without myelin sheath)/(radius of axon with myelin sheath)] and for the quantification of axons with decompacted myelin and of degenerating axons (Kremer et al, 1996). All data were analyzed using the Fiji/ImageJ, IMOD 3dmod and Photoshop programs.

2.6. Fluorescent Immunohistochemistry

Before harvesting the brain and optic nerves of male mice (4 CTR, 4 KO, 1 mTmG; PlpCre$^{ERT2\ (-)}$, 1 mT/mG; PlpCre$^{ERT2\ (+)}$), each animal was anesthetized using 70 mg ketamine/xylazine and then perfused with 4% paraformaldehyde (PFA) in 0.1 M Phosphate Buffer Saline (PBS). The extracted tissues were subsequently post-fixed in the same fixative for 30 min at 4^{o}C and cryo-protected in 30% sucrose (0.1 M PBS, 0.01% azide) for at least 24 hours. The embedding of the samples was achieved using a 7.5% gelatin/15% sucrose gel.

Depending on the tissue, cryosections of different thickness were obtained. More specifically, optic nerve sections had a thickness of 10 μm whereas sagittal or coronal brain sections were 15 μm and 14 μm thick, respectively. All sections were mounted on Superfrost Plus microscope slides and post-fixed in ice-cold acetone for 10 min. After being blocked in 5% BSA (Sigma-Aldrich) in 0.1 M PBS for 1 hour at RT, the samples were then incubated with the following primary antibodies overnight (at 4^{o}C): anti-platelet derived growth factor receptor alpha (PDGFRa) (1:100, rat), anti-adenomatous polyposis coli clone CC1 (APC/CC1) (1:100, mouse), anti-PLP (1:1000, rabbit), anti-Caspase 3 (1:200, rabbit), anti-GFP (1:2000, rat). The last step involved incubating the sections with fluorochrome labeled secondary antibodies Alexa Fluor 488, 555 and 633 (1:800) and DAPI (1:2000) for 1.5 hours at RT and the mounting of coverslips using the MOWIOL Reagent (Merk-Millipore).

The images that were acquired, using a confocal microscope (TCS SP8, LEICA DMI-8), were analyzed using the Fiji/ImageJ Cell Counter plugin.

2.7. Statistical analyses

The results were analyzed for statistical significance using the GraphPad Prism 8 software. Data in the respective graphs are presented as mean ± standard error of mean (SEM). The differences between groups in the tasks were examined using either parametric or non-parametric tests according to the normality of the distribution (examined with the Shapiro-Wilk test). Two-way ANOVA, followed by Tukey's or Sidak's post hoc test for multiple comparisons, or Unpaired t Test were performed when data fit in a Gaussian distribution. The Mann-Whitney test was performed when data did not fit a normal distribution. As far as the statistical significance is concerned, it was set at $p < 0.05$ and is indicated with an asterisk (*). Finally, a trend for statistical significance was set at $p < 0.1$ and is indicated with (#) in the graphs.

III. Results

3.1 Recombination efficiency of tamoxifen-inducible plpCre^{ERT2} line

Prior to investigating whether macro-autophagy is implicated with a sexually dimorphic manner in the maintenance of myelin in the CNS of adult mice, we firstly had to confirm that the administered dose of tamoxifen was sufficient for the achievement of efficient recombination.

For this purpose, immunohistochemistry analyses were performed using sagittal sections of optic nerves and whole brains of 6-months old mT/mG; plpCre^{ERT2} mice. The endogenous GFP signal of the respective animals, indicative of the existence of recombinant cells, was enhanced using antibodies against GFP. According to the results, GFP+ recombinant cells were detected only in myelin tracts of mT/mG; plpCre$^{ERT2\ (+)}$ mice (Figure 6B, C), thus verifying the recombination efficiency of the tamoxifen inducible plpCre^{ERT2} mouse line.

Figure 6

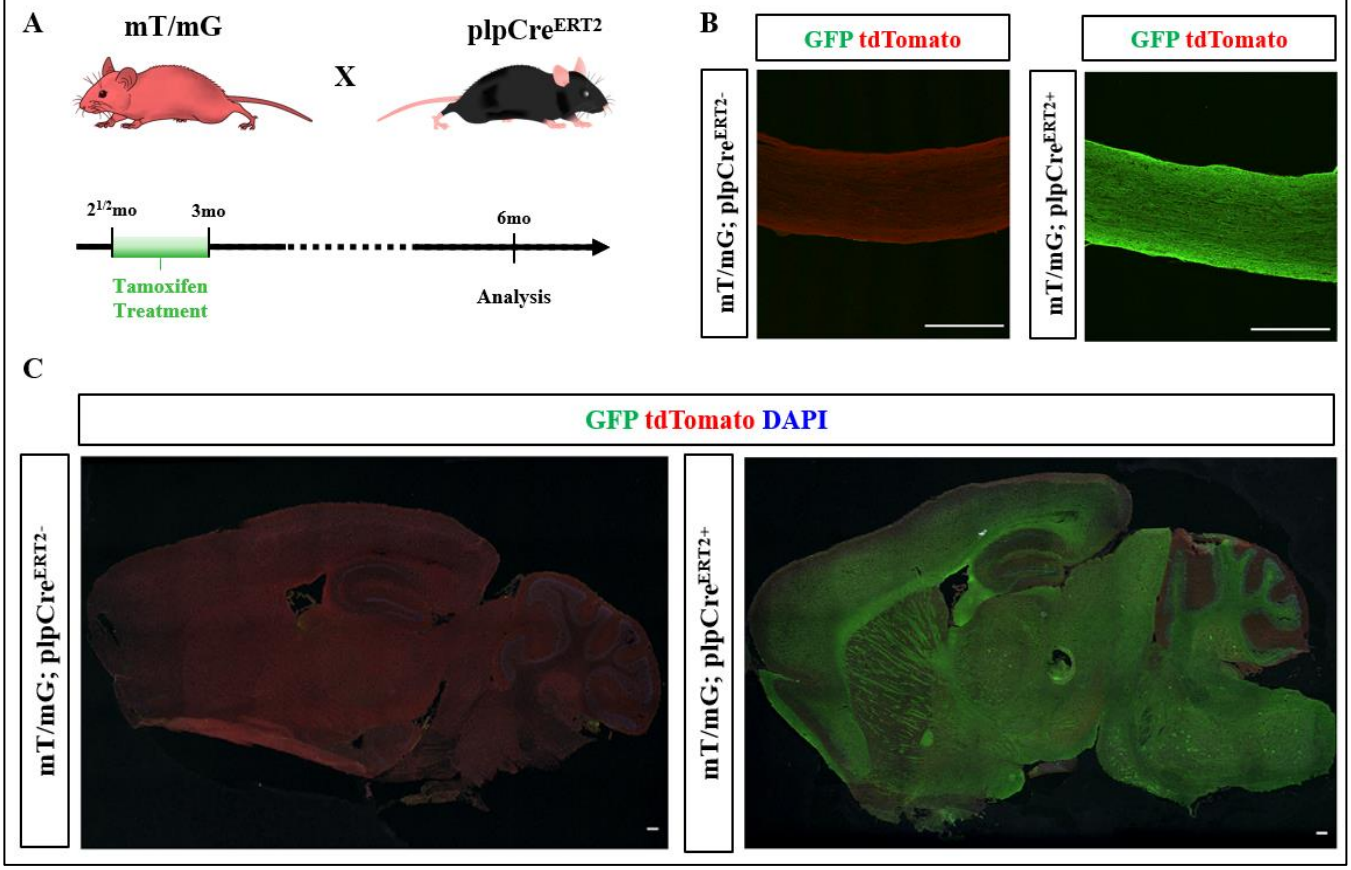


Figure 6: Recombination efficiency of tamoxifen inducible plpCre^{ERT2} line.

A) Experimental plan for verification of tamoxifen-induced recombination efficiency in plpCre^{ERT2}; atg5 $^{(fl/fl)}$ mice. All subjects received 10 intraperitoneal injections of 100 μg of tamoxifen per day for 10 days (with a 2-day break in between), at the age of 2.5 months. The respective analysis was performed at the age of 6 months.

B) Images of sagittal optic nerve cryosections of mT/mG; plpCre$^{ERT2\ (-)}$ and mT/mG; plpCre$^{ERT2\ (+)}$ mice depicting GFP positive (green) recombinant cells only in the former at the age of 6 months.

C) Confocal images of sagittal brain cryosections of 6 months old mT/mG; plpCre$^{ERT2\ (-)}$ and mT/mG; plpCre$^{ERT2\ (+)}$ mice. In the respective images, green fluorescence (i.e., GFP+ recombinant cells) is detected only in myelin tracts of plpCre$^{ERT2\ (+)}$ mice.

Scale bars: 200 μm (B, C).

3.2 Effect of AGT5-mediated macro-autophagy blockade on myelin protein levels

Based on preliminary data confirming the existence of myelin proteins in autophagosomes, we sought to answer whether ATG5-mediated macro-autophagy has a differential effect on myelin protein levels in the CNS of adult male and female mice (Ktena et al. 2022). Our specific focus on PLP, MBP and CNPase was based on both their abundancy and their importance for the maintenance of normal myelin structure.

For this purpose, Western blot analyses were performed in optic nerve lysates of 6-month-old control and cKO animals (Figure 7). The region of the optic nerve was specifically selected due to its high level of myelination. According to the respective results, male cKO mice displayed a significant increase of PLP protein levels, when compared to the controls (t-test, $p = 0.04$). No differences, however, were detected in either MBP (t-test, $p = 0.71$) or CNPase levels (t-test, $p = 0.67$) between the two groups (Figure 7A). As far as the female groups are concerned, cKO mice displayed similar levels of myelin proteins to that of their control counterparts (t-tests, $p = 0.24$ for PLP, $p = 0.95$ for MBP and $p = 0.77$ for CNPase) (Figure 7B).

Taking everything into account, it appears that the blockade of the AGT5-mediated macro-autophagy causes the accumulation of PLP only in males which, in turn, suggests a sex-dependent role of the respective pathway in the degradation of PLP.

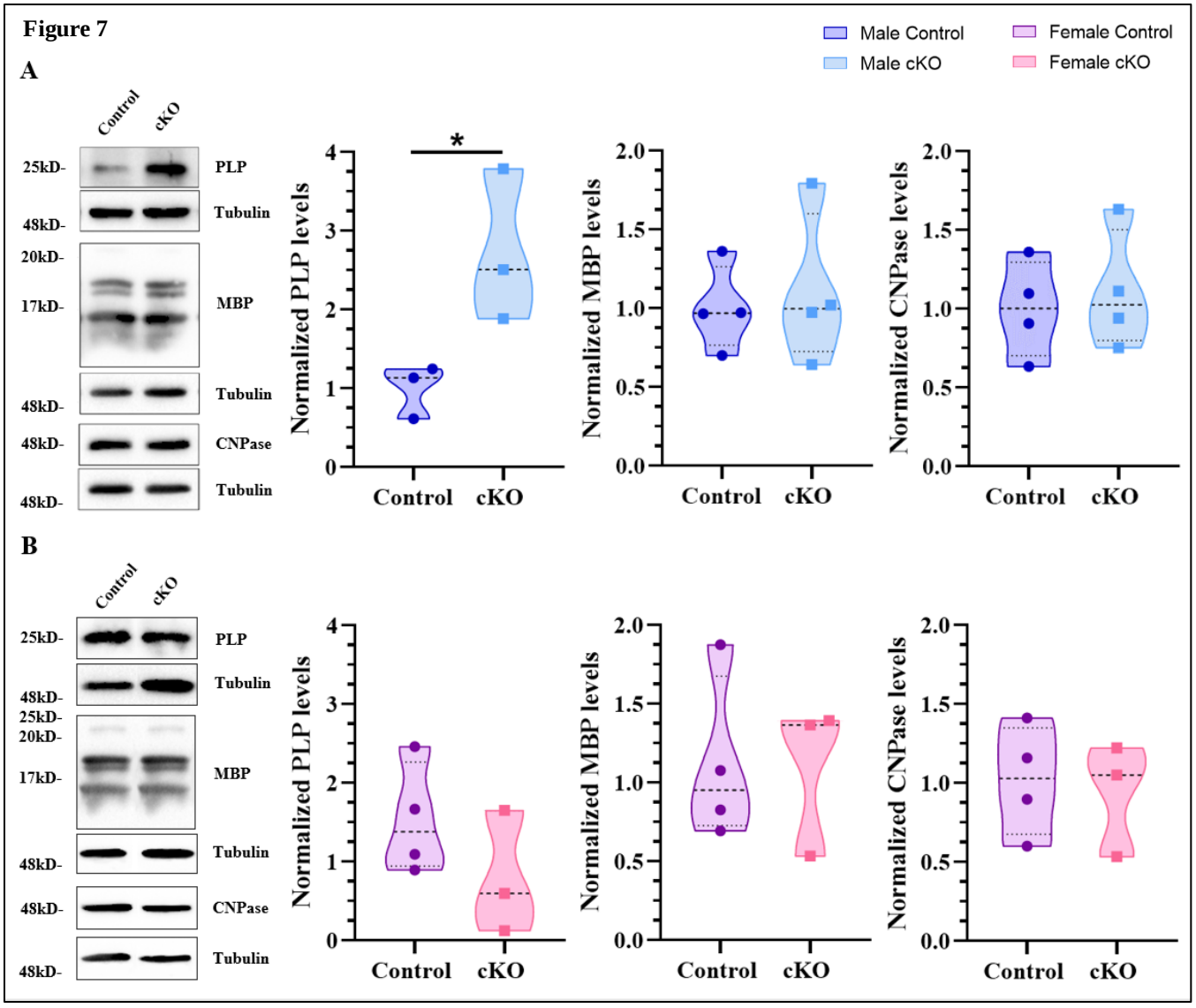


Figure 7: Effect of AGT5-mediated macro-autophagy blockade on myelin protein levels.

A) Western blot analysis in optic nerve lysates of 6mo control and cKO male mice (antibodies against PLP, MBP, CNPase) and quantification of normalized protein levels. Representative images are depicted.

B) Western blot analysis in optic nerve lysates of 6mo control and cKO female mice (antibodies against PLP, MBP, CNPase) and quantification of normalized protein levels. Representative images are depicted.

Data are shown as mean ± SEM. N=3-4 animals per genotype. *p < 0.05

3.3 Effect of ATG5-mediated macro-autophagy blockade on the integrity of CNS myelin

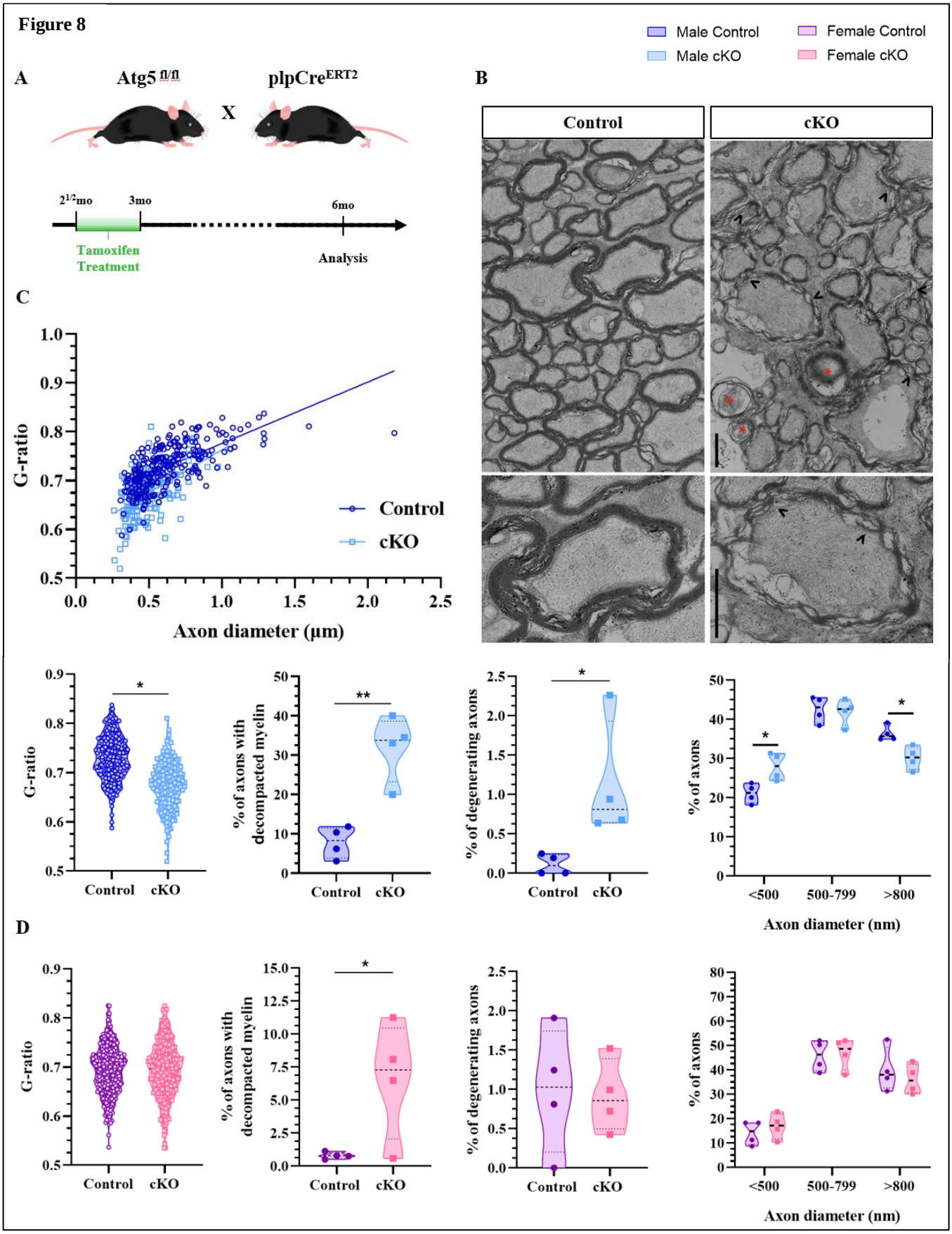
The aforementioned sexually-dimorphic accumulation of PLP urged us to investigate whether the ATG5-mediated macro-autophagic pathway has a sex-dependent role in the maintenance of normal myelin structure, and thus the integrity of myelinated axons, in the CNS of adult mice.

For this purpose, electron microscopic analyses were performed using optic nerves from 6-month-old control and cKO mice (Figure 8). According to the results, mice of the latter group had myelinated axons with smaller g-ratios than that of male controls (Linear regression, $p = 0.03$) (Figure 8C). To add to this, cKO males had a significantly larger percentage of axons with decompacted myelin (t-test, $p = 0.002$) and a greater number of degenerating axons (t-test, $p = 0.03$), when compared to their counterparts (Figure 8B, C). Lastly, male cKO mice displayed a higher percentage of small caliber axons (two-way ANOVA and Sidak's post-hoc test, $p = 0.01$) and a lower percentage of large caliber axons (two-way ANOVA and Sidak's post-hoc test, $p = 0.02$) when compared to mice of the control group (Figure 8C).

As far as the females are concerned, the g-ratio analysis did not reveal any differences between the two groups (Linear regression, $p = 0.23$) (Figure 8D). Moreover, despite the lack of differences in the percentage of degenerating axons (t-test, $p = 0.87$), cKO mice appeared to have significantly more axons with decompacted myelin (t-test, $p = 0.04$) when compared to controls (Figure 8D, E). Finally, contrary to male mice, cKO female mice displayed similar percentages of axons of specific caliber (two-way ANOVA and Sidak's post-hoc test, $p = 0.91$ for <500 nm, $p = 0.99$ for 500-799 nm, $p = 0.80$ for >800 nm) to that of control mice (Figure 8D).

Taking into consideration the aforementioned data, it appears that the blockade of the ATG5-mediated autophagic pathway affects the g-ratio, the efficiency of myelin compaction and the survival of myelinated axons in males (large caliber axons presumably being the most vulnerable). This is not the case, however, for females in which only the compaction efficiency was affected. These results support the idea that this specific autophagic mechanism manifests in a sexually-dimorphic fashion on the integrity of CNS myelin during adulthood.

Figure 8


Male Control
Male cKO
Female Control
Female cKO
A
Atg5 fl/fl
X
plpCreERT2
2 1/2mo
3mo
6mo
Tamoxifen Treatment
Analysis
B
Control
cKO
C
G-ratio
Axon diameter (µm)
Control
cKO
% of axons with decompacted myelin
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500-799
>800
Axon diameter (nm)
D

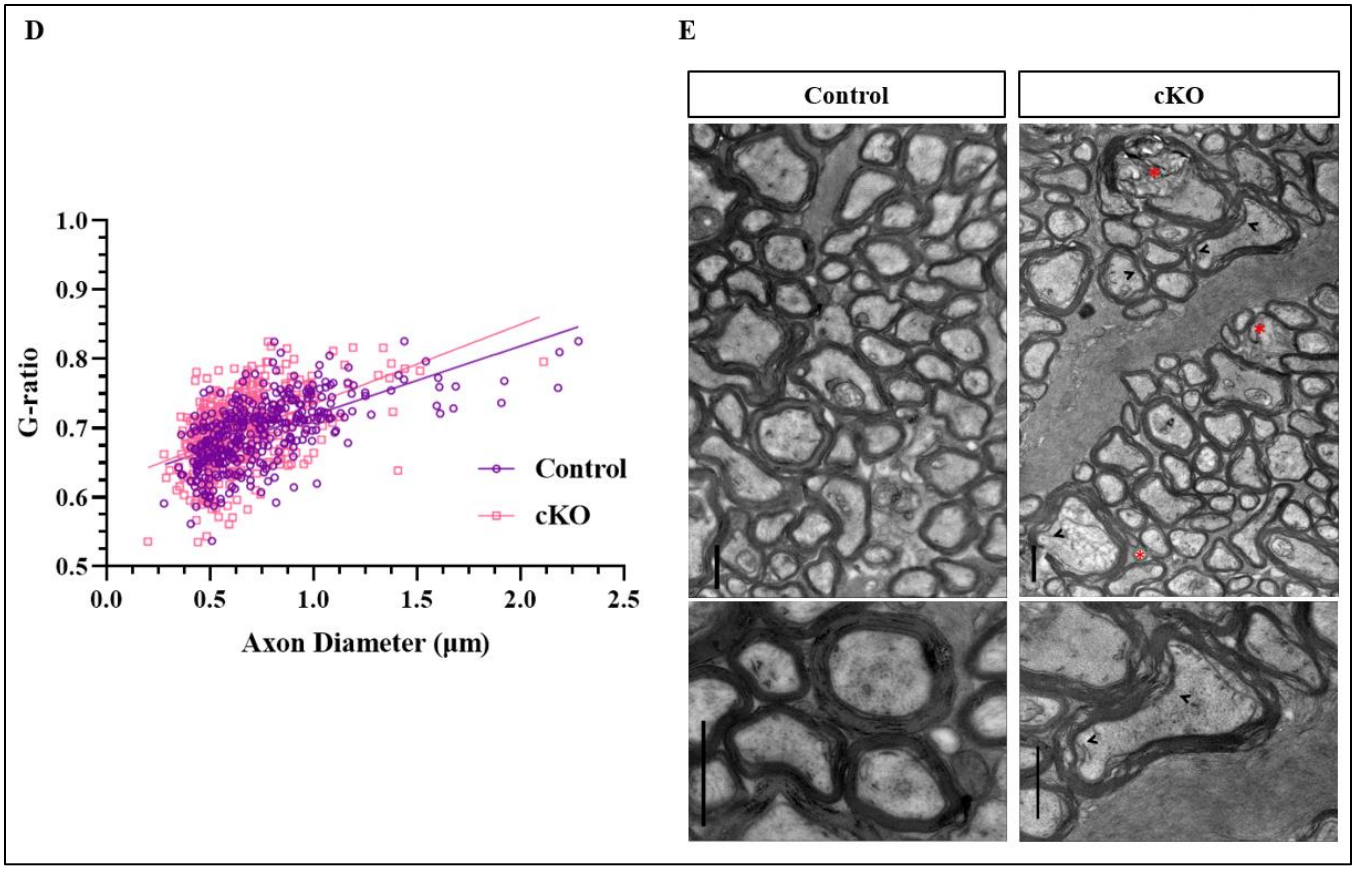


Figure 8: Effect of ATG5-mediated macro-autophagy blockade on the integrity of CNS myelin.

A) Experimental plan for tamoxifen-induced recombination in plpCre^{ERT2}; atg5 $^{(fl/fl)}$ mice. All subjects received 10 intraperitoneal injections of 100 μg of tamoxifen per day, for 10 days (with a 2-day break in between), at the age of 2.5 months. The respective analysis was performed at the age of 6 months.

B) Ultrastructural analysis of optic nerves from control and cKO male mice. In the respective electron micrographs of coronal sections, the presence of both degenerated axons (red stars) and decompacted myelin sheaths (arrowheads) is demonstrated. Scale bars: 1μm.

C) Assessment of myelin sheath thickness by calculating the g-ratio of axons in the optic nerve of control and cKO male mice. Plots of g-ratio distribution to different axonal diameters (linear regression, 260 axons measured for the control and 170 axons for the cKO group) and of the g-ratio values per animal group (each point represents an axon). Quantification of axons with decompacted myelin sheaths and of axonal degeneration events in optic nerves of control and cKO male mice. Quantification of the percentage of axons with small (< 500 nm), medium (500-799 nm) and large (> 800 nm) diameter in the optic nerve of control and cKO male mice (450 axons measured per sample).

D) Assessment of myelin sheath thickness of axons in the optic nerve of control and cKO female mice. Plots of g-ratio values per animal group and of g-ratio distribution to different axonal diameters (linear regression, 361 axons measured for the control and 356 axons for the cKO group). Quantification of axons with decompacted myelin sheaths and of degenerating axons. Quantification of the percentage of axons with small (< 500 nm), medium (500-799 nm) and large (> 800 nm) diameter in the optic nerve of control and cKO female mice (300 axons measured per sample).

E) Ultrastructural analysis of optic nerves from control and cKO female mice. In the respective electron micrographs of coronal sections, the presence of both degenerated axons (red stars) and decompacted myelin sheaths (arrowheads) is demonstrated. Scale bars: 1μm.

Data are shown as mean ± SEM. N=4 animals per genotype. Violin plots present individual values. *$p < 0.05$, **$p < 0.01$

3.4 Effect of ATG5-mediated macro-autophagy blockade on motor-related behavior and function

Taking into account both the relationship between OL-mediated myelination and motor learning and the myelin perturbations detected under conditions of defective macro-autophagy, we examined whether the dysfunction of the respective pathway also leads to motor-related deficiencies (McKenzie et al. 2014).

3.4.1 Open Field Test

The first step towards examining whether ATG5-mediated macro-autophagy has a sex-dependent effect on the motor-related behavior of adult mice, involved studying the results of the respective blockade on locomotion and anxiety (Figure 9B).

For this purpose, 6-month-old control and cKO mice of either sex were used. According to the results, males did not display any differences in either their ambulatory ability (t-test, $p = 0.95$) or their anxiety levels (t-test, $p = 0.39$) (Figure 9B). On the same side of the spectrum lied also the respective female groups (t-tests, $p = 0.29$ for total distance travelled and $p = 0.30$ for Thigmotaxis) (Figure 9B).

Based on these data, it appears that the specific autophagic mechanism does not have a sexually dimorphic effect on either anxiety or locomotion. Furthermore, since the former is inversely proportional to the latter, the similar locomotor activity of control and cKO mice further supports the previous notion.

3.4.2 Rotarod Test

In the next step we sought to investigate the relationship between myelin-dependent functions, such as motor coordination and learning, and the ATG5-mediated autophagic pathway.

For this purpose, age-matched control and cKO mice of either sex were used. As far as the males are concerned, the control group displayed significant motor learning capacity (two-way ANOVA and Tukey's post hoc test, $p = 0.004$ for Day1 vs Day 2, $p < 0.0001$ for Day1 vs Day 3) during the 3-day accelerating trial, something which was not the case for the cKO group (two-way ANOVA and Tukey's post hoc test, $p = 0.06$ for Day 1 vs Day 3) (Figure 9C). Motor coordination, on the other hand, did not differ between the groups in either the low-speed (Mann-Whitney test, $p = 0.40$) or the high-speed task (t-test, $p = 0.23$) (Figure 9C).

Interestingly enough, the comparison of female control and cKO mice did not display equivalent results to that of males. More specifically, neither group improved its performance during the accelerating trial which, in turn, translates to similar motor learning skills (Figure 9C). To add to this, no differences were also detected during the motor coordination testing phase (Mann-Whitney, $p = 0.49$ for Day 4 and $p = 0.16$ for Day 5) (Figure 9C).

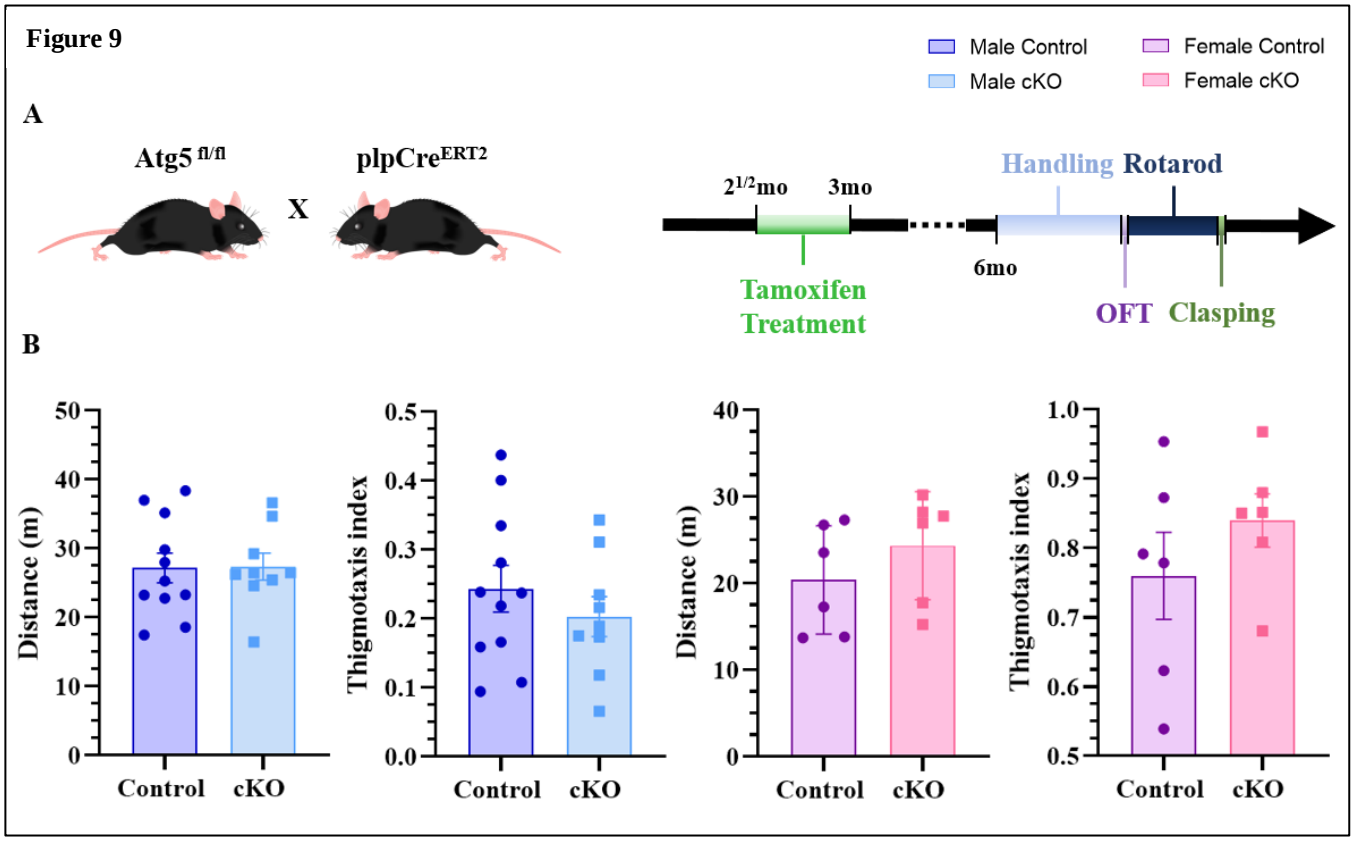
Taking everything into consideration, it appears that the ATG5-mediated autophagic mechanism has the capacity to alter motor learning, but not motor coordination, in mice. However, since only males were affected by the blockade of the respective pathway, it seems that the effect is at least partially sex-dependent.

3.4.3 Hindlimb Clasping Test

The last part of the behavioral testing entailed verifying whether the ATG5-mediated pathway has indeed no significant effect on the motor coordination of mice.

For this purpose, the Hindlimb Clasping test was conducted using the same groups of adult mice. According to the respective clasping scores, no significant differences were detected between control and cKO mice (Mann-Whitney tests, p = 0.33 for male groups and p = 0.39 for females) (Figure 9D).

So, keeping in mind the aforementioned results, it appears that specific autophagic mechanism does not play a significant role in the motor coordination of either male or female mice.



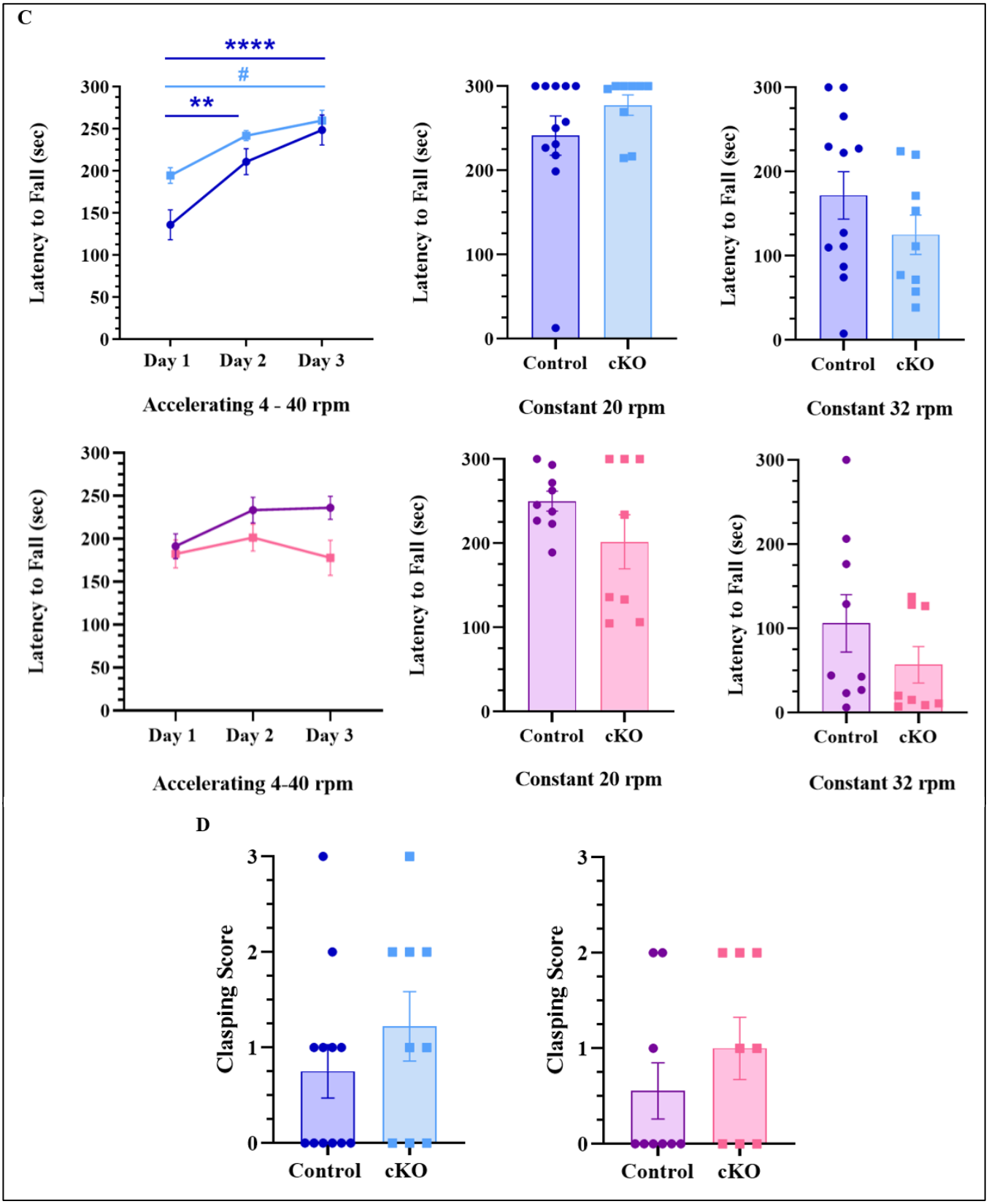

C
****
#
**
Latency to Fall (sec)
300
250
200
150
100
50
0
Day 1
Day 2
Day 3
Accelerating 4 - 40 rpm
Control
cKO
Constant 20 rpm
Constant 32 rpm
Accelerating 4-40 rpm
D
Clasping Score
3
2
1
0

Figure 9: Effect of ATG5-mediated macro-autophagy blockade on behavior and motor function.

A) Schematic illustration of experimental plan for behavioral tests. All subjects received 10 intraperitoneal injections of 100 μg of tamoxifen per day, for 10 days (with a 2-day break in between), at the age of 2.5 months. At the age of 6 months, all subjects were subjected to 4 days of handling before the conduction of the Open Field Test (OFT), the Rotarod test and lastly the Hindlimb Clasping task.

B) Assessment of locomotor activity and anxiety levels of male (n = 11 CTR and n = 9 cKO) and female (n = 6 CTR and n = 6 cKO) mice. Bar graphs representative of the total distance travelled and of the thigmotaxis index (time spent at outer zone/ total time). Only the first 5 min of each OFT session were analyzed.

C) Assessment of motor learning and motor coordination of male and female control and cKO mice. Line graphs representative of the performance of each genotype/sex group during the first 3 days of the experiment (i.e., motor learning testing phase). Bar graphs indicative of the latency of mice from each group to fall during the fourth and fifth day of the experiment (motor coordination testing phase), respectively.

D) Assessment of genotype effect on motor coordination of male and female mice. Bar graphs indicative of the clasping level of hindlimbs (0 = hindlimbs splayed outwards, 1 = one hindlimb retracted, 2 = two hindlimbs partially retracted, 3 = fully retracted hindlimbs).

Data are shown as mean ± SEM. N=8-12 animals per genotype for Rotarod and Clasping tests. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, $^{\#}p < 0.1$.

3.5 Effect of ATG5-mediated macro-autophagy blockade on Oligodendrocyte survival in male mice

In the last part of this study, we sought to determine whether the ATG5-mediated autophagic pathway plays a significant role in the survival of Oligodendrocyte-lineage cells in adult males.

For this purpose, a series of immunohistochemistry experiments took place in which coronal sections of the rostral Corpus Callosum (CC) of 6-month-old control and cKO male mice were exclusively used (Figure 10). The region of the CC was selected due to it having a high density of both mature oligodendrocytes (i.e., $CC1^+$ cells) and oligodendrocyte progenitor cells (i.e., $PDGFRa^+$ cells) (Figure 10A). According to the results, the two groups did not differ as far as the density of either subpopulation was concerned (Mann-Whitney test, $p = 0.68$ for OLs and t-test, $p = 0.79$ for OPCs) (Figure 10B). To add to this, the absence of cleaved Caspase-3 activity (marker of apoptosis) in the general region of the CC further supported the notion of no effect (Figure 10C).

Bearing in mind the respective results, it appears that the ablation of the aforementioned autophagic pathway in OLs does not affect their survival nor the OPCs' in adult males, at least for the following period of 3 months.

Figure 10

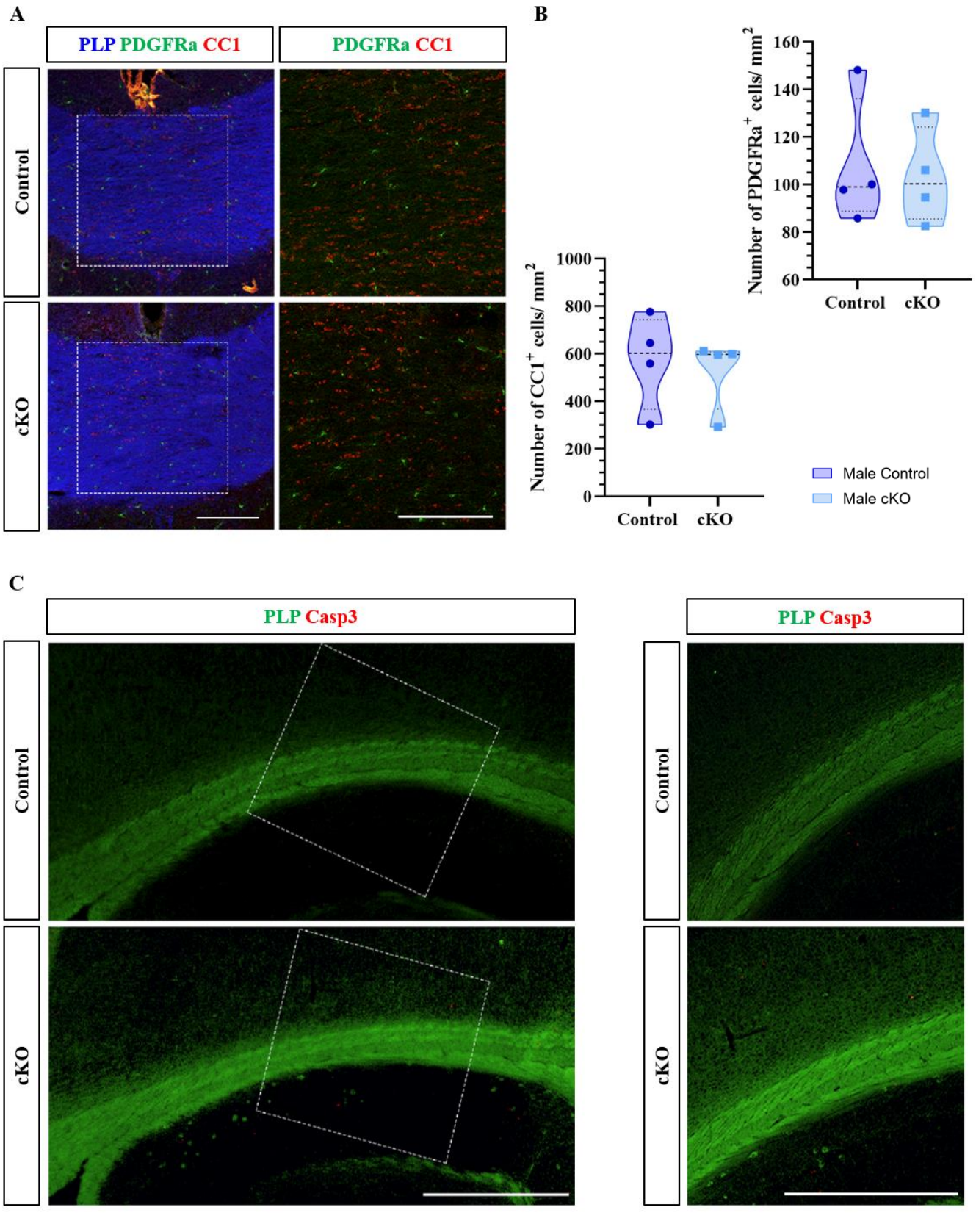

A
PLP PDGFRa CC1
PDGFRa CC1
Control
cKO
B
Number of CC1+ cells/ mm2
1000
800
600
400
200
0
Control
cKO
Number of PDGFRa+ cells/ mm2
160
140
120
100
80
60
Control
cKO
Male Control
Male cKO
C
PLP Casp3
PLP Casp3
Control
cKO
Control
cKO

Figure 10: Effect of ATG5-mediated macro-autophagy on Oligodendrocyte survival in male mice.

A) Immunohistochemical analysis of rostral Corpus Callosum cryosections from 6 months old control and cKO mice. PDGFRa (green) and CC1 (red) are OL lineage markers for OPCs and mature OLs, respectively. PLP immunostaining is used for the identification of the area of interest.

B) Density of mature OLs (i.e., $CC1^+$ cells) and OPCs (i.e., $PDGFRa^+$ cells) in the rostral Corpus Callosum.

C) Confocal images of sagittal Corpus Callosum sections from control and cKO mice immunostained for PLP (green) and cleaved Caspase-3 (red). Boxes indicate the areas magnified on the right.

Data are shown as mean ± SEM. N= 4 animals per genotype; Scale bars: 200 μm (A), 500 μm (C).

IV. Discussion

The purpose of this study was firstly to underline the significance of well-regulated macro-autophagy for the maintenance of normal myelination in the adult mouse brain, and secondly to elucidate whether basal autophagy has a sexually dimorphic effect on CNS myelin during adulthood. Both the importance of macro-autophagy and the existence of a sex-dependent effect on myelination were investigated through the conditional deletion of ATG5, specifically in OL-lineage cells of young adult male and female mice. The results of the respective ablation were examined 3 months later on a cellular, structural and behavioral level. Interestingly, our data revealed the existence of a sexually dimorphic effect of autophagy blockade on protein levels, myelin integrity and motor-related processes.

4.1 Sexually dimorphic effect of macro-autophagy blockade on PLP levels

The analyses of myelin protein levels, after ablation of the ATG5-mediated macro-autophagic pathway, revealed the existence of a sex-dependent effect. More specifically, while the levels of MBP and CNPase appeared relatively similar between control and cKO mice of either sex, there was a significant increase in the levels of PLP in cKO males when compared to their age- and sex-matched counterparts.

Recent evidence confirms an autophagy-mediated degradation of myelin proteins (i.e., PLP and MBP incorporation in OL-derived autophagosomes) (Ktena et al. 2022). Compared to the other myelin proteins (i.e., MBP, CNPase), PLP is considerably more abundant in the CNS (consists ~50% of CNS myelin protein) (Boggs et al. 2006). The higher levels of PLP could, in turn, contribute to it being more prone to accumulation in conditions of defective autophagy. To add to this, alternative mechanisms (i.e., proteasome) have been found to mediate the specific degradation of myelin proteins such as MBP (Bacheva et al, 2009; Belogurov et al. 2009; Belogurov et al. 2014). This mechanism of action could also play a role in the degradation of CNPase, under both physiological and pathological conditions (i.e., the absence of a functional macro-autophagic machinery). Were that to be true, it could sufficiently explain the observed increase in PLP levels and the respective absence of MBP and CNPase accumulation.

Male rodents presumably have higher levels of myelination in their CNS (including the optic nerve), based on the aforementioned CC data, which practically translates into them having greater amount of

myelin proteins (e.g., PLP) compared to females (Yates and Juraska, 2007; Patel et al., 2013; Cerghet et al., 2006; Pesaresi et al, 2015). To add to this, a higher density of myelinating OLs has been detected throughout the brain and spinal cord of males, when compared to females (Cerghet et al., 2006; Patel et al., 2013). These sex-dependent differences could mean that in males a proportionally larger amount of PLP is due for macro-autophagy-mediated degradation. Interestingly, the data associated with basal autophagy levels between males and females are rather controversial. More specifically, while a higher degradation rate of proteins (e.g., myelin proteins) in the CNS of adult females has been proposed by Cerghet et al (2006), we must take into account that the respective data referred to both OLs and neurons (Cerghet et al., 2006). Other studies support the notion that males have higher levels of autophagy compared to females (data from neonatal rat brains) (Demarest et al. 2016). Finally, a recent study by Olivan et al. (2014) revealed a sex- and tissue-specific difference in the levels constitutive autophagy that is also age-dependent (Olivan et al. 2014).

In relation to that, sex hormones appear to play a significant role not only in the regulation of OL- and myelin-related processes, but also in the regulation of autophagy. In combination with the fact that males and females differ substantially as far as their hormonal environment in the CNS is concerned (i.e., expression level of sex-steroids and respective receptors), a sexually dimorphic regulation of autophagy is highly possible. This, in turn, could lead to a sexually dimorphic dependency on the autophagic pathway for the degradation of myelin proteins such as PLP (e.g., PLP also degraded by proteasome), which could explain why only males were affected by the ablation. Nonetheless, these results suggest that the clearance/degradation of PLP in males is greatly dependent on macro-autophagy.

4.2 Sexually dimorphic effect of macro-autophagy blockade on myelin structure and axon integrity

Structural analyses of the optic nerve of adult mice revealed a sexually dimorphic effect of ATG5-mediated macro-autophagy blockade on both myelin structure and the integrity of the respective axons.

As far as the female groups are concerned, only an increased percentage of axons with decompacted myelin was detected in cKO mice, when compared to controls. Males, on the other hand, were more severely affected. To begin with, the myelinated axons of cKO male mice were characterized by smaller g-ratios, when compared to those of controls, which practically translates into them having either thicker or less compacted myelin sheaths. The latter seems more plausible considering the higher percentage of myelinated axons with decompacted myelin that was detected in the respective group. Furthermore, the frequency of axonal degeneration events was significantly increased in the absence of a working autophagic machinery. Last but not least, a marked drop in the number of large caliber axons was accompanied by an increase in the percentage of small caliber axons in cKO mice.

While both male and female cKO groups displayed increased levels of axons with myelin decompaction, when compared to their same-sex counterparts, only males displayed a decrease in mean g-ratio. Considering that the evaluation of mean g-ratio was based only on normal-looking axons, it is

possible that this difference results from the inclusion (in the male samples) of axons that were in the first stages of myelin decompaction (consequences of inefficient compaction are not yet visible). In turn, the similar g-ratios of cKO and control females could indicate that a lower percentage of seemingly normal axons was included in the respective samples. Taking this into consideration, a closer examination of myelin lamellae should be conducted in order to confirm the source of the discrepancy.

The significance of well-regulated macro-autophagy for normal myelination during the early stages of life was proven by Bankston et al (2019), considering that neonatal deletion of autophagy in OL-lineage cells at the OPC stage resulted in both poor myelin compaction and reduced number of axons with normal myelin (Bankston et al, 2019). Although autophagy seemingly has an equivalent role during adulthood, at least for males, the visual comparison of optic nerves from male and female cKO mice revealed that the former group is characterized by a severe state of demyelination (i.e., decompaction with increased incidence of axonal death), whereas the latter displays a relatively mild state of dysmyelination (i.e., decompaction without events of axonal lesions). To add to this, the accumulation of PLP (due to abnormal protein expression) has the capacity to cause, in time, both demyelination and the degeneration of axons in CNS myelin tracts (e.g., optic nerve) (Anderson et al 1998, Kagawa et al. 1994). Combined with the fact that macro-autophagy appears to play a particularly significant role in the degradation of PLP only in males, it is highly plausible that the observed sex-dependent accumulation is responsible for the respective sexual-dimorphism in phenotype severity (i.e., males with myelin decompaction and significant levels of axonal degeneration).

In male cKO mice, the high levels of axonal degeneration were accompanied by a shift in the percentages of axon caliber diameter towards smaller values. This is indicative of a higher susceptibility of larger caliber axons to degeneration elicited by autophagic dysfunction. Since under normal conditions myelin thickness seems to increase proportionally to axon caliber in the CNS (e.g., spinal cord, hindbrain), it is possible that thicker myelin sheaths may have greater autophagy-mediated maintenance requirements, compared to thinner myelin sheaths (Ishii et al, 2012). Were that to be true, large caliber axons would indeed be more vulnerable to degeneration, something which would sufficiently explain the afore-mentioned results.

As we mentioned previously, by regulating myelin-related processes, sex hormones influence the level and pattern of myelination (Acs et al., 2009; De Nicola AF et al., 2006; El-Etr et al., 2015; Patel et al., 2013; Cerghet et al., 2006; Pesaresi et al, 2015; Abi Ghanem et al., 2017; Abi Ghanem et al., 2017). To add to this, both male (e.g., testosterone) and female (e.g., 17-beta-estradiol, progesterone) sex hormones protect axons from demyelination/degeneration and positively assist to their remyelination under various pathological conditions (e.g., ALS, SCI, Cuprizone-induced demyelination, LPC, EAE) (Patel et al., 2013; Lee et al. 2012; Labombarda et al. 2011; Labombarda et al.2008; Bielecki et al. 2016; Breton et al. 2021; Lee et al. 2012). Of particular interest is the fact that, although sex hormones exert their neuroprotective effect irrespectively of sex, their potency appears to be different (Labombarda et al. 2011; Labombarda et

al.2008; Taylor et al. 2010; Groeneveld et al. 2004; Choi et al. 2007; Heitzer et al. 2017; Kim et al. 2013; Toung et al. 1998; Rusa et al. 1999; Garay et al. 2011; Hussain et al. 2013; Patel et al., 2013). Lastly, although TBI and MCAO models present female hormones as more neuroprotective, we should bear in mind that all models display sex-dependent and pathology-specific vulnerability (Roof et al. 1993; Alkayed et al. 1998).

Taking everything into account, a sexually dimorphic dependency on macro-autophagy (for the degradation of PLP) in combination with the sexually dimorphic hormonal environment (i.e., sex- and pathology-dependent neuroprotection), could explain why males were more severely affected by the respective ablation. Nonetheless, these results confirm the role of macro-autophagy in myelin maintenance during adulthood in both males and females.

4.3 Sexually dimorphic effect of macro-autophagy blockade on motor learning

Both motor learning and motor coordination are closely associated with myelin integrity. In relation to this, the respective analyses showed that the ablation of ATG5-mediated macro-autophagy had a sex-dependent effect only in motor learning.

As far as anxiety and locomotion are concerned, no differences were detected between cKO and control mice of either sex. To add to this, females appeared to be completely unaffected by the respective blockade considering the absence of motor learning and the similar levels of motor coordination (examined via rotarod and hindlimb clasping test) between the two groups. This, however, was not exactly the case for male groups. More specifically, although no differences were detected in motor coordination between cKO and control mice, only the latter displayed significant learning capacity during the first 3 days of the rotarod task (i.e., Day1 < Day2 and Day1 < Day3).

Motor learning has been associated with a variety of areas in the adult murine brain including the motor cortex (M1/M2), the cerebellum and the striatum (Scholz et al. 2015). As far as the striatum is concerned, dysregulation of its neuronal activity has been linked to reduced learning in the accelerated rotarod task (Hirata et al. 2016). In relation to this, mice harboring extra copies of PLP have impairments of myelin that disrupt the thalamocortical neuronal activity which, apparently, is also necessary for efficient motor learning (i.e., the synchrony of cortical spike time arrivals through long range axons facilitating the propagation of information required for learning is not maintained) (Kato et al. 2019). The significance of normal myelination is further supported by the fact that associated perturbations (e.g., hypomethylation, disorganization of nodes of Ranvier) ultimately result in impaired motor learning (Luo et al. 2018). Evidently, the capacity for active myelination during adulthood (i.e., capacity to generate OLs and form new myelin) is the most critical factor in determining the efficiency of motor learning (McKenzie et al. 2014; Xiao et al. 2016). To add to this, although defects in active myelination resulted in inability of mice to master the respective skill (i.e., running on a complex wheel), motor coordination was not affected (McKenzie et al. 2014).

Since male cKO mice are characterized by both myelin abnormalities and a marked decline in their motor learning capacity, we can safely say that there are significant myelin perturbations in CNS regions related to this process (e.g., striatum, motor cortex, cerebellum, thalamus). As we mentioned previously, it is possible that the respective abnormalities are the result of PLP accumulation caused by the ablation of ATG5-mediated autophagy. To add to this, since only motor learning was affected (but not motor coordination), a greater dependency of adult-born OLs on the macro-autophagic machinery for their normal function could sufficiently explain our results. Finally, the proposed region-specific vulnerability could be attributed to the region-specific expression levels of sex hormones and receptors (i.e., differential level of neuroprotection).

In the case of females, the lack of differences on a behavioral level is in line with the less severe phenotype displayed in the previous experiments (i.e., no PLP accumulation, insignificant axonal degeneration, dysmyelination). Of particular interest, however, is the fact that control females did not show any signs of motor learning. Future analyses of the respective estrus cycle samples are due as they could provide a valuable insight to the aforementioned discrepancies (i.e., clarify if females were synchronized, in which stage was each subject), providing there is indeed a correlation of hormone concentrations with motor learning performance. Nevertheless, it appears that a sexually dimorphic dependency on autophagy, combined with a sex-dependent neuroprotection, could sufficiently explain the observed results.

4.4 Blockade of macro-autophagy does not affect the survival of OL-lineage cells in males

The immunohistochemical analyses that were performed showed that the ablation of the ATG5-mediated macro-autophagic pathway, in myelinating OLs of adult males, did not affect the survival of OL-lineage cells. More specifically, cKO and control mice displayed similar densities of both OPCs and OLs, in the rostral corpus callosum. To add to this, no cleaved caspase 3-related activity was detected in the respective region.

Based on these results, it appears that the accumulation of PLP is not toxic to OLs under these specific conditions of defective autophagy. In relation to this, sex hormones are involved in the regulation of OL cell death and the end result is critically dependent on both the combination of existing hormones and their respective concentrations (Swamydas et al., 2008). Taking also into account that the castration of male mice results in decreased OL density and that hormone administration (i.e., testosterone, DHT, 17-beta-estradiol) can restore to some extent the number of OPCs/OLs after normal and demyelination conditions, a hormone-mediated protection effect could also be contributing to the maintenance of OL-lineage density (Cerghet et al., 2006; Hussain et al. 2013; Patel et al., 2013).

Since a well-regulated autophagic machinery appears to be nonessential for the survival of OL-lineage cells in males, which were ultimately the most affected by the blockade, it is highly possible that this is also the case for females. Further experiments, however, testing the importance of ATG5-mediated macro-

autophagy for the survival of female-derived OL-lineage cells should be conducted so as to ensure the validity of our assumptions.

V. Conclusion

To sum up, our data confirm the involvement of macro-autophagy in the maintenance of normal myelination in the adult CNS of both male and female mice. To add to this, the survival of OL-lineage cells is seemingly independent of the respective pathway. Of utmost importance is the fact that a sexually dimorphic role of the macro-autophagic mechanism was also confirmed, considering that the respective blockade in mature OLs resulted in males with a different pathologic phenotype (i.e., PLP accumulation, demyelination, axon degeneration and motor learning deficits in males) than females (i.e., dysmyelination). Our hypothesis is that the hormonal environment contributes to the emergence of a sexually dimorphic regulation of autophagy in OLs. As a result, male- and female-derived OLs are differentially dependent on the respective machinery (e.g., PLP degradation) for their normal function (i.e., maintenance of myelin homeostasis). Considering the observed sex-dependent vulnerability to the ablation of autophagy (i.e., males > females), we propose that myelin maintenance of only adult males is critically dependent on a well-regulated macro-autophagy. Overall, the aforementioned results highlight the necessity of gender control in neuro-experimental studies.

VI. References

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