UNIVERSITY OF CRETE

MEDICAL SCHOOL

LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY

IMBB-FORTH

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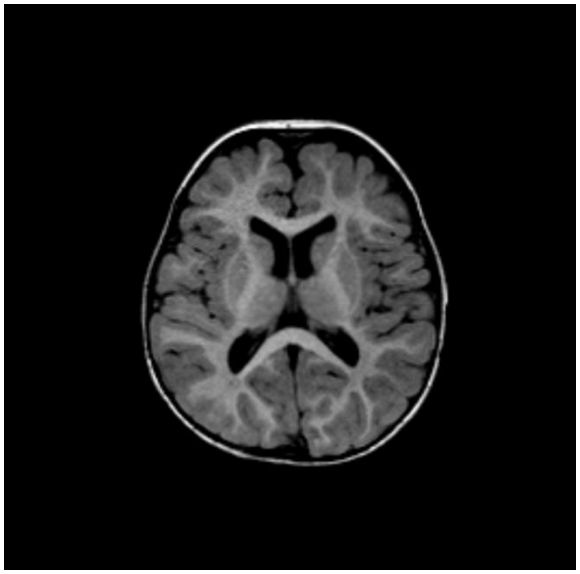

MASTER THESIS

"The effect of the absence of TAG-1 in the expression level of specific myelin genes and transcription factors"

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ABSTRACT

Myelination is a very important procedure of the nervous system. The main purpose of a myelin sheath is to increase the speed at which impulses propagate along the myelinated fiber. Myelin decreases capacitance and increases electrical resistance across the cell membrane. Thus, myelination prevents the electrical current from leaving the axon and helps in the transmission of the electrical signal that is required for the coordination of the vertebrate nervous system between different brain areas. Schwann cells supply the myelin for the peripheral nervous system, whereas oligodendrocytes myelinate the axons of the central nervous system. Loss of myelin in the axons is one of the main reasons for a lot of neurodegenerative diseases.

The cell adhesion molecule TAG-1 is expressed by neurons and glial cells and plays a very important role in axon outgrowth, migration and fasciculation during development. It also plays a very important role in the production and maintenance of myelin. Absence of TAG-1 leads to a lot of abnormalities and axon dysfunctions. One of these is the hypomyelination that occurs in axons.

This hypomyelination may occur either from defects in oligodendrocytes development or from defects during myelination process. In our study we focus in the possible defects during myelination process. The goal of our experiments was to examine if there is any difference in the expression level of specific myelin genes (*plp, mbp, cnp, mag, nogo-a*) and transcription factors (*zfp, mrf*) in WT and KO mice for TAG-1 in three different time points: in P10 (initiation of myelination in mice), P21 (pick of myelination in mice) and P30 (end of myelination).

Our results indicate that in TAG-1 KO mice there is an overall delay in the production of myelin genes (*plp,mbp, cnp, nogo-a*) and probably a delay in myelination. There is also a mechanism that tries to repair this defect through the up-regulation of the transcription factor MRF.

ΠΕΡΙΛΗΨΗ

Η μυελίνωση είναι μια πολύ σημαντική λειτουργία του νευρικού συστήματος. Ο κύριος σκοπός του στρώματος μυελίνης είναι να αυξηθεί η ταχύτητα με την οποία οι ώσεις διαδίδονται κατά μήκος των εμμύελων ινών. Η μυελίνη μειώνει την χωρητικότητα και αυξάνει την ηλεκτρική αντίσταση κατά μήκος της κυτταρικής μεμβράνης. Έτσι, η μυελίνωση αποτρέπει την έξοδο του ηλεκτρικού ρεύματος από τον νευράξονα και βοηθάει στην σωστή μετάδοση του ηλεκτρικού σήματος για την φυσιολογική λειτουργία του νευρικού συστήματος. Τα κύτταρα Schwann παρέχουν την μυελίνη του περιφερικού νευρικού συστήματος , ενώ τα ολιγοδενδροκύτταρα παρέχουν μυελίνη στους άξονες του κεντρικού νευρικού συστήματος .

Το μόριο κυτταρικής συνάφειας TAG-1 εκφράζεται από νευρώνες και ολιγοδενδροκύτταρα και παίζει πολύ σημαντικό ρόλο κατά την ανάπτυξη των νευραξόνων, όπως επίσης και κάτα την διαδικασία της μυελίνωσης. Απουσία της πρωτείνης TAG-1 έχει σαν αποτέλεσμα την εμφάνιση ορισμένων ανωμαλιών και δυσλειτουργιών όσον αφορά τους άξονες. Μία από αυτές είναι και η υπομυελίνωση που παρατηρείται στους νευράξονες.

Η υπομυελίνωση αυτή μπορεί να οφείλεται είτε σε κάποια δυσλειτουργία κατά την διάρκεια της ανάπτυξης των ολιγοδενδροκυττάρων, είτε σε κάποια δυσλειτουργία κατά την διάρκεια της διαδικασίας της μυελίνωσης. Ο στόχος των πειραμάτων μας ήταν να εξετάσουμε αν υπάρχει κάποια διαφορά μεταξύ ποντικιών WT και KO για την TAG-1 στην έκφραση συγκεκριμένων γονιδίων (*plp, mbp, cnp, mag, nogo-a*) και μεταγραφικών παραγόντων (*zfp, mrf)*που παίζουν σημαντικό ρόλο κατά την διαδικασία της μυελίνωσης. Τα πειράματα μας έγιναν σε 3 διαφορετικές ηλικίες, στη ηλικία P10 (έναρξη μυελίνωσης), στη ηλικία P21 (pick της μυελίνωσης) και στην ηλικία P30 (τέλος της μυελίνωσης).

Τα αποτελέσματα των πειραμάτων μας έδειξαν πως στα KO ποντίκια υπάρχει μία καθυστέρηση στην έκφραση ορισμένων από τα γονίδια που αναφέραμε παραπάνω (*plp, mbp, cnp, nogo-a)* και ως εκ τούτου παρατηρείται μια καθυστέρηση και στην διαδικασία της μυελίνωσης. Επίσης με βάση τα αποτελέσματα των πειραμάτων μας είναι πιθανόν η υπερέκφραση του μεταγραφικού παράγοντα MRF να λειτουργεί σαν ένας αμυντικός μηχανισμός προσπαθώντας να επιδιορθώσει αυτή την δυσλειτουργία.

INTRODUCTION

Myelination

Myelin is an electrically insulating material that forms a layer usually around only the axon of a neuron. This layer is called myelin sheath (figure 1). Myelin is essential for the proper functioning of the nervous system. It is an outgrowth of a type of glial cells [1].

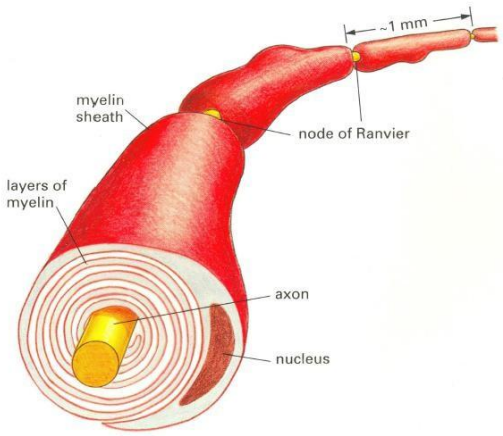

Figure 1: Myelination

The production of the myelin sheath is called myelination. In humans, myelination begins in the 14th week of fetal development, although little myelin exists in the brain at the time of birth. During infancy, myelination occurs quickly, leading to a child's fast development, including crawling and walking in the first year. Myelination continues through the adolescent stage of life [1].

Schwann cells supply the myelin for the peripheral nervous system (figure 2), whereas oligodendrocytes myelinate the axons of the central nervous system (figure 3).

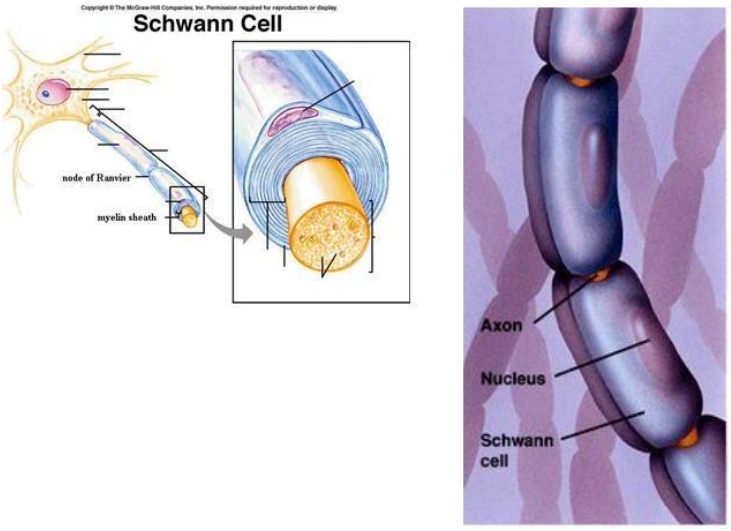

Figure 2: Schwann cells in myelinating axons

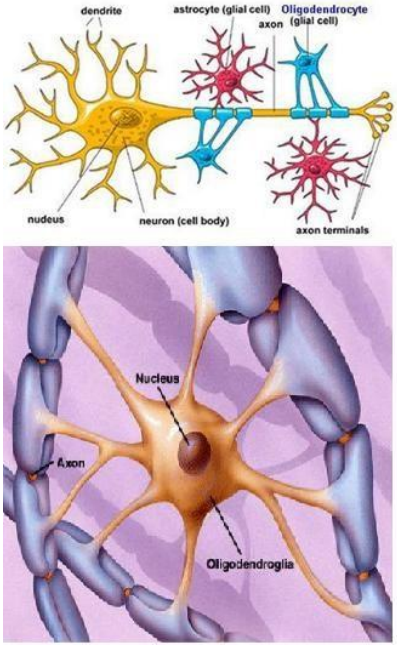

Figure 3: Oligodendrocytes in myelinating axons

Myelin is made by different cell types and performs the same insulting function, although it varies in chemical composition and configuration. Myelinated axons are white in appearance. Myelin insulates the axons from electrically charged atoms and molecules. These charged particles are found in the fluid surrounding the entire nervous system [1].

Cholesterol is an essential constituent of myelin. Myelin is about 40% water and 60% dry mass. From this dry mass about 70–85% are lipids and about 15–30% are proteins. Some of the proteins are myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP). The primary lipid of myelin is a glycolipid called galactocerebroside. The intertwining hydrocarbon chains of sphingomyelin serve to strengthen the myelin sheath [2].

The main purpose of a myelin sheath is to increase the speed at which impulses propagate along the myelinated fiber. Along unmyelinated fibers, impulses move continuously as waves, while in myelinated fibers they propagate by saltatory conduction. Myelin decreases capacitance and increases electrical resistance across the cell membrane. Thus, myelination prevents the electrical current from leaving the axon. Loss of the myelin sheath is called demyelination and is the hallmark of some neurodegenerative autoimmune diseases, including multiple sclerosis, acute disseminated encephalomyelitis, neuromyelitis optica, transverse myelitis and chronic inflammatory demyelinating polyneuropathy [1].

Oligodendrocytes development:

Oligodendrocytes or oligodendroglia are a type of neuroglia. As mentioned above, their main functions are to provide support and insulation to axons in the central nervous system of some vertebrates by creating the myelin sheath. A single oligodendrocyte can extend its processes to 50 axons, wrapping approximately 1 μm of myelin sheath around each axon. Each oligodendrocyte forms one segment of myelin for several adjacent axons [3].

Oligodendrocytes arise during development from oligodendrocyte precursor cells (OPCs) (figure 4). They are found only in the central nervous system which comprises the brain and spinal cord. These cells were originally thought to have been produced in the ventral neural tube, however, it has been shown that oligodendrocytes originate from the ventral ventricular zone of the embryonic spinal cord and possibly have some concentrations in the forebrain. They are the last cell type to be generated in the CNS [4].

OPCs are marked by PDGFRα and NG2 expression, while oligodendrocytes express *adenomatous polyposis coli* (APC) and proteolipid protein (PLP) (figure 4) [3]. Proteolipid protein (PLP) is a 4 transmembrane domain protein which binds other copies of itself on the extracellular side of the membrane. PLP has a very important function during myelination. It may play a role in the compaction, stabilization, and maintenance of myelin sheaths, as well as in oligodendrocyte development and axonal survival. In a myelin sheath, as the layers of myelin wraps come together, PLP will bind itself and tightly hold the cellular membranes together [5].

Myelinating oligodendrocytes express myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and myelin associated glycoprotein (MAG), while making compact myelin (figure 4) [3]. MBP is a protein believed to be important in the process of myelination of nerves in the nervous system. The myelin sheath is a multi-layered membrane, unique to the nervous system that, as already mentioned, functions as an insulator to greatly increase the velocity of axonal impulse conduction. MBP maintains the correct structure of myelin, interacting with the lipids in the myelin membrane. It is supposed to promote compaction of the myelin membrane by electrostatic interaction of its basic amino acids with negatively charged membrane interfaces. The pool of MBP in the central nervous system is very diverse with several splice variants being expressed and a large number of post-translational modifications on the protein, which include phosphorylation, methylation and deamination [6].

MAG was the first identified potent inhibitor of neurite out- growth. It is a transmembrane protein with five immunoglobulin-like domains in its extracellular region, and is localized in both PNS Schwann cells and CNS oligodendrocytes of myelin sheaths. MAG is required for the formation and maintenance of myelin. It is found that it has bidirectional effects on axonal growth [3]. In young neurons, MAG promotes axonal growth, whereas in older neurons, it inhibits axonal growth. This bidirectional effect of MAG on neurons seems to depend on intra- cellular levels of cyclic AMP [7].

During embryogenesis, ongoing exposure to Shh is required until the time of OPC migration from the ventricular zone. *Gli2,* Nkx6.1, and Nkx6.2 play also essential role in this stage, as well as temporal-dependent cell fate specification mechanisms (figure

4). These activities are required so that the pMN domain is competent to produce motor neurons and oligodendrocytes. pMN domain is a restricted domain of the ventral ventricular zone of the spinal cord, where motor neurons and oligodendrocytes are produced [8].

The switch of OPC production involves down regulation of neurogenic factors (Ngn2) at the end of motor neuron production and maintenance of glial precursors until the second wave by Delta–Notch signaling, Id2/4 and proglial activity of Sox9. Later phases of OPC maturation are Shh-independent and require Sox10, Nkx2.2, and Olig1 function (figure 4) [3].

2',3'-Cyclic-nucleotide 3'-phosphodiesterase also known as CNPase, which is encoded by the *cnp* gene, is a myelin-associated enzyme that makes up 4% of total CNS myelin protein. CNPase is thought to undergo significant age-associated changes. It is named for its ability to catalyze the phosphodiester hydrolysis of 2',3'-cyclic nucleotides to 2'-nucleotides. It is expressed exclusively by oligodendrocytes in the CNS and the appearance of CNPase seems to be one of the earliest events of oligodendrocyte differentiation. CNPase is thought to play a critical role in the events leading up to myelination [9].

One more protein that plays an important role during oligodendrocytes development is NOGO-A. It is found that NOGO-A is one more marker for mature oligodendrocytes like APC and CNP [10]. Due to studies it was shown that absence of NOGO-A leads to a delay in oligodendrocytes differentiation, in myelin sheath formation and in axonal caliber growth within the first postanatal month. These abnormalities lead to hypo myelination. It was also found that NOGO-A is an inhibitor of axonal outgrowth. Blocking NOGO-A during neuronal damage can help in the protection or restoration of the damaged neurons by reducing the local inflammatory processes. NOGO-A neutralization may have a beneficial effect early during the inflammatory process or for regenerating axons after CNS trauma, but it could be negative for the process of myelin repair at a later stage. Due to the latest studies regarding NOGO-A, it was shown that cell type-specific gene ablation of NOGO-A results in the promotion of axonal regeneration in injured adult optic nerve. As it is already known NOGO-A is not only expressed from oligodendrocytes, but also from some subpopulations of neurons including retinal ganglion cells (RGCs). So

due to the latest studies it was proposed that inactivating NOGO-A in oligodendrocytes while preserving neuronal NOGO-A expression may be a successful strategy to promote regeneration in CNS [11]. The investigation into the mechanisms of this protein presents a great potential for the treatment of auto-immune mediated demyelinating diseases and spinal cord injury regeneration [12].

Several key regulators of developmental myelination have been reported, including transcription factors like for example YY1, MRF, ZFP191, and TCF4, intracellular signaling pathways such as Wnt and also posttranscriptional control via miRNAs (figure 4) [3].

Myelin regulatory factor (MyRF) also known as myelin gene regulatory factor (MRF) is a protein that in humans is encoded by the *myrf* gene. MRF is a transcription factor that promotes the expression of many genes important in the production of myelin. It is therefore of critical importance in the development and maintenance of myelin sheaths. The expression of MRF is specific to mature, myelinating oligodendrocytes in the CNS and it has been shown to be critical for the maintenance of myelin by these cells. In MRF deficient mice the expression of myelin genes such as PLP, MBP, MAG and MOG drops rapidly. Therefore, MRF is a key regulator and likely a direct activator of the expression of these genes [13]. Furthermore, mice lacking MRF in the oligodendroglial lineage continue to generate oligodendrocytes, but these cells do not fully mature and display defects in myelin gene expression and myelin internode formation [14].

Zinc finger protein-191 (ZFP) is a widely expressed, nuclear-localized protein that belongs to a family whose members contain both DNA-binding zinc finger domains and protein-protein interacting SCAN domains. According to previous studies it was found that *Zfp191* mutants express an array of myelin-related genes (MBP, MAG) at significantly reduced levels and it is indicated that mutant ZFP191 acts in a cell-autonomous fashion to disrupt oligodendrocyte function. It was also shown that absence of ZFP191 leads to hypo myelination. For all these reasons it is demonstrated that ZFP191 is an important transcription factor that is required for the myelinating function of differentiated oligodendrocytes [15].

The understanding of how these multifaceted transcription factors, intracellular signaling pathways, and posttranscriptional mechanisms interact in an integrated

fashion to coordinate myelin generation and regeneration represents an important future area of integrative research.

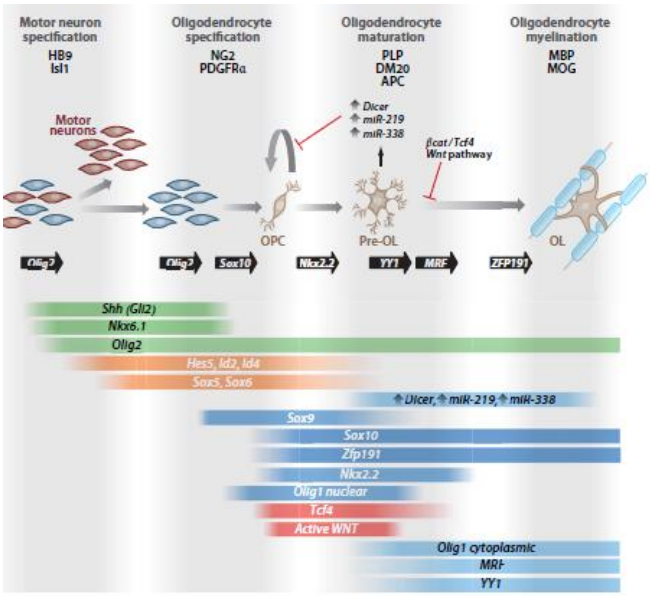

Figure 4: Schematic illustrating multiple mechanisms influencing oligodendrocyte development and differentiation at various developmental stages [3]

Axo-Glia interactions:

High velocity signal transmission is required for the coordination of the vertebrate nervous system between different brain areas. High speed nerve conduction is achieved in the myelinated fibers due to the myelin sheath that acts as an insulator of the axon. The interactions between the glial cell and the adjacent axon segregate the fiber in distinct molecular and functional domains. These domains are the node of Ranvier, the paranode, the juxtaparanode and the internode and are characterized by multiprotein complexes between voltage-gate ion channels, cell adhesion molecules, members of the Neurexin family and cytoskeletal proteins [16] (figure 5).

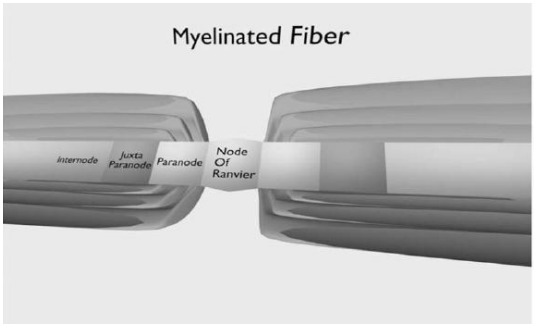

Figure 5: Schematic representation of a myelinated fiber cross-section [16]

Nodes of Ranvier:

In myelinated fibers the myelin sheath is interrupted by a 1μm, unmyelinated axonal segment, which is called node of Ranvier. The node is the area where ion flow across the membrane occurs, resulting in the propagation of action potentials along the axon, via saltatory conduction[17] (figure 6).

Paranodes:

Paranodes serve as a membrane barrier for the segregation of sodium and potassium channels, which are found at nodes of Ranvier and juxtaparanodes, respectively. Paranodes morphological characteristic are the septate-like junctions formed at the axo-glial contact sites. Three molecules are implicated in paranodal junction formation, CONTACTIN and the 155 kDa isoform of neurofascin (NF155), which belong to the IgSF family and are detected in the glial and axonal cell membrane and contactin associated protein (CASPR), which is a member of the Neurexin family. In the absence of any of these molecules, paranodes are disrupted with progressive loss of axo-glial interactions, defective ion channel segregation and impaired nerve conduction [18-21] (figure 6).

Juxtaparanodes:

The juxtaparanodal region is adjacent to the paranode comprising part of the internodal compact myelin. Its organization and maintenance depends on the combination of two distinct processes. First the lateral diffusion barrier created by the paranodal domain and second, the formation of the juxtaparanodal membrane complex and its linkage to the cytoskeleton. The juxtaparanodal complex consists of TAG-1, which is a GPI anchored adhesion molecule of the IgSF and is present on the glial and axonal membranes as well as the Neurexin protein Caspr2 and the Shaker-type voltage-gated potassium channels (VGKCs) on the axon. The absence of either TAG-1 or Caspr2 results in the disruption of this complex and the subsequent diffusion of VGKCs towards the internode. It is also important to mention that there are also some other proteins present in juxtaparanodes like for example 4.1B, PSD-93, PSD-95 and ADAM-22 with important function each [16, 22] (figure 6).

Internodes:

The internode comprises the largest domain of the myelinated fiber and is the area of compact myelin between adjacent nodes of Ranvier. PNS internodal myelin sheath is characterized by small parts of looser myelin compaction, the Schmint-Lanterman incisures, which are rare in the CNS fibers. Characteristic proteins that are found in paranodes are MAG, NECL1, NECL2 and NECL4 and they play an important role in axo-glia interactions [23, 24] (figure 6).

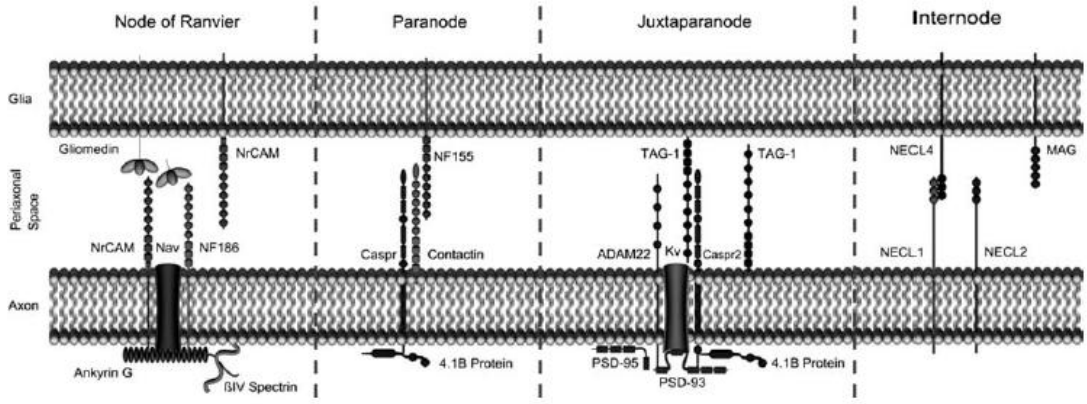

Figure 6: The molecular organization of a PNS myelinated

fiberIn and the key molecules of each domain in the axo-glial interactions [16]

The multiprotein complexes formed at contact sites between glial cells and neurons lend specific properties in each domain that result in an upright myelinated fiber function and proper nervous system coordination. Disruption of axo-glial interactions leads to a number of pathologies like multiple sclerosis (MS), a chronic inflammatory demyelinating disease of the CNS [16].

TAG-1:

The cell adhesion molecule TAG-1/CONTACTIN-2 is expressed by developing neurons and in adult CNS and PNS by glial cells and axons. It plays a very important role in axon outgrowth, migration and fasciculation during development, while it also has a crucial role in the production and maintenance of myelin. TAG-1 is required for the clustering of Kv1.1/1.2 potassium channels and CASPR2 at the juxtaparanodes of myelinated fibers. Experiments that have been done in the past have shown that the absence of Tag-1 causes abnormalities in the caliber distribution and cytoskeletal

defects of RGC axons. TAG-1 deficiency showed also impaired juxtaparanodal clustering of CASPR-2 and Kv1.1/1.2 in the hippocampus, entorhinal cortex, cerebellum and olfactory bulb, with diffusion into the internode. CASPR2 and Kv1.1 levels were reduced in the cerebellum and olfactory bulb, while shorter internodes were observed in the cerebral and cerebellar white matter. It was also shown that absence of TAG-1 leads to hypomyelination. This could be a result of a defect during oligodendrocytes development or a defect during the myelination process. Lastly, it is also important to mention that TAG-1 is recognized as an autoantigen by autoantibodies and T cells in MS patients, contributing to the development of gray matter pathology [25-27].

AIM OF THE STUDY

As mentioned in the introduction, the cell adhesion molecule TAG-1 is expressed by neurons and glial cells and plays a very important role in axon outgrowth, migration and fasciculation during development. It also plays a very important role in the production and maintenance of myelin. Absence of TAG-1 leads to a lot of abnormalities and axon dysfunctions. One of these is the hypomyelination that occurs in axons.

This hypomyelination may occur either from defects in oligodendrocytes development or from defects during myelination process. In our study we focus in the possible defects during myelination process. The goal of our experiments was to examine if there is any difference in the expression level of specific myelin genes (*plp, mbp, cnp, mag, nogo-a*) and transcription factors (*zfp, mrf*) in WT and KO mice for TAG-1 in three different time points: in P10 (start of myelination in mice), P21 (pick of myelination in mice) and P30 (end of myelination).

MATERIALS AND METHODS

Animals:

For our experiments we used WT mice and KO mice for Tag-1

Genotyping:

DNA extraction from mouse tail by using following protocol:

1. Add to the tube which contains the mouse tail 400µl tail lysis buffer(100mM NaCl, 10mM Tris HCl pH=8, 25mM EDTA pH=8, 0.5%SDS) and 8µl proteinase K (10mg/ml)
2. Incubation at 55 °C overnight
3. Fast spin down
4. Add 1µl RNase 10mg/ml
5. Incubation at 37 °C for 10 minutes
6. Fast spin down and transfer the supernatant to a new clean tube
7. Add 400µl of PhOH and mild agitation for 10 minutes
8. Add 400µl of CHCl3 and mild agitation for 10 minutes
9. Centrifuge for 5 minutes at13000rpm and transfer the supernatant to a clean tube
10. Add 400µl CHCl3 and mild agitation for 10 minutes
11. Centrifuge for 5 minutes at13000rpm and transfer the supernatant to a clean tube
12. Add ½ volume Ammonium Acetate 10M and 2 volumes of 100% Ethanol
13. Centrifuge for 10 minutes at 4°C at 13000rpm
14. Remove carefully the supernatant and keep the pellet
15. Dry the pellet
16. Resuspend the pellet in 100µl dH20

PCR for Tag-1:

Primers used:

TAG 5: 5’-CTCGATCTGAGGAAGATGAG-3’

TAG3: 5’-CTTTGCCACATTGTGCTGTG-3’

NEO3: 5’-GAAGACAATAGCAGGCATG-3’

Program used:

1. 94°C for 3 minutes
2. 94°C for 30 seconds
3. 59°C for 30 seconds
4. 72°C for 1 minute
5. Repeat steps 2-4 for 32 cycles
6. 72°C for 5 minutes
7. 4°C

For every PCR sample we used:

1. 1µl DNA
2. 2µl 10x buffer
3. 2µl 2mM dNTPs
4. 1µl Neo3(50ng/µl)
5. 1.3µl Tag5(50ng/µl)
6. 1.3µl Tag3(50ng/µl)
7. 1µl DMSO
8. 0.6µl Taq polymerase
9. 9.8µl dH2O

Dissection:

We sacrifice the mice by cervical dislocation and we isolate carefully the cortex.

RNA extraction:

RNA extraction from brain cortex according to the protocol of Takara RNAiso plus

cDNA synthesis:

cDNA synthesis according to the protocol of Affinity Script Multiple Temperature cDNA synthesis kit

Real-Time PCR analysis:

We did real-time PCR for following genes: *plp, zfp, mbp, mag, nogo-a, cnp* and *mrf*

Real-time PCR was performed in total RNAs extracted from brain cortex using a StepOne Plus Real-Time PCR system from Applied Biosystems and we analyzed them in StepOne Plus software. The relative gene expression was normalized to *gapdh* which served as an internal control. Reactions were performed using the Biorad iTaq Universal SYBR Green Supermix.

Each reaction included 7.5 μl of the master mix, 2 μl of each primer (2pmol/μl), 3.5 μl of cDNA.

For *plp, zfp, mbp, mag, nogo-a* Real-time PCR we used 50ng of cDNA for each sample, while for *cnp* we used 100ng of cDNA and for *mrf* 200ng.

PCR conditions were 95 ^{o}C for 20 seconds, followed by 40 cycles of 95 ^{o}C for 3 seconds, 58 ^{o}C (*plp, zfp, mbp, mrf, cnp, nogo-a*)/ 57 ^{o}C (*mag*) for 45 seconds and 95 ^{o}C for 15 seconds, and a final step of 60 ^{o}C for 1 minute and 95 ^{o}C for 15 seconds.

For every of these genes we used following specific primers:

PLP FORWARD:

5’-TCAGTCTATTGCCTTCCCTA-3’

PLP REVERSE:

5’-AGCATTCCATGGGAGAACAC-3’

MBP FORWARD:

5’-CACACACGAGAACTACCCA-3’

MBP REVERSE:

5’-GGTGTTCGAGGTGTCACAA-3’

ZFP FORWARD:

5’-CTGGTCAGCCGGTTTCTCT-3’

ZFP REVERSE:

5’-TTCCCAGGATGCCCACTTGA-3’

MRF FORWARD:

5’-GCACTACAGATACAAGCCTGAG-3’

MRF REVERSE:

5’-AAGATTCGCTCCTTGTTCACT-3’

CNP FORWARD:

5’-AGGAGAAGCTTGAGCTGGTC-3’

CNP REVERSE:

5’-CGATCTCTTCACCACCTCCT-3’

NOGO-A FORWARD:

5’-AGAGTCACCTGCGACCCTTA-3’

NOGO-A REVERSE:

5’-TAGCTCTAGCAGCCAGCACA-3’

MAG FORWARD:

5’-CTGCTCTGTGGGGCTGACAG-3’

MAG REVERSE:

5’-AGGTAGAGGCTGTTGGCAACT-3’

GAPDH FORWARD:

5’-ATTGTCAGCAATGCATCCTG-3’

GAPDH REVERSE:

5’-ATGGACTGTGGTCATGAGCC-3’

For all the genes we did comparative Real-Time PCR, except for *mrf* and *cnp* genes in which we did relative standard curve PCR.

Protein extraction:

1. We add in the tube which contains our tissue 1ml pyranoside lysis buffer and 1μl protease inhibitors(1000x)
2. We mix well with the help of the vortex
3. We homogenize the tissue if needed
4. Sonication (5 seconds sonication (60-65), 10 seconds incubation on ice and we repeat many times)
5. After sonication we centrifuge our samples for 20 minutes at 4^{o} C and 12000rpm and we transfer the supernatant in a new clean tube
6. We repeat this procedure for 3-4 times till our sample has a lucid color

Pyranoside lysis buffer:

85mM Tris-HCl pH=7.5

30mM NaCl

1mM EDTA

120mM glucose

1% Triton X-100

60mM octyl β-D glucopyranoside (SIGMA)

Western Blot analysis:

Bradford protein assay:

1. We add in a tube 799μl dH2O and 1μl from our protein sample

2. Then we add 200μl Bradford
3. Incubation for 15 minutes at room temperature
4. Then we measure the optic density of each sample at 595nm
5. The protein concentration of our samples is determined by comparison of them with already known BSA measurements at 595nm

Sample preparation:

1. After the appropriate measurements we transfer the appropriate quantity(100μg) of proteins in a new tube and we add 2x sample buffer and DTT so that we have a final concentration of 100mM
2. We boil our samples for 5 minutes at 95 ^{o}C
3. We make a quick spin down

SDS-PAGE:

1. We prepare the polyacrylamide gel and and we transfer it in the Biorad electrophoresis and blotting apparatus
2. We add 1Lt 1x running buffer
3. We load our samples in the polyacrylamide gel
4. We use the apparatus at 80V in the first 30 minutes while our samples are still in the stacking gel and afterwards we adjust it to 100V for 2 hours

Transfer:

1. We prepare 1x transfer buffer 1 hour before usage and we incubate it at 4 ^{o}C
2. When the running of the gel ends, we remove the running buffer and we isolate the gel
3. We remove the stacking gel and we keep the separating gel
4. For the transfer we need 4 whatman papers, 2 sponges and 1 nitrocellulose membrane
5. We place all the above into the cold transfer buffer
6. We make a sandwich with the following specific order: 2 whattman papers, polyacrylamide gel, nitrocellulose membrane, 2 whattman papers
7. We carefully remove the bubbles with the help of a Pasteur pipette
8. We place the sandwich in the specific biorad apparatus and we fill it with transfer buffer

9. We also place ice near the apparatus
10. We adjust the apparatus at 310mA for 1 hour so that the proteins get transferred from the polyacrylamide gel to the nitrocellulose membrane

Western Blotting:

1. After the transfer we place the nitrocellulose membrane in PBSMT(0.1%PBST with 5% non-fat dry milk)
2. Incubation for 1 hour while moving for Blocking
3. Incubation of the membrane with the primary antibody diluted in PBSMT O/N at 4^{o}C while moving
4. We remove the primary antibody
5. We make 3 washes with 0.1% PBST (15 minutes each wash)
6. Incubation of the membrane with the secondary antibody(linked with peroxidase) diluted in PBSMT for 1 hour while moving
7. We make 3 washes with 0.1% PBST (15 minutes each wash)
8. We dry the membrane with the help of a whattman paper
9. Incubation of the membrane for 4 minutes with a peroxidase substrate
10. Colorimetric detection

0.1% PBST(500ml)

500ml 1x PBS

500μl Tween 100%

10x Running-Transfer Buffer (1L)

900ml 10x Tris-Glycine

100ml SDS 10%

(For 1L 1x Running Buffer: 100ml 10x Running-Transfer Buffer plus 900ml ddH2O)

(For 1L 1x Transfer Buffer: 100ml 10x Running-Transfer Buffer plus 200ml 100% Methanol plus 700ml ddH2O)

10x Tris-Glycine(1L, pH=8.3)

30.2gr Tris-base

188gr glycine

2x Sample Buffer

100mM Tris-CLpH=6.8

4% SDS

0.2% bromophenol blue

20% glycerol

Gel preparation:

Separating gel 7.5% (5ml)→

- 2.5ml dH2O
- 1.25ml 30% acrylamide
- 1.25ml separating gel buffer (1.5M Tris-Cl pH=8.8 plus 0.4% SDS)
- 50µl 10% APS
- 2.5µl TEMED

Stacking gel (3ml)→

- 1.8ml dH2O
- 450µl 30% acrylamide
- 750µl stacking gel buffer (1M Tris-Cl pH=6.8 plus 0.4% SDS)
- 30µl 10% APS
- 3µl TEMED

Primary antibodies used in our Western Blots:

Anti-MBP (Rat) 1/5000)

Anti-Actin (Mouse) 1/5000

Anti-Gapdh (Rabbit) 1/1000

Anti-NogoA Bianca (Rabbit) 1/20000

Anti-TUI/Anti-γ tubulin (Mouse) 1/5000

Anti-TG-2/Anti-Tag-1 (Rabbit) 1/6000

Secondary antibodies used in our Western Blots:

Anti-Rat HRP 1/10000

Anti-Mouse HRP 1/5000

Anti-Rabbit HRP 1/6000

RESULTS

In the beginning of our study we decided to focus in the mRNA levels of the following myelin genes and transcription factors in P10 (start of myelination), P21 (pick of myelination) and P30 (end of myelination) WT and KO mice: *plp*, *mbp*, *mag*, *cnp*, *nogo-a*, *zfp* and *mrf*. For this reason we performed Real-Time PCR using mRNA isolated from mouse brain cortex. Before proceeding with the Real-Time PCRs for our experiments we designed and tested all the primers we would use. For *plp*, *mbp*, *mag*, *nogo-a* and *zfp* the primers were working with high efficiency so we decided to perform comparative Real-Time PCR and make the analysis with the help of Cts. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold (backround level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (this actually means that the lower the Ct level is, the greater the amount of the target nucleic acid in the sample is). For *mrf* and *cnp* the primers were working with lower efficiency so we decided to perform relative standard curve Real-Time PCR so we made the analysis with the help of the quantities. In order to easily compare our results for all the genes we also analyzed with the help of the fold change of our KO samples in compare with the WT samples. As internal control we always used *gapdh*. After the analysis of the Real-Time PCR we got following results:

P10:

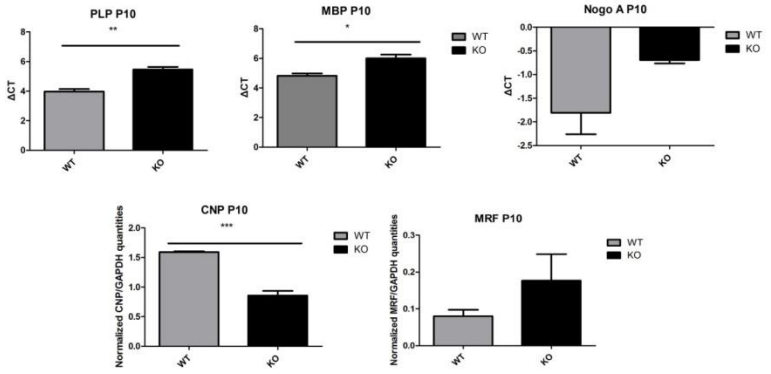

Figure 7: ΔCTs of *plp, mbp, nogo-a* and normalized quantities of *cnp, mrf* between WT and KO P10 samples after the analysis of Real-Time PCR (error bars represent SEM)

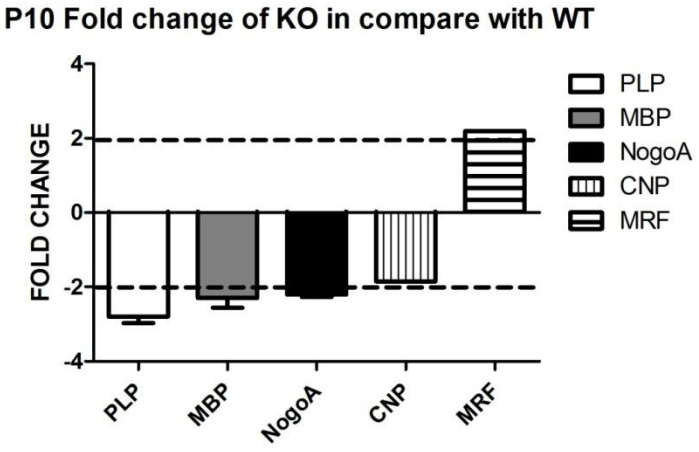

Figure 8: Fold change of *plp, mbp, nogo-a, cnp* and *mrf* in KO samples in compare with WT samples in P10 stage of life (error bars represent SEM)

In P10 stage of life we observed a reduction in the transcripts of *plp*, *mbp*, *nogo-a* and *cnp* in KO mice in compare with the transcripts of the WT mice. We also observed an increase in the expression of *mrf* in KO in compare with the WT mice. As for *mag* and *zfp* we did not observe any difference (figure7, 8).

P21:

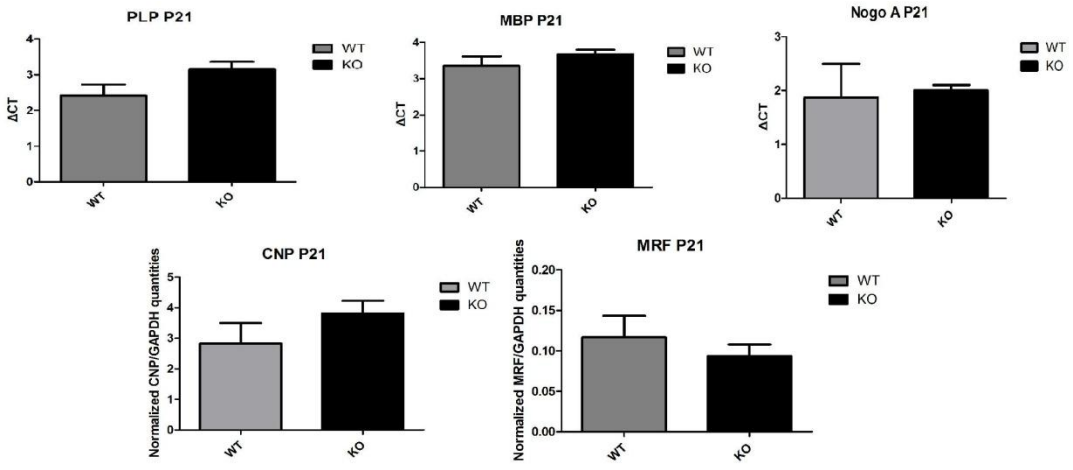

Figure 9: ΔCTs of *plp, mbp, nogo-a* and normalized quantities of *cnp, mrf* between WT and KO P21 samples after the analysis of Real-Time PCR (error bars represent SEM)

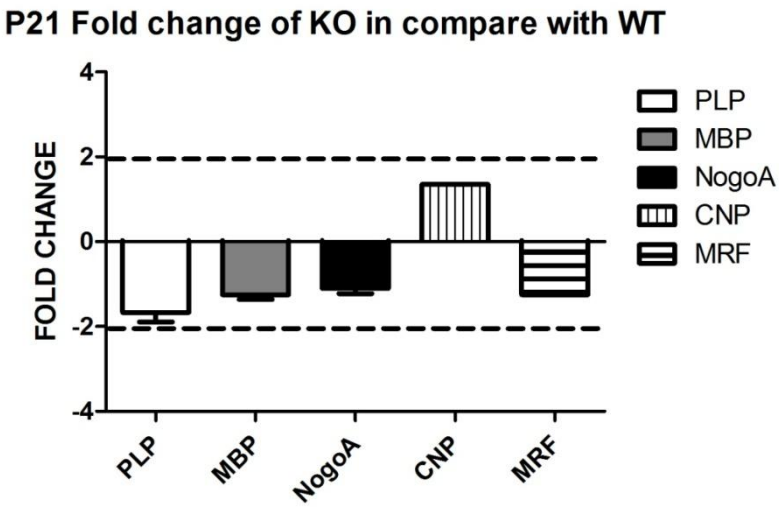

Figure 10: Fold change of *plp*, *mbp*, *nogo-a*, *cnp* and *mrf* in KO samples in compare with WT samples in P21 stage of life (error bars represent SEM)

In P21 stage of life we did not observe a statistically significant difference in any of these myelin genes and transcription factors as we can see from the figures above. The only difference we could observe is a slight reduction in *plp* transcripts in KO compared with the controls, but we were not sure that this reduce is statistically significant (figure 9, 10).

P30:

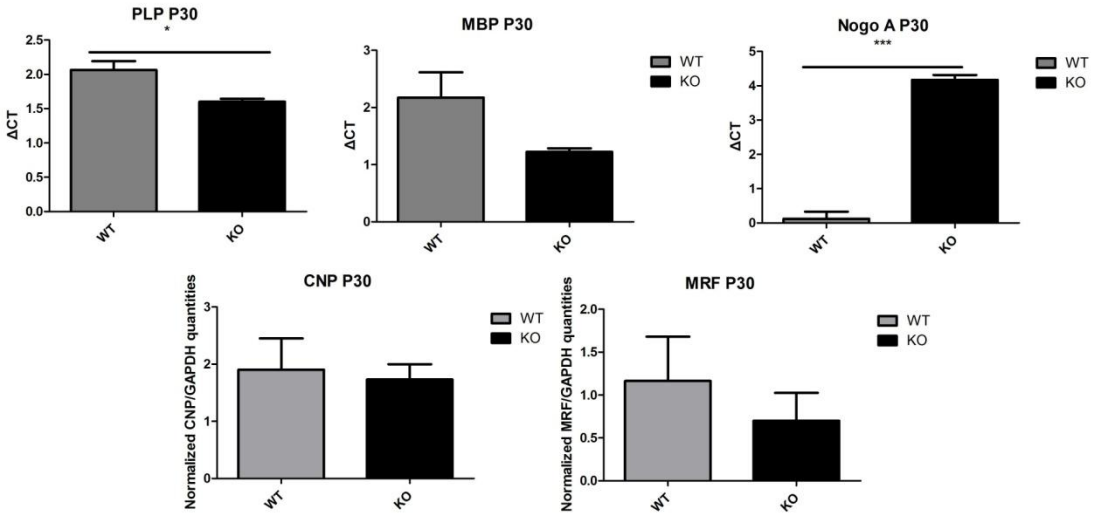

Figure 11: ΔCTs of *plp*, *mbp*, *nogo-a* and normalized quantities of *cnp*, *mrf* between WT and KO P30 samples after the analysis of Real-Time PCR (error bars represent SEM)

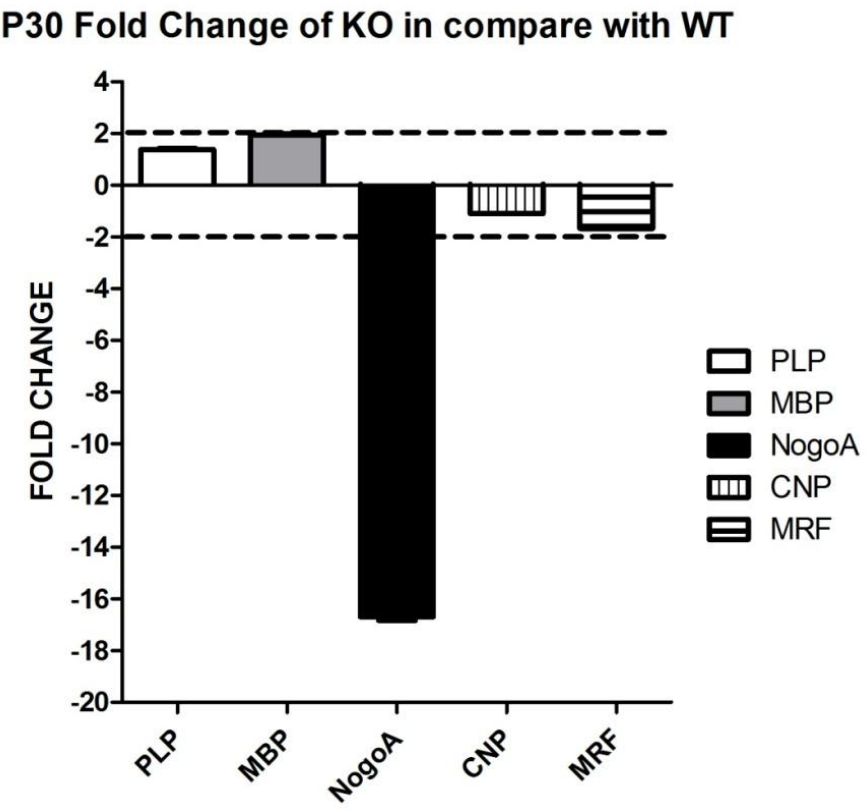

Figure 12: Fold change of *plp*, *mbp*, *nogo-a*, *cnp* and *mrf* in KO samples in compare with WT samples in P30 stage of life (error bars represent SEM)

In P30 stage of life we observed an increase in the transcripts of *plp* and *mbp* in KO samples in compare with the WT, while we also observed a huge reduction in *nogo-a* expression in KO mice in compare with WT mice. As for *cnp*, *mrf*, *mag* and *zfp* there were not any statistically significant differences (figure 11, 12).

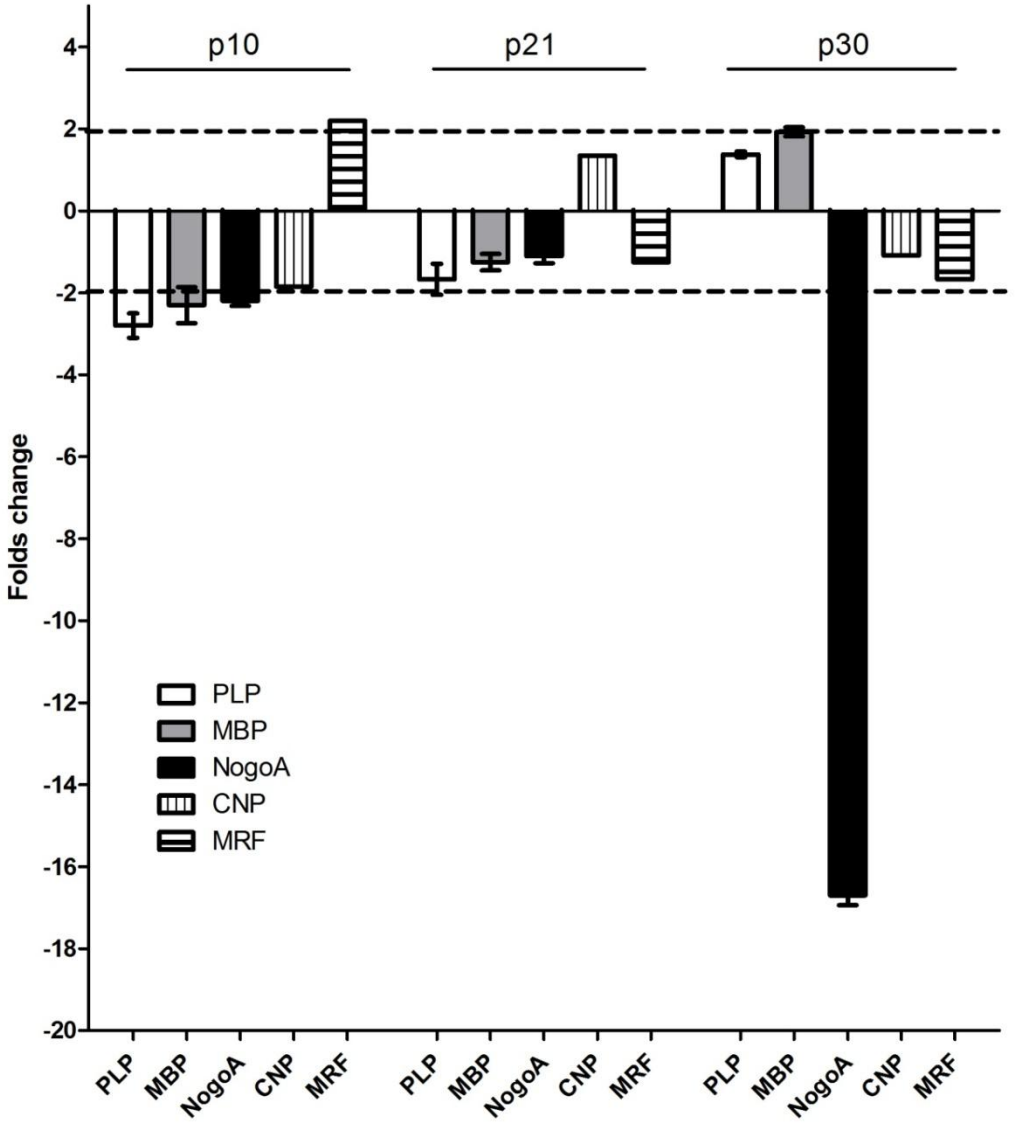

Figure 13: Fold change of *plp*, *mbp*, *nogo-a*, *cnp* and *mrf* in KO mice in compare with the WT mice in 3 different stages of life (P10, P21, P30) (error bars represent SEM)

	P10	P21	P30
plp	-	(-)	+
mbp	-	No difference	+
nogo-a	-	No difference	-
cnp	-	No difference	No difference
mrf	+	No difference	No difference
mag	No difference	No difference	No difference
zfp	No difference	No difference	No difference

Table 1: Expression pattern of specific myelin genes and transcription factors in KO mice compared with WT in P10, P21 and P30 stage of life. – means reduction in the transcript level of KO compared with WT. + means increase in the transcript level of KO compared with WT.

So to sum up our real-time pcr results we have the following conclusion (figure 13, table 1):

In P10 stage of life we observe a reduction in the expression of *plp*, *mbp*, *nogo-a* and *cnp* and an increase in the expression of *mrf* in KO mice in compare with the WT.

In P21 stage of life we observe some small changes in the expression of these genes in KO mice in compare with the WT but none of them is statistically significant. We can only observe a slight reduction of *plp* in KO mice compared with WT mice.

In P30 stage of life we observe an increase in the expression of *plp* and *mbp* and a huge reduction in the expression of *nogo-a* in KO mice in compare with the WT. In *cnp* and *mrf* we do not observe any statistically significant differences.

As for *mag* and *zfp* we do not observe any differences in the expression in KO in compare with the WT in any of these 3 different stages of mouse life

After these experiments we decided to focus in MBP and NOGO-A and try to observe if there is a difference between WT and KO mice in the protein level in P21 and P30 stage of life since at these ages we have the pick and the end of myelination respectively. For this reason we performed Western Blot analysis for these 2 proteins mentioned above using protein extracts from mouse brain cortex.

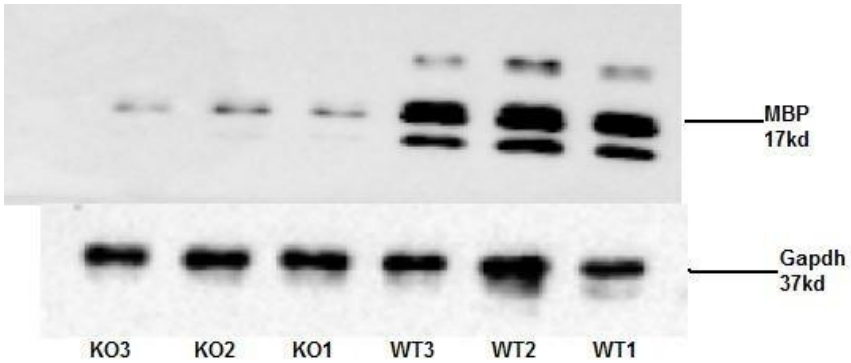

Figure 14: Western Blot performed from proteins extracted from P21 mouse brain cortex. In this blot it has been used antibody for MBP and for GAPDH as internal control

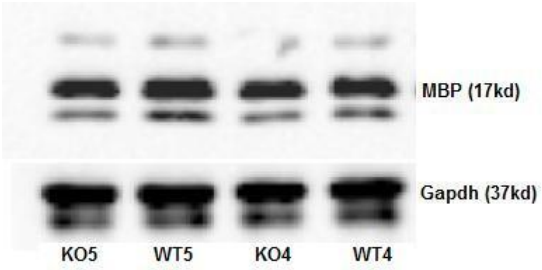

Figure 15: Western Blot performed from proteins extracted from P21 mouse brain cortex. In this blot it has been used antibody for MBP and for GAPDH as internal control

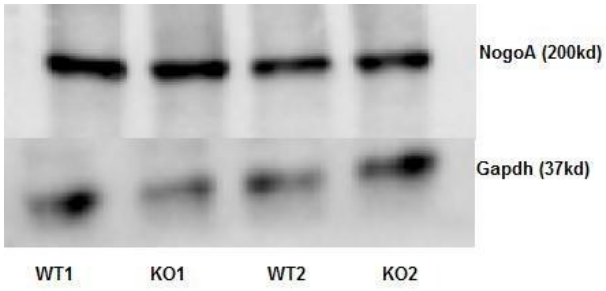

Figure 16: Western Blot performed from proteins extracted from P21 mouse brain cortex. In this blot it has been used antibody for NOGO-A and for GAPDH as internal control

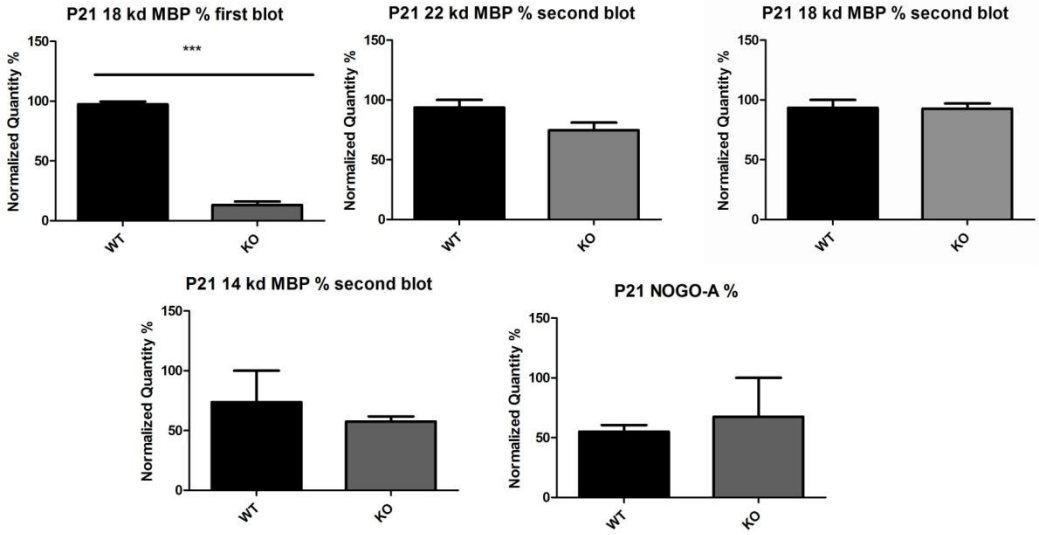

Figure 17: Normalized Quantities % from the Blots in P21 stage of life (error bars represent SEM)

It was shown from the blots above (figure 14, 15) and from the normalized quantities (figure 17) that in P21 stage of life MBP is reduced in KO mice in compare with the

WT mice. MBP has 3 isoforms, so we expected that our antibody for MBP detects 3 bands (one in 22kd, one in 17-18kd and one in 14kd). The difference in the expression of MBP between the WT and the KO mice is pretty clear in figure 14. In figure 15 we can also observe a difference in the MBP expression but only if we compare the first and the third band of MBP. In these 2 different Blots we used protein samples from different animals, so this is an explanation why we get a bigger difference in the first Blot compared with the second one.

It was also shown that in P21 stage of life NOGO-A is similarly expressed in WT and KO mice (figure 16, 17).

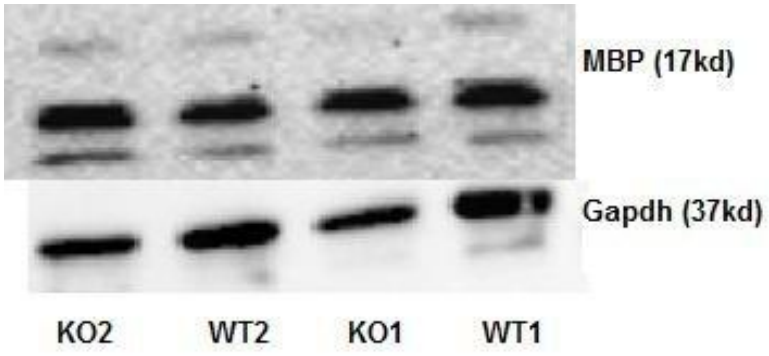

Figure 18: Western Blot performed from proteins extracted from P30 mouse brain cortex. In this blot it has been used antibody for MBP and for GAPDH as internal control

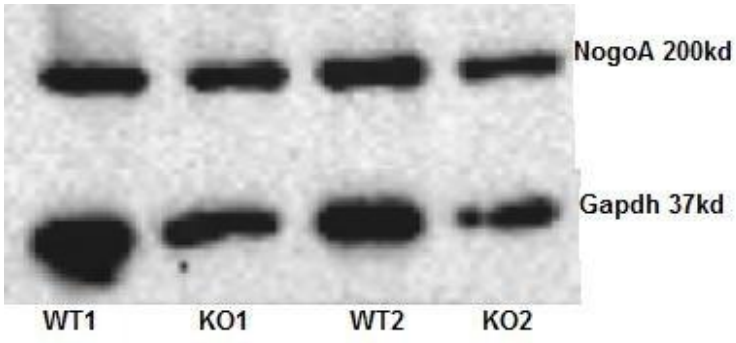

Figure 19: Western Blot performed from proteins extracted from P30 mouse brain cortex. In this blot it has been used antibody for NOGO-A and for GAPDH as internal control

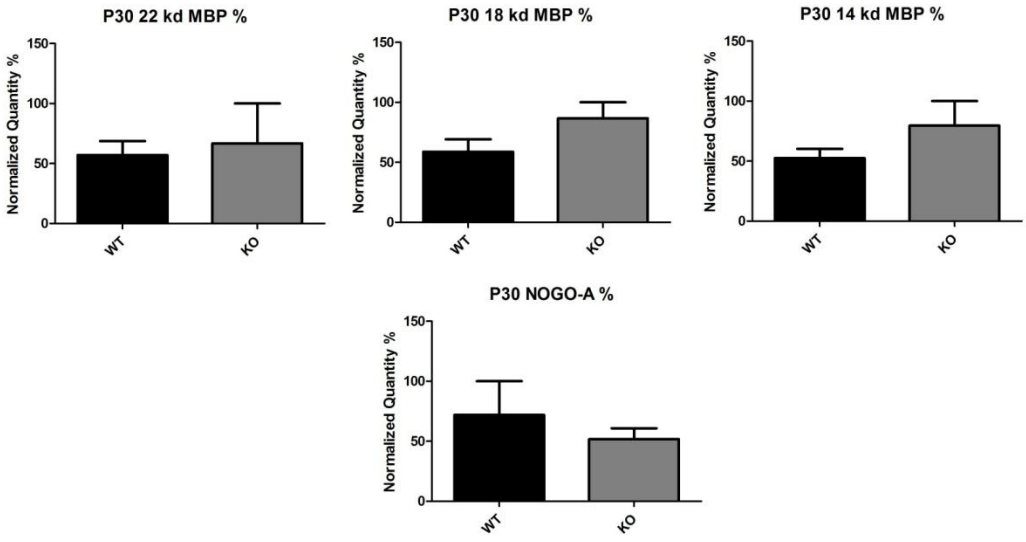

Figure 20: Normalized Quantities % from the Blots in P30 stage of life (error bars represent SEM)

In P30 stage of life it was observed that MBP and NOGO-A is similarly expressed in both WT and KO mice (figure 18, 19, 20).

DISCUSSION

As already mentioned, the cell adhesion molecule TAG-1 is expressed by neurons and glial cells and plays a very important role in axon outgrowth, migration and fasciculation during development [26]. It is also important for the production and maintenance of myelin. Absence of Tag-1 leads to a lot of abnormalities and axon dysfunctions. One of these is the hypomyelination that occurs in axons [27].

This hypomyelination may occur either from defects in oligodendrocytes development or from defects in myelination process. In our study we were interested in the possible defects during myelination process in vivo. For this reason we examined if there is any difference in the expression level of specific myelin genes and transcription factors in WT and KO mice for TAG-1 in three different time points: in P10 (initiation of myelination in mice), P21 (pick of myelination in mice) and P30 (end of myelination).

Our results showed that there is a reduction in the transcript levels of *plp*, *mbp*, *nogo-a* and *cnp* in P10 KO mice compared with the P10 WT mice, while it was also observed an increase in the transcript level of *mrf* in P10 KO mice compared with the control. As we already know *plp, cnp* and *nogo-a* are genes that start to be expressed in mature oligodenfrocytes, while *mbp* starts to be expressed in myelinating oligodendrocytes. A possible meaning of this reduction is that there is a lower number of mature and myelinating oligodendrocytes in KO mice compared with WT in P10 stage of life or there is the same number of oligodendrocytes, but there is a down-regulation in the expression of all these myelin genes in KO mice. MRF, which is a very important transcription factor responsible not only for the transition of pre-myelinating oligodendrocytes to myelinating oligodendrocytes but also for the regulation of specific myelin genes like *plp* and *mbp*, may be upregulated in KO mice compared with the control because of a possible mechanism in order to face this down regulation of the genes mentioned above.

In P21 stage of life we didn't observe any difference in transcripts of most of myelin genes and transcription factors. What we observed was only a slight reduction in *plp* transcripts in KO samples compared with the controls. The expression level of all transcripts in all myelin genes was similar between KO and WT mice something that

means that maybe our hypothesis of the reason of the up-regulation of *mrf* gene in P10 stage of life is right. Due to this mechanism *mrf* tries to rescue the phenotype of KO samples and bring the expression of *plp, mbp, cnp* and *nogo-a* transcripts in normal levels. What we also noticed in P21 stage of life was a difference in MBP protein levels. MBP was reduced in KO mice compared with WT mice something that is normal according our observation in the transcript levels of P10 stage of life.

Last but not least in P30 stage of life we showed that there is an increase in the transcript level of *plp* and *mbp* in KO mice compared with WT mice, while there is also a reduction in the transcripts of *nogo-a*. These results show the mechanism of oligodendrocytes, which try to express in high levels PLP and MBP because of the defect that existed in the initiation of myelination in P10 stage of life. The fact that we did not observe a different expression level of MBP and NOGO-A in P30 stage of life shows that the phenotype in KO mice was rescued.

Our results indicate an overall delay in the production of myelin genes and probably a delay in myelination. MRF, which is a very important transcription factor responsible not only for the transition of pre-myelinating oligodendrocytes to myelinating oligodendrocytes but also for the regulation of specific myelin genes like *plp* and *mbp*, may be upregulated in KO mice compared with the control because of a possible mechanism in order to face this myelination defect. So our results showed that Tag-1 deficiency leads to a defect during myelination process (delay in the expression of specific myelin genes and maybe a delay in the expression of these myelin proteins) that has as a consequence the observed hypomyelination.

What we should do in the future is to examine if there is a different in the expression of MBP and Nogo-A in WT and KO mice in P10 stage of life, which is the initiation of myelination as mentioned above. What we expect is a reduction in the protein level of both genes in this stage of life according to our results from the transcripts. Furthermore we should examine the expression level of PLP between WT and KO mice in all 3 different stages of life so that we can compare these results with the results we have already taken from the trascripts. What we expect here is a reduction of PLP in P10 and P21 stage of life in KO mice compared with WT and a similar expression of PLP in P30 between WT and KO samples.

As we mentioned above, hypomyelination in TAG-1 deficient mice may be a result not only because of defects in myelination process, but also because of possible defects during the oligodendrocyte development. For this reason we should check in the future if there is a difference in the number of oligodendrocytes (OPCs, mature and myelinating oligodendrocytes) in WT and KO mice in all 3 different stages of myelination process. What we also could do and we already started is to have oligodendrocyte cultures from WT and KO samples and to examine if there is a difference not only in differentiation of them but also in the production of extensions in myelinating phase.

Another experiment we can design in the future is to generate transgenic mice that will express TAG-1 only in the axons and not in oligodendrocytes. As we already know from previous experiments, the expression of TAG-1 in oligodendrocytes is sufficient for the formation of the juxtaparanodes complex and for the phenotypic rescue of TAG-1 homozygous mutants in the CNS [25]. So we can try finding out if the expression of TAG-1 from the axons is sufficient for the phenotypic rescue and if this expression is enough for the proper myelination process.

Last but not least we have to examine what is happening with NOGO-A. As we already know from previous experiments, NOGO-A is expressed not only from oligodendrocytes, but also from axons. Absence of this protein leads to a delay in differentiation of oligodendrocytes and in hypomyelination [11]. In P10 stage of life we observed a reduction in the *nogo-a* transcripts in KO samples compared with WT something that was expected since we have hypomyelination in our KO cases. What we have to examine is if there is also a difference in protein level between the WT and KO samples. In P30 we observed a high reduction in the *nogo-a* transcripts in KO samples compared with WT, although we did not observe a difference in the expression of NOGO-A protein. So since we are in the end of myelination, maybe TAG-1 in this stage of life regules the axonal NOGO-A and not the glial NOGO-A expression. If TAG-1 was regulating also the expression of glial NOGO-A we would possibly observe a difference between the WT and the KO samples.

As a conclusion of our experiments we can say that TAG-1 deficiency leads to hypomyelination due to defects during the myelination procedure. More specifically, our results indicate an overall delay in the production of myelin genes (*plp,mbp, cnp,*

nogo-a) and probably a delay in myelination. There is also a mechanism that tries to repair this defect through the up-regulation of the transcription factor MRF.

What we should do in the future is to check if this hypomyelination due to TAG-1 deficiency occurs due to defects in oligodendrocytes development too. We should examine if there is a diference in the number of OPCs, mature and myelinated oligodendrocytes and if there is a difference in the differentiation of oligodendrocytes and the production of extensions of them between the WT and the KO situation.

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