AALBORG UNIVERSITY

IMMUNOHISTOCHEMICAL ASSESSMENT OF EFFECTIVENESS OF CARNITINE PALMITOYL TRANSFERASE 1 (CPT1) BLOCKING IN RAT MODEL OF EXPERIMENTAL AUTOIMMUNE ENCEPHALITIS (EAE)

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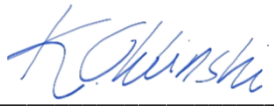
Author:	 Kirsten Egelund Oklinski
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ABSTRACT

Introduktion: Multipel sklerose (MS) er karakteriseret som en kronisk inflammatorisk nervedegenererende sygdom. Dog er den specifikke patologi og sygdommens forløb ikke klarlagt. De seneste år, er en ny hypotese vedrørende MS-patologien blevet fremført. Hypotesen bygger på at teorien omkring en ubalanceret lipidmetabolisme grundet af oxidativt stresset i mitokondriet.

Etomoxir blokere carnitin palmitoyltransferase 1a (CPT1a) irreversible. CPT1a transporterer acetyl-CoA. Acetyl-CoA er grundstenene i adenosin trifosfat (ATP) dannelsen. Blokade af CPT1a medfører af mitokondriet tvinges til at producere ATP af andre metabolitter.

Metode: Effekten af etomoxir, ekspressionen af CPT1a samt tilstedeværelsen af IgE og IgG blev undersøgt ved brugen af immunhistokemisk fluorescens og peroxid farvninger af behandlede og placebo rotte cerebellum og hjernestamme. Etomoxir effekt blev testet ved brugen af myelin basic protein (MBP) antistoffer. Hvorefter en kvantitative analyse blev udført. Peroxid farvningen blev udført ved brugen af IgE og IgG antistoffer hvorved etomoxir´s effekt på Ige og IgG antistoffer blev undersøgt.

Data og dataanalyse: Den kvantitative behandling af fluorescensfarvningerne, viste en signifikant (p-værdi 0.0073) forskel i MBP ekspressionen i cerebellum i de etomoxir behandlede rotter og sammenlignet med placebo og raske kontrol rotter. Ingen signifikant forskellig var fundet i hjernestammen (p-værdi 0.7859). CPT1a ekspression blev fundet til at være signifikant (p-værdi 0.0001 og < 0.0001) lavere i de etomoxir behandlede rotter i forhold til placebo, både i cerebellum og hjernestammen.

Analyse af IgE og IgG farvninger, viste en generel mærkering af IgE i både cerebellum og hjernestammen. IgG kunne identificeres i højere grad i placebo-rotterne.

Konklusion: Den kvantitative analyse af MBP ekspression klarlagte at etomoxirs gavnlige effekt på myelin regeneration. Dette var signifikant i cerebellum. Etomoxir effekt på mitokondriet blev påvist indirekte af nedreguleringen af CPT1a var signifikant i både hjernestamme og cerebellum.

IgE ekspression kunne ses i cellernes nuclei uafhængigt af celletype og væv. IgG ekspression i sektionerne var identificeres til at være højst i strukturer som blev påvirket af neuron skader som ses i MS.

INDHOLD

ABBREVIATIONS

ATP	Adenosine triphosphate
BDI	Beck Depression Inventory
BMS	Benign Multiple sclerosis
BSA	Bovine serum albumin
CNS	Central nervous system
CT	Cortical thickness
CPT	Carnitine palmitoyoltransferase
DAB	Diaminobenzidine
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded Disability Stats Scale
F-RRMS	Fatigue relapsing-remitting multiple sclerosis
FSS	Fatigue Severity Scale
GABA	Gamma-aminobutyric acid
GPxs	Glutathione peroxidase
HRP	Horseradish peroxidase
IHC	Immunohistochemical staining
MFI	Modified Fatigue Impact scale
ML	Medial lemniscus
MS	Multiple sclerosis
NF-RRMS	Non-fatigue relapsing-remitting multiple sclerosis
PNS	Peripheral Nerve system
RRMS	Relapsing-remitting multiple sclerosis
SOD	Superoxide dismutase
SPMS	Secondary Progressive Multiple Sclerosis
ST	Spinothalamicus tract
TL	Trigeminal lemniscus

INTRODUCTION

Multiple sclerosis (MS) is characterized as a multifocal neurodegenerative brain disease [1]. MS was described as an autoimmune disease, where the disease progression was initiated by autoreactive T and B-cells thus leading to an immune response [1]–[3]. In recent years, this hypothesis has been questioned and widely discussed [4]. New evidences showed the possibility of a new concept for understanding of multiple sclerosis and its underlying mechanism which is the basis for this report [5].

What is multiple sclerosis?

The origin of MS is unknown, and the pathophysiology of MS is not yet clarified [5], [6]. Additionally, clinical symptoms of MS are unspecific and with a lot of variation[3], [7]. Hence, MS is difficult to diagnose in its initial phases[6], [7]. However, the symptoms worsening during the MS progression occurs due to the increased axonal damage [5].

To further understand the development of the disease and its symptoms, the gross anatomy of the central nervous system will be summarized. Further, new MS hypotheses, and a novel MS treatment will be described.

GROSS ANATOMY OF CENTRAL NERVOUS SYSTEM

In general, the central nervous system (CNS) consists of the brain and the spinal cord. The brain can be divided into four regions; cerebrum, diencephalon, the brain stem, and the cerebellum. Each of these regions has a specific role in the human body. The neocortex is controlling for instance muscle movements and sensory input. Furthermore, the neocortex is responsible for thinking, consciousness, and intelligence. Survival functions such as breath, autonomous nervous system, and hormonal control are managed by the diencephalon and the brainstem [8]. The cerebellum is called the motor coordination center, due to its role in the coordination of signals from the other brain parts [9].

The subcortical basal ganglia have relation to the motoric cortex, thereby having an involvement for the control of voluntary movements together with thalamus [10]. These connections between basal ganglia and cerebral cortex are called indirect and direct circuits.

The mesencephalon has a strong association with the cerebellum and contains a high concentration of neuronal tracts [11]. Neuronal tracts such as medial lemniscus (ML), spinothalamic tract (ST) and the trigeminal lemniscus (TL) tracts can be observed on axial sections [11]. These tracts in ML are composed of heavy myelinated axons. ML cross in the mesencephalon and carry vibratory, proprioception, and touch-pressure signals from the skin to the thalamus [11]. Another sensory tract which synapses in the ventral posteriolateral nucleus of the thalamus is ST. ST consists of two tracts; the anterior spinothalamic which responsible for impulses from non-discriminative touch and the lateral spinothalamic tract which ascend pain and temperature impulses [11]. The TL tracts are responsible for the pain, touch and temperature signal from the facial skin, eye, and mucous membranes. Furthermore, the tracts also caring out the proprioceptive information from the facial and jaw nerve ending. TL terminates at the thalamus and is a part of the lemniscus [11]. Further neuronal tracts can be identified in CNS, e.g. the pyramidal tracts in the pons [11].

HISTOLOGY OF THE CENTRAL NERVOUS SYSTEM

The CNS consists of two major structures, white and grey matter. A high concentration of myelin and lipids gives a white appearance to the white matter and a high concentration of cell bodies, dendritic connections, axonal terminals, and nerve synapses results in a grey color of gray matter. The white matter gives rise to the myelin

encloses the ascending and descending nerves tracts. Asceending nerve tracts travel towards the CNS from the peripheral nervous system (PNS) and descending nerves travels from the brain to PNS [12].

The central nervous system consists of neurons and supporting cells. The neurons are responsible for signal transmission either within the CNS or to the peripheral nervous system (PNS). The signaling travels along the neuron´s axon. The neurons consist of one axon, a nuclear body, and several dendrites. Dendrites have the function of creating synapsis where information is exchanged via release and uptake of neurotransmitters in the synaptic cleft. Neurotransmitters are molecules which either evokes or inhibits an action potential in the neurons. Some neurotransmitters, e.g. glutamate, have an excitatory function, and promotes the electrical impulses and initiates the action potential. Whereas, gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter, which subsides the action potential [13] [11 pp. 4-8].

Neurons are divided by their anatomic structure or their function. Neurons with myelin-coated axons are defined as a high-speed transmitting neuron, here the electric current can only run in the nodes of Ranvier. As a result, a forward push is created in these nodes and the signal moves along the axon. Thus, increasing the speed also known as conduction velocity. This is called saltatory conduction. The nerve fibers which runs towards the skeletal muscles synapses in the lower motor neuron have a conduction velocity of 80-120 m/s and a myelin diameter of 12-20 µm [13] [11 pp.63-65].

Myelin has an isolating function and is synthesized by oligodendrocytes which are the supporting cells in the CN. Myelin is essential for saltatory conduction. Overall, four supporting cell types can be distinguished; (1) oligodendrocytes, (2) microglia, (3) astrocytes, and (4) ependymal cells. Microglia, astrocytes, and oligodendrocytes belongs to the category of neuroglial cells. They are highly represented in CNS, where their purpose is to support the neurons via nutrients, maintenance, and by facilitating the synaptic interaction between the dendrites. Neuronal glia contributes to the synaptic interactions and the electrical signaling, only indirectly [11 pp. 8-9].

MULTIPLE SCLEROSIS SYMPTOMS

When the neuronal damage and degeneration occurs in a specific area of the brain such as frontal lobe, brainstem and cerebellum. Patients most often will experience symptoms as attention deficit, loss of memory together with walking and coordination difficulties, and autonomic symptoms. Manifestation of these clinical symptoms can perpetuate the development of mental illnesses such as depression and anxiety.

In addition to the neocortex, motor cortex is also located in pars frontalis and can be identified in gyrus precentralis posterior. Gradually, MS patients will develop muscle spasticity related with the demyelination of the upper motor neuronal tracts. Damage of the upper motor neurons will cause spasticity, hyperreflexia, positive Babinski sign, and heighten muscle tonus, due to the loss of regulating signal from the motor cortex [14]. A positive Pronator drift test can indicate upper motor neuron syndrome. The positive symptoms will be a dropping hand or balance difficulties due to impaired proprioception [15]. Furthermore, because of the degeneration of inhibitory nerves, which are projecting to the micturition centers in the pons, the patient can suffer of bladder control impairment [16].

As mentioned before, the brainstem interacts with the basal ganglia in voluntary movements, via the pigmented neurons in the brainstem [8]. Damage to the upper neurons in the brainstem will result in motor movement impairment, wobbly walk and hyperkinesia. Furthermore, damaged direct and indirect circuits will result with either hypokinetic or hyperkinetic symptoms. Hypokinetic symptoms are caused by the greater output from the basal ganglia, causing insufficient movements due to the high concentration of inhibitory stimuli from the basal ganglia. A hyperkinetic symptom is noticed as tremor or slightly spastic movements such as ataxia when walking. The decrease in stimulation results in an inefficient inhibition of the thalamus, giving an uprise to these symptoms. [13][pp.428-431]

Moreover, neuron degeneration in the cerebrum and the basal ganglia gives additional cognitive symptoms [17]. This was investigated by M. Calabrese et al. (2010) [18] by using medical tests and self-report questionnaires such as Fatigue Severity Scale (FSS), Expanded Disability Stats Scale (EDSS), Modified Fatigue Impact scale (MFI) and the Beck Depression Inventory (BDI). These evaluations were then compared to brain volume MRI images. This study had a total of 152 patients, all suffering from relapsing-remitting MS (RRMS)[18].

When the brain volumes were compared between the RRMS patients and healthy control, it was found that the overall cortical thickness (CT) was lower in the RRMS patients. The lower CT was especially found in the medial and superior temporal gyri, thinning of the cortex was also observed in the frontal lobe. There was no significant difference in volumes of occipital and parietal lobe between the two group. A significant decrease in volume was found in the basal ganglia with an emphasis on thalamus, putamen and caudate[18].

Patients (71) having a score of FFS ≥ 4, were evaluated to suffer of fatigue (F-RRMS), and patients (81) under 4 were non-fatigue (NF-RRMS). F-RRMS patients had a higher score of EEDSS when compared to NF-RRMS. Furthermore, a significant decrease in the volume of putamen, thalamus, and caudate was observed in the F-RRMS when compared to NF-RRMS patients. Additionally, CT was observed in superior frontal gyri and inferior parietal gyri of the F-RRMS patients group [18].

The findings in the study of M. Calabrese et al (2010) [18]showed that both the frontal- and the parietal lobe together with the basal ganglia are affected by MS. Furthermore, M. Calabrese et al. (2010) showed that the clinical symptoms were in correlation to the lower cortical volume of specific parts of the brain.
However, due to the cortical projections, demyelination of e.g. the centers for sleep and autonomic function can result in constipation, drooling, or/and insomnia. Other autonomic symptoms such as choking, coughing and gagging are also present in these patients. These symptoms will occur when there is a plaque in the center for swallowing in the brain stem. Moreover, coughing and drooling can occur if the hypoglossal and/or the trigeminus nerve is affected by demyelination. Progressing demyelination, can affect the optic nerve and result in optic neuritis. The symptoms will manifest as a partial or complete loss of eyesight of an eye, often with an acute onset. Constipation may occur if the vagus nerve signaling is disturbed [18].

The impact of the autonomic symptoms on the patients was investigated by A.J. McDougall and J.G McLord (2003) [19]. Sixty-three MS patients were tested for autonomic functions such as; micturition, impotence, and gastrointestinal function. The severity of MS was found to be correlated with the amount of abnormal autonomic functions in 18% of the patients. Furthermore, autonomic symptoms were found in 73% of the patients. Micturition and incontinent (65%), was the two symptoms found in the autonomic control of the urinary system. In 50% of the male patients, impotence was reported which significantly correlated with bladder symptoms. In 33% of the patients, gastrointestinal dysfunctions were reported. The common dysfunctions were; compromised urge of defecation, upper gastrointestinal symptoms, constipation and fecal incontinence [19].

Urinary symptoms and bladder control were also studied by C. D. Betts et al (1993). In the study of 170 patients, it was reported that 85% of the patients had micturition dysfunction, while urge incontinence was found in 63% of the patients. furthermore, C. D. Betts et al. (1993) notated that 95% of the patients had pyramidal symptoms in the lower limbs [16].

When cerebellum and especially the cerebellar circuits, are affected by neurodegenerative diseases such a Parkinson´s Disease or MS, the most common symptoms that will arise are ataxia and tremor or similar incoordination problems; some patients will also develop speech problems as dysarthria [20], [21].

For further clarification of the cerebellar damage during MS, D`Ambrosio et al (2017) investigated 95 MS patients and 32 healthy controls using MRI scans, as well as motor, and cognitive performance tests [21]. The MRI scan was

used to collect; total cerebellar-, posterior cerebellar- and anterior cerebellar volume measurements together with T2 lesion volume. T2 is a MRI-method which detects MS lesions both active and inactive [22]. Patients with relapsing-remitting multiple sclerosis (RRMS), benign multiple sclerosis (BMS) and secondary progressive multiple sclerosis (SPMS) was represented in the study. D`Ambrosio et al. (2017) found within the MS group, the correlation between motor impairments, high volume of T2 lesion and lower total cerebellar-, posterior cerebellar-, and anterior cerebellar volumes[21].

There was a significant correlation between worse fine motoric skills with higher volumes of T2 lesion, lower total cerebellar-, posterior cerebellar-, and anterior cerebellar volumes. When the cognitive skills were tested, a significant correlation between high volumes of T2 lesion, lower total cerebellar-, and anterior cerebellar volumes. A lower brain- and white matter volume was observed in the MS group when tested against healthy controls. The patients with SPMS had a lower cerebellar volume compared to the controls, BMS and RRMS groups. Furthermore, a correlation between symptoms such as pyramidal, cerebellar, brainstem motoric impairment and observed lower volumes of total cerebellar-, anterior cerebellar-, and posterior cerebellar volumes was found [21].

The occurrence of clinical symptoms happens due to demyelination mediated by the patient´s immune system what can be seen in active MS lesions [3]. The immune cells, especially B- and T-cells attack the lipids attached to the myelin ensheated neurons. This mechanism is driven by the secretion for cytokines and activated by immunoglobulins such as IgE and IgG [3]. A. S. Mørkholt et al. (2017) found the presence of autoreactive antibodies against MBP in rat EAE disease models [23]. Autoreactive antibodies are synthesized and secreted when the immune system recognizes its own body molecules as foreign. Autoreactive antibodies against MBP were found in brain tissue samples from human patients, indicating that immunoglobulins and B-cells activation plays an important role in MS pathophysiology [24]. Furthermore, presence of immunoglobulins was also found in the meninges and in lesions of the blood-brain barrier [25], [26]. MBP can become immune reactive, when it undergoes citrullination. In this process, the amino acid arginine is exchanged into citrulline within the MBP molecule [5], [27]. This results in cleaving MBP from the myelin sheets around the neurons by the immune cells what perpetuates myelin degeneration.

The new and the old MS disease hypothesis

Previously, it had been suggested that MS was an autoimmune disease due to activation of the B- and T cells with a sudden onset of the disease often connected with appearance of optic neuritis [5] An autoimmune reaction to myelin or the lipids localized on myelin ensheathing can be starting point of MS. Further leading to an inflammatory response, resulting in nerve and axonal damage, thus further perpetuating the damages and disease progression [5].

In 1989, Paul W. E. et al (1989) published the article "Autoimmunity and autoimmune diseases" where autoimmune disease was defined by following criteria: (1) Specific immune response; (2) lesion reproducibility by administration autoantibody or T-cells; (3) lesions reproducibility in animals; (4) Autoantibody or T-cell isolation from serum or lesion; (5) titers or T-cell levels associated with disease progression; (6) specific autoantigens; (7) Immune absorption with purified autoantigen abrogates pathogenic autoantigen or T-cell; (8) Reduction of immune response after clinical interference [28]. When compared to this definition of autoimmune disease, MS only fulfills two out of the mentioned eight conditions; (3) lesion are reproducible in animals and (8) Reduction of the immune response after clinical interference. However, presence of antigens in MS was reported, it was not present in all MS diagnosed patients. After injection with autoantibodies or T-cell, MS lesions were not reproduced in the animal studies. No specific autoantibody or titers were described [7]. Furthermore, A. Corthals (2011) describes other perspectives of why MS is not an autoimmune disease [6]. The genetic correlations between MS and immune response have been found, but as A. Corthals (2011) describes, no genetic correlation was found in a large enough population allowing for verifications of this hypothesis. In some older hypothesis, it was suspected that vitamin D deficiency and some viruses could trigger a debut of MS [3], but also her not enough of evidence were found to prove it [6].

MS progression is characterized by relapses and attacks where the disease worsens. This indicates that a damage to the axon and myelin occurs first, and then the immune system is activated – which is known as the inside-out model [5]. Furthermore, as seen in figure 1, the axonal loss continues even though the inflammation is decreasing, thus suggesting that MS is not a chronic inflammatory disease [5]

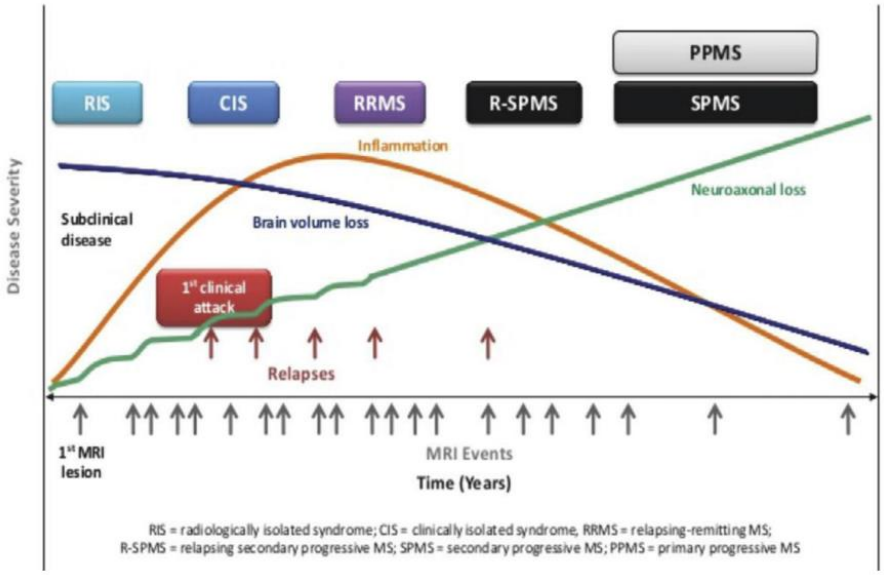

Figure 1: Correlation between inflammation response and MS. It is shown that inflammation is increasing when MS is in the first stages of the disease development. The first MS stages are used as a treatment window for anti-inflammatory drugs. As MS progresses, the inflammation decreases. The progression of MS is not correlated with inflammation, due to the increase in neuronal and brain volume loss. The inflammation is a secondary and natural response to damage of the neuronal fibers and the brain structures. Figure from: A. G. Solimanda *et al.*, "**B-Cell Therapies in Relapsing Remitting and Primary Progressive Multiple Sclerosis: A Short Clinical Review**" Biochem. Pharmacol. (Los Angel), vol. 5, 2016.

In figure 1, the lack of correlation between inflame-mation and MS progression is shown [29]. The neuronal loss, which is marked in green, shows the increase of demyelination and thereby loss of neuron

function. This correlates with brain volume loss, which is marked in blue. The correlation between loss of neuronal function and brain volume is also found in previously mentioned studies [18], [21]. The inflammation debut prior to the 1st clinical attack and increases during the periods of MS. Notably, the inflammation decreases during the years of disease [5], [29].

In the years of increasing inflammatory response, it is possible to manage MS with anti-inflammatory pharmaceuticals. However, this does not stop the MS from progressing. This strategy can suppress MS motion, giving fewer relapses and a lower inflammatory response. Nevertheless, a great number of side effect have been reported when using the anti-inflammatory treatments, what often lead to a need of premature treatment termination. The effectiveness of these treatments often decreased during MS progression requiring an increase in drug dosage [30].

METABOLIC STRESS, ALTERED LIPID CATABOLISM, THE PATHOLOGY OF MS PROGRESSION, AND TREATMENT

AS mentioned above, neuroglial cells provide an essential environment for neurons and facilitate fast neuron signal transmission. Myelin ensheating neurons i synthesized by oligodendrocytes and composes in 80% of lipids. It is hypothesized that an increase in unbalanced lipid catabolism may affect the quality and concentration of myelin in CNS. Thus, impaired upregulation in lipid catabolism and impaired or dysregulated glucose metabolism - can be one of the important factors contributing to the onset of MS [5]. Basing on this approach, a novel treatment strategy for MS is targeted to switch off the lipid metabolism and force the cells to restore their glucose metabolism.

In 1965, B. Gerstl et al (1965) [31], reported that a lower concentration of lipid in the white matter was found in MS patient when compared with healthy controls. The greater decrease in total lipid concentration was observed in the myelin and white matter. In the white matter, a decrease in lipid-phosphorus and carboxyl esters was observed [31]. Saturated fatty acids were showed not to be affected in MS, whereas, phospholipids and hydroxy fatty acids levels showed a great decrease. In addition, a high protein concentration together with low fatty acid concentration was found in the MS patients. B. Gerstl et. al. proposed that the high protein content were due to a selective degeneration of the myelin [31].

Decreased lipid catabolism was found together with increased neuronal protective mechanisms was reported in a study by M. J. Noga et al (2012) [32]. The aim was to clarify the mechanisms in the progression of MS. High levels of immune cells and immune response was reported in the onset of Experimental autoimmune encephalomyelitis (EAE), without clinical signs like muscle tone, paralysis, or tail reflex in the rats. At the peak of the disease progression, it was reported that levels of metabolites related with lipid metabolism, cytotoxicity and oxidative stress such as glutamic acid, asparagine, taurine, citrulline, and leucine were altered. An increase in GABA, taurine, and phosphoethanolamine was observed at the peak of the disease, which can indicates a stress reaction and points to an increased lipid catabolism [32].

The altered lipid catabolism has been suspected of being the origin of MS due to the mitochondrial damage which results in neuronal lipid loss and neuronal damage [5], [31], [32]. High concentration of superoxide and reactive oxygen species (ROS) can result in mitochondrial damage, such as ferritin deposits and alteration in the energy metabolism [5].

PATHOLOGY OF MULTIPLE SCLEROSIS

The MS pathophysiology which will be described below is according to the new MS disease hypothesis. The reasoning behind this hypothesis is based on the described experimental etomoxir results by L. P. Shriver et al (2011) and A. S. Mørkholt et al. (2017) [33], [34], low lipid concentration that is found within patients [31] and the that MS do not fulfills required autoimmune criteria by Paul W. E. et al (1989) [28]

The catabolism of lipids can be imbalanced due to a variety of reasons such as high concentration of free radicals, oxidative stress, imbalance in enzyme activity, and deposits of iron. This all together can contribute to mitochondria dysfunction [35]–[37].

These events are a result of cellular stress caused by to gene mutations or oxidative stress or both together. The gene mutations and oxidative stress arise from an overload of oxidative stress-induced damages [37]. These types of damages are proportional to the age of the patient – the older the patient is, the more cellular damages can be suspected [38]. A study by Z. Du et al. (2015) showed that oxidative damages in the brain were found to be more prevalent in aging rats than in younger rats [37]. These mutations like lower transcription of superoxide dismutase (SOD) and glutathione peroxidase (GPxs) can lower the mitochondrial tolerance for oxidative stress by insufficient production of antioxidants and chaperones thus leading to a decreased ATP production [36]. Furthermore, mutations affecting productions of mitochondrial transcriptional factors can lead to dysfunction of the mitochondria [36]. Peroxisome proliferator-activated receptors (PPARs) are transcriptional factors with a lipid metabolic regulatory function. At mitochondrial level, PPARS regulates acetyl CoA synthetase (ACS) and carnitine palmitoyltransferase (CPT). Mutations in this system could cause a dysfunction of lipid metabolism, further increase oxidative stress, activating of T-cells, and decrease of remyelination [5]. All these events leads to an increased myelination, imbalance in the lipid metabolism and mitochondrial homeostasis. Consequently, additional processes are initiated; (1) Enhancement of prostaglandin E2 which (2) stimulates and actives T- and B-cells. (3) Dysfunction of mitochondria and creation of free radicals. (4) Accumulation of iron deposits in the outer mitochondrial membrane, (5) upregulated of carnitine palmitoyltransferase 1a (CPT1a) triggers, (6) removal of myelin lipids due to increased demand energy substrates [5]. These events are illustrated in fig. 2.

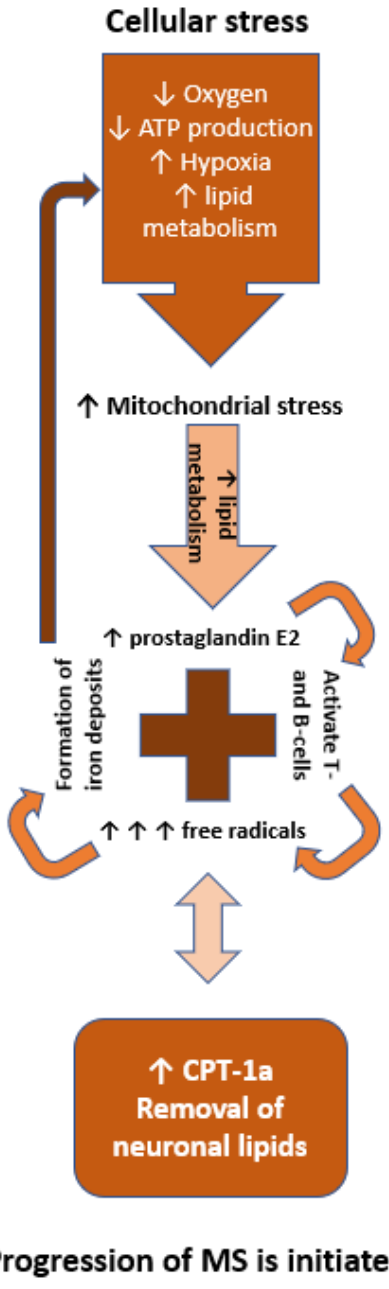

Figure 2 Flowchart illustrating the onset of events in proposed MS development and mitochondrial damage.

When the myelin is removed due to increased lipid catabolism, the microenvironment is disturbed creating an imbalance between ions and metabolites. As a result, the major neuronal function, transmission becomes impaired. Further damage to the microenvironment develops, when surface protein like, myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) are exposed to the immune system. The myelin sheet becomes a target for B- and T-cells since, MBP and MOG are no longer protected by the lipid layer. The B-

and T-cells binds to these surface proteins and becoming activated via an autoimmune reaction. The autoimmune reaction s caused by the activation of the major histocompatibility complex (MHC), especially HLA-B27 is eften a hallmark of autoimmunity. This event leads to the synthesis and secretion of inflammatory factors e.g. cytokines and immunoglobulins [5], [7]. Significant amounts of nitric oxide (NO) is released by the microglia in response to the stress. Nitric oxide is normally secreted as a signal molecule in CNS, but in pathophysiological events, NO is synthesized in a higher concentration as a result of intracellular hypoxia and is damaging to the intercellular environment. This mechanism is hypothesized to result in an abnormal oxidative metabolism and lead axon demyelination [39].

Background of Etomoxir

Production of adenosine triphosphate (ATP) aside of glycolysis, can occur by lipids are catabolized via beta-oxidation [40]. Beta-oxidation takes place in the peroxisome organelle and in the mitochondria of all cells where ATP is synthesized via the electron-chain reaction. Peroxisomes can regulate lipid catabolism by either preparing of long-chain fatty acid to be absorbed by the mitochondria, breaking down free radicals, synthesis of cholesterol [41]. Long chain and branched fatty acids are metabolized via beta-oxidation by the peroxisome to form acetyl-CoA. Acetyl-CoA is then transformed into carnitine derivatives which then are transported to the mitochondria.

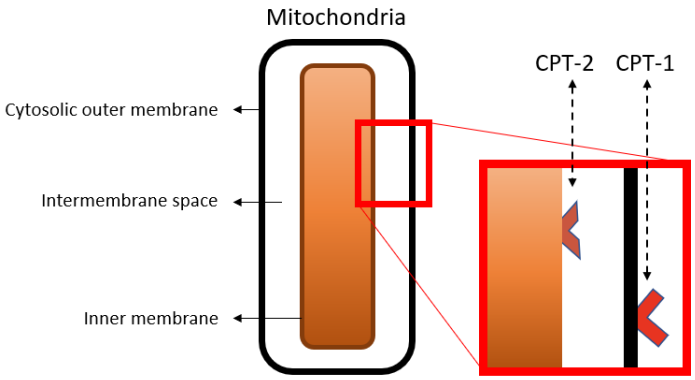

Figure 3 placement of CPT-1 and CPT-2. The mitochondria are characterized by the two membranes. The CPT-1is sitting on the cytosolic membrane while CPT-2 is sitting on the inner membrane of

The transport of acetyl-CoA into the mitochondria is performed by CPT1 and CPT2 enzymes. CPT1 is placed on the cytosolic outer membrane of the mitochondria and CPT2 is placed on the inner membrane [40]. The mitochondrial beta-oxidation has three main outcomes; (1) production of NADH-H+ and FADH2, (2) production of ATP via re-oxidation of NADH-H+ and FADH2, (3) generation of shorter fatty acyl-CoA chains [5].

The data illustrating occurrence of MS in population with naturally altered lipid catabolism can further confirm presented inside-out theory, and to clarify the connection between MS and the lipid catabolism.
The Innuits of the North American Arctic have adapted a high fat and protein diet, they live mainly of seals, walrus, whales, and caribou. This adaption mutation promoted a downregulation of CPT1a activity which in turn them to store more fat. This gives the Innuits a higher chance of surviving the cold weather and periods of famine. Another similar mutation of CPT1a was found among the Hutterites population.
The Hutterites and the Inuit population have shown a low activity or complete loss of CPT1a activity. Relating, these findings to the occurrence of MS in these populations, it suggests that low functioning or loss of CPT1a results in a neuron protective function. The MS prevalence was 1/1.000 for the Hutterites and only a single case of MS case was reported so far in the Inuit population [5].

To stop the progression of MS, the pathological CNS lipid catabolism needs to be pushed in the direction of glucose metabolism. This can be achieved by blocking CPT1a thus lowering the lipid transport over the mitochondrial membrane, thereby blocking beta-oxidation and reducing acetyl-CoA production from lipids. Consequently, forcing the mitochondria to switch over to glucose metabolism. CPT1a can irreversibly be blocked by etomoxir. Etomoxir

was a drug initially developed for the treatment of diabetes and congestive heart failures. Under the human trials, it was reported that the effect of etomoxir and beta-blockers counteract each other, resulting in a risk for the human participants [42]. Beta-blockers are used for arrhythmic heart diseases like hypertension by blocking the glucose metabolism. Combing a beta-blocker with etomoxir was found to increase the risk of liver damages [5].

In recent years, etomoxir has been tested on EAE-mice and -rats. Results, from these studies, have shown a decrease in neuronal damage and increase in remyelination and improvement of cognitive functions.

L. P. Shriver and M. Manchester (2011) [33] administrated etomoxir two times to EAE mice within 20 days. Results showed the higher function of hind-limb with the etomoxir treated group compared to the control mice. Furthermore, lowering of CNS inflammation was found in the etomoxir-treated animal together with intact myelin sheet. These findings indicated the possibility of etomoxir being used for MS treatment [33]. L. P. Shriver and M. Manchester also notated that etomoxir had a negative effect on T-cells, leading to a decrease of inflammatory response in the CNS [33].

In 2017, at the Consortium of Multiple Sclerosis Centers 31st Annual Meeting in New Orleans by AS. Mørkholt, K. Kastaniegaard, M. Trabjerg et al. (2017) [43] presented a comparison of MS interferon-beta treatment with etomoxir treated rats. The etomoxir treated animal showed a lowering of the disease score when compared to placebo and a greater improvement in the animal disease score then interferon-beta treatment [43].

PROJECT AIM

The aim of the project is to investigate etomoxir effect on EAE brain model in rats by assessing the neuron myelination and CPT1a expression by comparing placebo, etomoxir treated, and, wildtype rat group. The primary investigation methods used in this project is; immunohistochemical of IgE and IgG, and immunofluorescence staining of MBP in connection with fluorescent signal quantitative assessments of MBP.

METHODS AND MATERIALS

Immunofluorescence staining was performed to clarify the progession of MS in placebo-treated and etomoxir-treated rats. These two groups were compared with wildtype rats.

Myelin-basic protein (MBP) is an abundant protein in CNS and constitutes a major part of the myelin sheath together with other proteins such as actin, tubulin, and, lipids. Furthermore, it has been suggested that MBP possesses signaling properties [44].

Assessing of MS progression and effectiveness of etomoxir was performed by immunohistochemical staining (IHC) with the use of anti-MBP antibody. The signal intensity from staining with MBP antibody was an indicator for the MBP attachment to the myelin and thereby an evidence of myelin sheet regeneration. It is reported that in MS the lipid concentration decreases in conjugation with the disease progression [5], [45]. If etomoxir will have a regenerative effect, it is suspected that a higher myelin presence can be observed in the treated animals when compared with placebo.

Fluorescence immunostaining of FFPE sections

ANIMALS

Lewis rats were used for inducing EAE model and IHC were conducted according to National Institutes of Health (NIH) guidelines. The procedures were accepted by the Danish National Committee of Ethics on Animal Experimentation (2007/561-1364 and 2015-15-0201-00647). The rats were kept at a conventional animal facility at the University of Copenhagen with standardized conditions of light, temperature and with a 12h day/night cycle. Food and water were provided ad libitum. The rats were EAE-immunized and were monitored daily for clinical symptoms and weight. Under the progression of EAE, the mice developed motor disabilities. The rats were offered water in a petri dish and soaked chow to ensure the proper amount of food and water intake.
The EAE-immunizing of the rats was preformed over 4 days. The first day, the rats were administrated with 200 µg of MOG35–55 peptid in 0.1 ml PBS, and 0.1 ml CFA containing 0.1 mg Mycobacterium tuberculosis (Becton Dickinson) together with an intraperitoneal injection of 200 ng Pertussis toxin (List Biological Laboratories Inc.). The same procedure was performed on day 4. Day 2 and 3 were rest days.

The treated rats received etomoxir every other day (1mg/kg/day in 37C olive oil), whereas rats in the placebo group received an injection with either saline water or olive oil.

TISSUE OBTAINING AND PREPARATION

The tissues were stored in paraformaldehyde for 3 years (since 2015). Therefore, collected brains had to be washed due to the crosslinking properties of paraformaldehyde [46]. As first the brains were cut into subsequent 2 mm thick coronal sections in the rat brain cutting matrix. The sections were then numbered starting with 1 at the frontal part of the brain together with the olfactory bulb. Afterward, the sections were placed in histological cassettes and washed with cold running tap water for 4 hours. The cassettes were then placed in the blue cap bottle (500mL) and washed with cold 0.01M PBS for 5 days with a PBS exchange after every 24 hours. Further, dehydration of the tissue was performed by submerging in increasing ethanol concentrations starting from 70%, 96% and 99% for 4 hours at each step followed up with an overnight incubation in tissue clear. Immersion in paraffin was performed overnight and at the following day, tissues were embedded paraffin and stored at -18 until cutting on the microtome.

FLUORESCENT IMMUNOLABELLING

All sections were acquired from formalin-fixed paraffin-embedded (FFPE) tissue blocks, which were cut in 2µm thick sections using a rotating microtome (Leica RM2255, Leica Microsystems, Germany) and mounted on superfrost glass ((Menzel-Gläser, Superfrost Plus) from Thermo Scientific.

Before the sections could undergo fluorescent immunolabeling; the sections were incubated overnight in tissue Clear () for dewaxing. The following day, the sections were washed once more in fresh tissue clear and subsequently rehydrated. The rehydration process started with 99% ethanol 3 times for 10 minutes, afterward the section was placed in 96% ethanol two times for 10 minutes. At last the sections was placed in 70 % ethanol for 10 minutes. An additional washing step was performed with miliQ water.

The antigen retrieval was performed by heat treatment of the sections in the microwave. The sections were submerged in the citric buffer (10 mM Sodium Citrate, 0.05% Tween-20, pH 6.0), incubated for 10 minutes at room temperature (RT) followed by a boiling session in a microwave oven for 10 minutes. The cooldown of the sections took at least 45 minutes. Afterwards, the sections were outlined with a hydrophobic marker (Dako pen, Dako) and incubated with 50 mM NH_4Cl in PBS for 30 minutes. The sections were washed with blocking solution 3 times for 10 minutes. The blocking buffer consisted of 1% bovine serum albumin (BSA), 0.2% gelatin and 0.05% saponin in PBS, and was used to prevent non-specific antibody binding.
Afterwards, the primary bodies were diluted in antibody diluent (1% BSA, 0.3% Triton X-100 in PBS) and added on the sections and where incubated overnight at 4◦C. Used primary antibody commercially obtained was anti-Myelin Basic Protein (Abcam #ab62631) antibody in a concentration of 1:500. The anti-MBP antibody from Abcam which is used in this project, have been widely used by various research groups and with 40 references are registered on Abcam webpage [47].

The following day, sections were incubated at RT for 1 hour prior to proceeding with fluorescent immunolabelling. The sections were washed with washing buffer 3 times for 10 minutes. The washing buffer was composed of 0.1% BSA, 0.2% gelatin and 0.05% saponin in PBS and was used for washing the primary antibody residue off.
Afterwards, a PBS wash for 10 minutes was performed before applying the secondary antibodies on the sections. The dilution secondary antibodies used for fluorescence immunolabelling 1:200 in PBS. Secondary antibodies are sensitive light thus all further steps were performed in a shady spot. Secondary antibody used: anti-mouse AlexaFluor 555 (ThermoFisher Scientific #A-21422)

After an hour of incubation with secondary antibodies, the sections were washed with PBS three times for 10 minutes. To-ProTM-3 (Thermo Fisher Scientific #T3605) in a concentration of 1:1000 PBS was used for counterstaining of cells nuclei. Afterwards, the sections were mounted with coverslips using SlowFade™ Diamant mounting medium (ThermoFischer Scientific #S36963) prior for confocal microscopy using Leica DMI6000CS (Wetzlar, Germany) along with the LAS AF software.

The same procedure was performed by M. K. Oklinski Ph.D. for immunofluorescent staining using 1:100 mouse CPT1a antibody (Abcam, #ab128568) in the concentration of 1:100 with and donkey anti-mouse Alexa Flour 488 (ThermoFisher Scientific #A-21202) as secondary antibody in the concentration of 1:500. The commercially bought mouse CPT1a antibody have been validated in knock-out mouse cell line by Abcam [48].

PEROXIDE IMMUNOSTAINING OF IGE AND IGG OF FFPE SECTIONS

Staining with anti-rat IgE and IgG was performed to assess the immune system reaction in the rat model of EAE. In the literature, IgE and IgG have been registered in serum samples from rats MS disease models as well as in samples from human patients diagnosed with MS [23], [49]. However, the presence of immunoglobulins in the brain have not been investigated thoroughly. This procedure was performed on sections of placebo and etomoxir rats, as described above.

Prior to incubation with IgE and IgG, there procedure was performed as described above for immunofluorescent staining. After overnight incubation with primary anti IgE and anti IgG antibodies. Sections were then with 50 mM NH4CL in PBS for 30 minutes. Followed up by blocking with buffer (1% BSA, 0.2% gelatine, 0.05% saponin in PBS pH 7.0). This were prefomed 3 times for 10 minutes. The primary antibody used anti-rat IgG horseradish peroxidase (HRP) conjugated and goat anti-rat IgE at the concentration of 1:100 diluted in 0.1% BSA, 0.3% Trinton-X in PBS.

On the following day, the sections were incubated at RT for one hour prior to further proceedings. The sections were washed 3 times for 10 minutes using washing buffer solution (0.1 BSA, 0.2% gelatine and 0.05% saponin in 0.01M PBS pH 7.0). The sections stained with anti-rat IgE were incubated with a secondary rabbit anti-goat HRP conjugated antibodies diluted 1:200 in PBS for 1 hour at RT. Detection for both IgE and IgG was done with diaminobenzidine (DAB) (5mg/ml) for 4 minutes. Afterward, the section was washed 3 times for 10 minutes with the washing buffer solution prior to counter-staining with Mayer's hematoxylin (2 minutes). The sections were washed with miliQ water 2 times for 2 minutes and then washed for additional 15 minutes under running tap water.

Dehydration steps were performed in graded alcohol (70%, 96% and 99% for 10min in each) followed, clearing in tissue clear and coverslips were mounted with mounting medium. The sections were examined using bright field microscopy (Leica DM5500B).

DATA AQISITION AND ANALYSIS

The progression of MS in the placebo and etomoxir-treated rats were investigated by immunofluorescence staining with anti-MBP and anti-CPT1a antibodies and histochemical staining using IgE and IgG specific antibodies

Intensity quantification of immunofluorescent labeling

The immunofluorescent staining performed with anti-MBP antibody was used as an indicator for regeneration of the damaged nerves. Fluorescent signal intensity in the sections labeled with anti-CPT1a antibody was used to assess CPT1a expression in placebo and etomoxir treated rats. A total number of 196 images was obtained from 4 etomoxir-treated animals, 4 placebo animals, and 4 wildtypes. 8 images were taken of each sample; 4 from the brainstem and 4 from the cerebellum. The brainstem and cerebellum were selected. The images were taken in a non-randomized manner (targeting possibly similar locations in the sections). Images designated for fluorescent labeling intensity quantification were taken the resolution of 1024 x 1024 pixels. All microscopy settings, such as laser power, gain, offset, gamma values intensity and pinhole size were kept identical during the whole acquisition process. Single channel gray images of MBP or CPT1a-labeling sections were used, and fluorescent intensity were measured using ImageJ software as mean gray intensity after setting a threshold level covering desired labeling. Afterward, GraphPad Prism (version 5 for Windows, GraphPad Software, San Diego, California, USA) was used for the statically analysis and the creation of the figures.

STATISTICAL ANALYSIS

After the integrated density analysis was performed in ImageJ, the data were used to perform a t-test to find to the p-value. In experiments with more than 2 groups, a one-ANOVA was used for this purpose. the results are considered significant when the p-value is under 0.5.

Results

IHC STAINING DESCRIPTION

Several antibodies were tested with the purpose of finding a set of 3 to 4 antibodies which could be used to clarify the effects of etomoxir. S100 beta, TU-20, and 3 different Ferritin primary antibodies and 4 secondary antibodies were tested. All of these except a pair of secondary antibodies were excluded due to due low affinity. Afterward, immunofluorescent staining of CPT1a was performed by M. K. Oklinski Ph.D. The purpose of using an antibody against CPT1a was to assess the expression of this mitochondrial transporter. Hence, indirectly showing the lower amount of lipid that is catabolized by the mitochondria of etomoxir treated animals.

Figure 4 shows a selection of MBP immunofluorescent staining performed on etomoxir-threated, placebo, and wildtype rat brainstem. The rats treated with etomoxir (Fig 4. A, D, G) showed an increase in MBP labeling signal intensity when compared to both placebo (Fig 4, B, E, H) and wildtype rat brainstem (Fig. 4. C, F, I). The MBP labeled neuronal fibers can be identified easily on the etomoxir-threated rats. The brain stem of the wildtype rats showed higher labeling intensity then the placebo-treated rats.

Figure 5 shows a selection of the images of immunofluorescent staining acquired from the cerebellum from etomoxir-threated, placebo, and wildtype rats. The general signal intensity from these 9 images shows a slight difference. The greatest signal intensity comes from the etomoxir-threated rats (Fig. 5, A, D, G), this could indicate

that etomoxir has a less effect on the MBP regeneration in the cerebellum. Furthermore, the neuronal fibers appear more defined and detailed on the images from etomoxir-threated rats (Fig. 5 A, D, G)

Overall, results presented on figure 4 and figure 5 indicate that neuronal tracts and fibers visualized in etomoxir treated sections appear to be more similar to wildtypes. This could indicate etomoxir efficacy for MBP regeneration or protection especially in the brainstem.

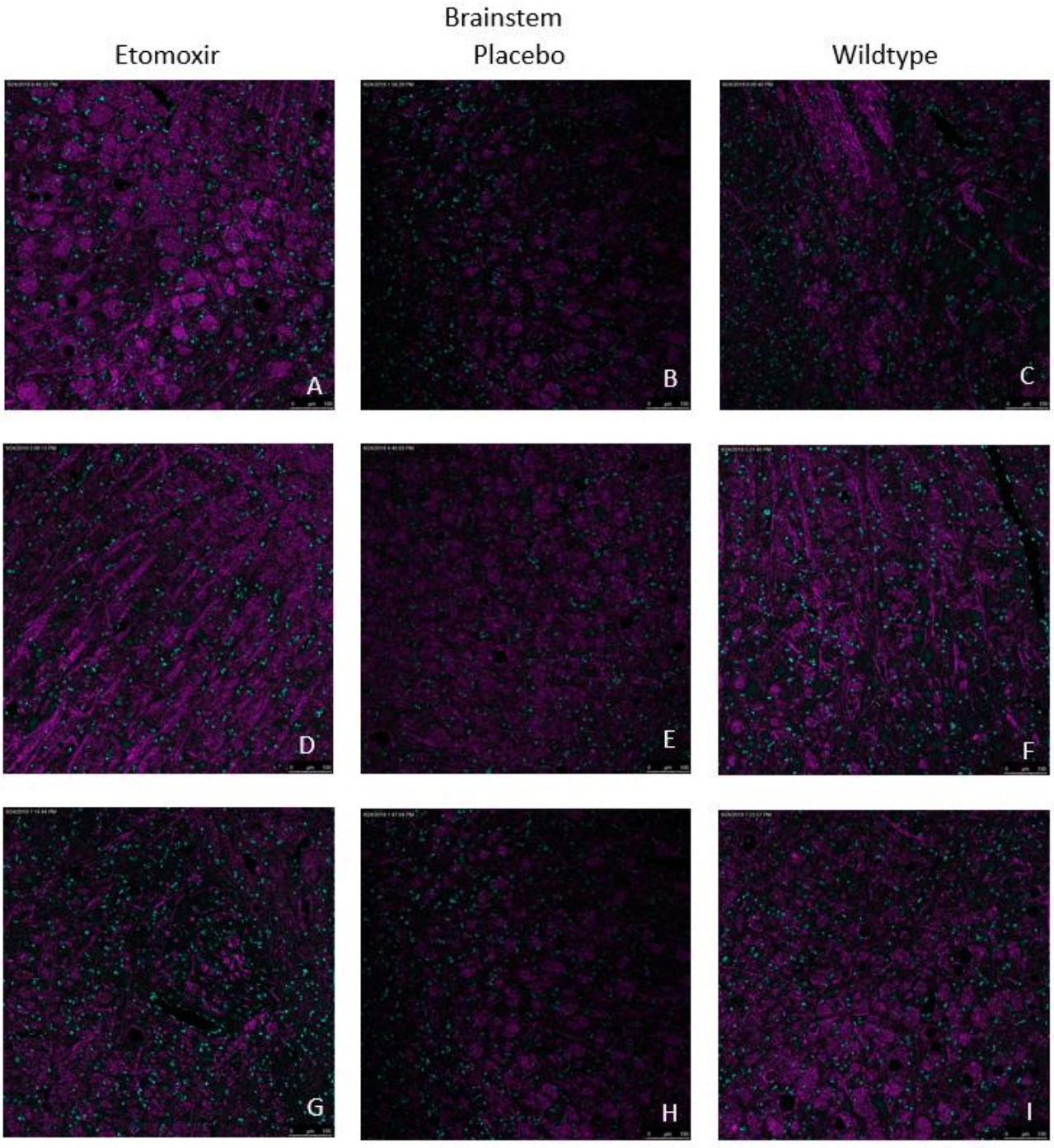

Figure 4 Immunofluorescent staining of rat brainstem. Etomoxir-threated, placebo, and wildtype rats were stained with MBP fluorescent to determent the effect of etomoxir. The purple color is the MBP labeling, where the turquoise is TOPro-3 counterstain by the cell nuclei. A, D, G shows a greater signal intensity than both placebo (B, E, H) and wildtype (C, F, I). Magnification 20X.

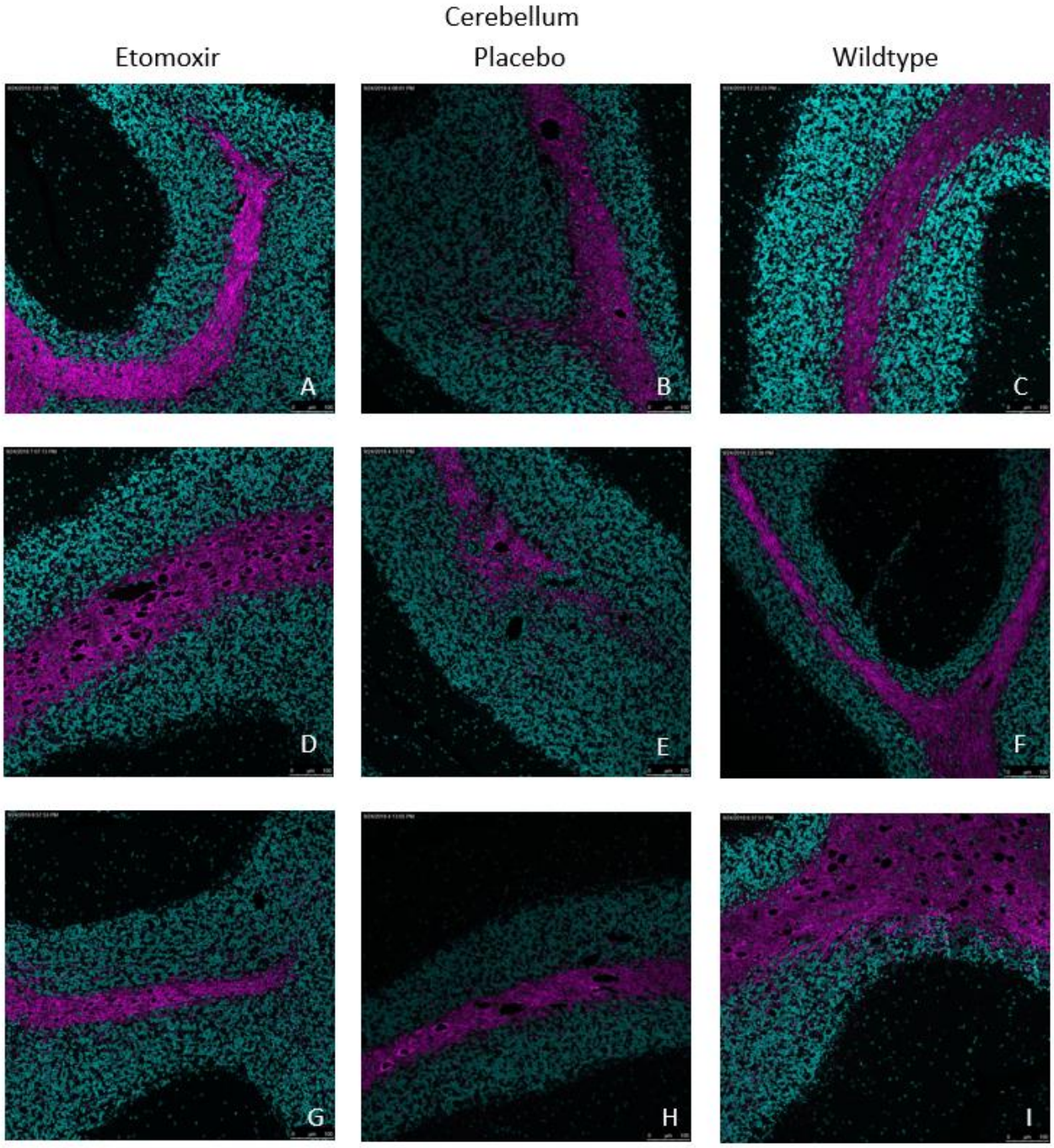

Figure 5 Immunofluorescent staining of rat cerebellum. Etomoxir-threated, placebo, and wildtype rats were stained with MBP fluorescent to determent the effect of etomoxir. The purple color is the MBP labeling in the white matter of cerebellum, where the turquoise is ToPro-3 counterstain by the cell nuclei. The MBP labeling is identified to be in the white mater. The counterstain highlights the nuclei in the granular layer. Equal signal intensity is seen between etomoxir-threat (A, D, G), placebo (B, E, H), and wildtype rats (C, F, I).

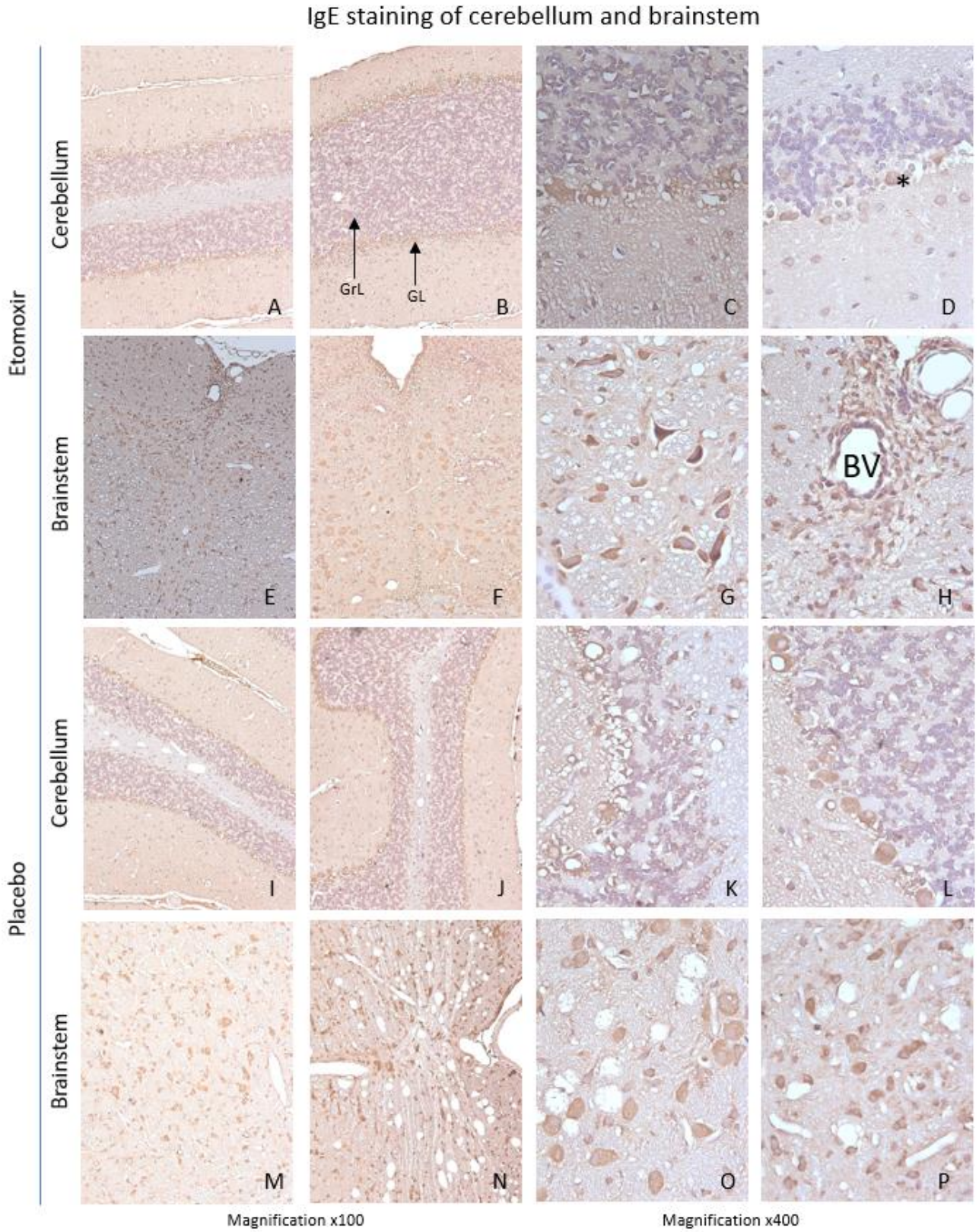

Figure 6 Immunohistochemistry staining of IgE in cerebellum and brainstem. The IgE labeling is colored brown and the nuclei are counterstained using Mayer´s hematoxylin. Overall a great signal is identified in both etomoxir threated and placebo treated rats. A slight greater signal can be observed in the placebo threated rats, this is especially seen in image N. however, the labelling does not show a homogeneous labelling in between the groups. Images A, B, E, F, I, J, M, and, N are taken with x100 magnification. Images C, D, G, H, K, L, O, and, P are taken with x400 magnification. The granular layer (GrL) and the ganglionic layer (GL) are marked in 6B, the purkinje cells are marked with asterix. The blood vessel in 6H is marked with BV.

The immunohistochemical staining of IgE is shown in figure 6. Overall, observed labeling, indicates that autologous IgE are present in both etomoxir-threated and placebo rats. Figure 6, A, B, C, and D shows cerebellum from etomoxir-threated rats. The IgE labeling can be observed along the ganglionic layer matter and along the nerves. Labeling can be identified in both molecular layer and the granular layer of the cerebellum. In the molecular layer, labeling cell bodies can be identified. In the ganglionic layer, labeled the cell bodies of purkinje cells can be observed. On figure 6C and D, the labelling can be localized in the neuronal cell bodies along the grey/white matter transition. In the brainstem of the etomoxir treated rats, stronger labeling was identified in the neuronal cell body throughout the whole section. At X400 magnification, an intense labeling was observed around blood vessels and the protrusions of the neuronal cells.

In the placebo threated rats, the signal appears to be slightly greater than the in etomoxir threated rats which may indicate a higher concentration of IgE. In the placebo threated cerebellum, the labeling is also identified along the white/grey matter transition. The higher signal intensity can especially be seen on figure 6J. In the brainstem, the signal intensity is strong, and can be identified along the whole section. The labelling can be observed especially along the blood vessels and in a great number of neuronal cell bodies and their protrusions. On figure 6N, the nerve tracts showed strong labelling. However, when figures 6G, H, O and P are compared the signal intensity seem to be the strongest in the etomoxir threated rats, but the reversed can be observed on figure 6E, F, M and N.

Throughout the whole panel of images in figure 6, it is noticeable that the signal intensity is not equal in the brain sections. In figure 6A and figure 6B, a difference in signal intensity and labelling can be seen. On figure 6 A, that labeling is narrow and the signal intensity is light. Whereas, in figure 6B, the labeling is broad fulling up the whole ganglion layer and the signal intensity is strong. This difference is emphasized in figure 6 C and figure 6, where the strong signal with broad labeling can be seen and in figure 6D, the weaker signal intensity is seen in a narrow pattern. Similar tendency, can be seen in the brainstem in figure 6E, F, G, and, H. In figure 6 in the placebo treated rats, the labelling and signal intensity is similar throughout the images. The difference in signal intensity can easily been seen in figure 6M, and figure 6N. the signal intensity in figure 6 M is light and less defined than what can be seen in figure 6N. Figure 6O and figure 6P, shows similar intensity of the signal, however the labeling in figure 6O is more defined. However, the peroxidase intensity on these sections are inconclusive. Both labeling in the cerebellum (Fig. 6) and brainstem (Fig. 7) are very variable in the groups. Therefore, concluding from such a small number of animals in the groups will be inaccurate.

In figure 7, brainstem sections from placebo and etomoxir threated rats is shown. Overall, the labelling is heterogenous. Little to no labelling is observed in the image from etomoxir treated rats on the fig. 7A. A slightly higher signal intensity is noted in another etomoxir treated rat fig. 7B, where the nerve tract can be identified. The strongest signal is seen in fig 7C, where the nerve tracts are identified like for instance in tractus corticospinals. The same tendency is seen in the placebo threated rats, no to little signal were identified in some section whereas, strong signal was observed in others. On fig. 7 D, minimal labelling is observed, whereas on fig7E, the signal intensity is the strongest and the nerve tracts can easily be observed. The strong intensity is seen in fig7C and F, the labeling in medial lemnicus can be observed from both groups. The tractus corticospinalis labeling can easily be noted due to a high signal intensity on fig. 7F.

The cerebellum of etomoxir- and placebo treated rats can be seen in figure 8. Little to no signal can be seen in all sections except for fig 8C. In fig. 8C, labelling can be observed in the cells of the granular layer of the cerebellum.

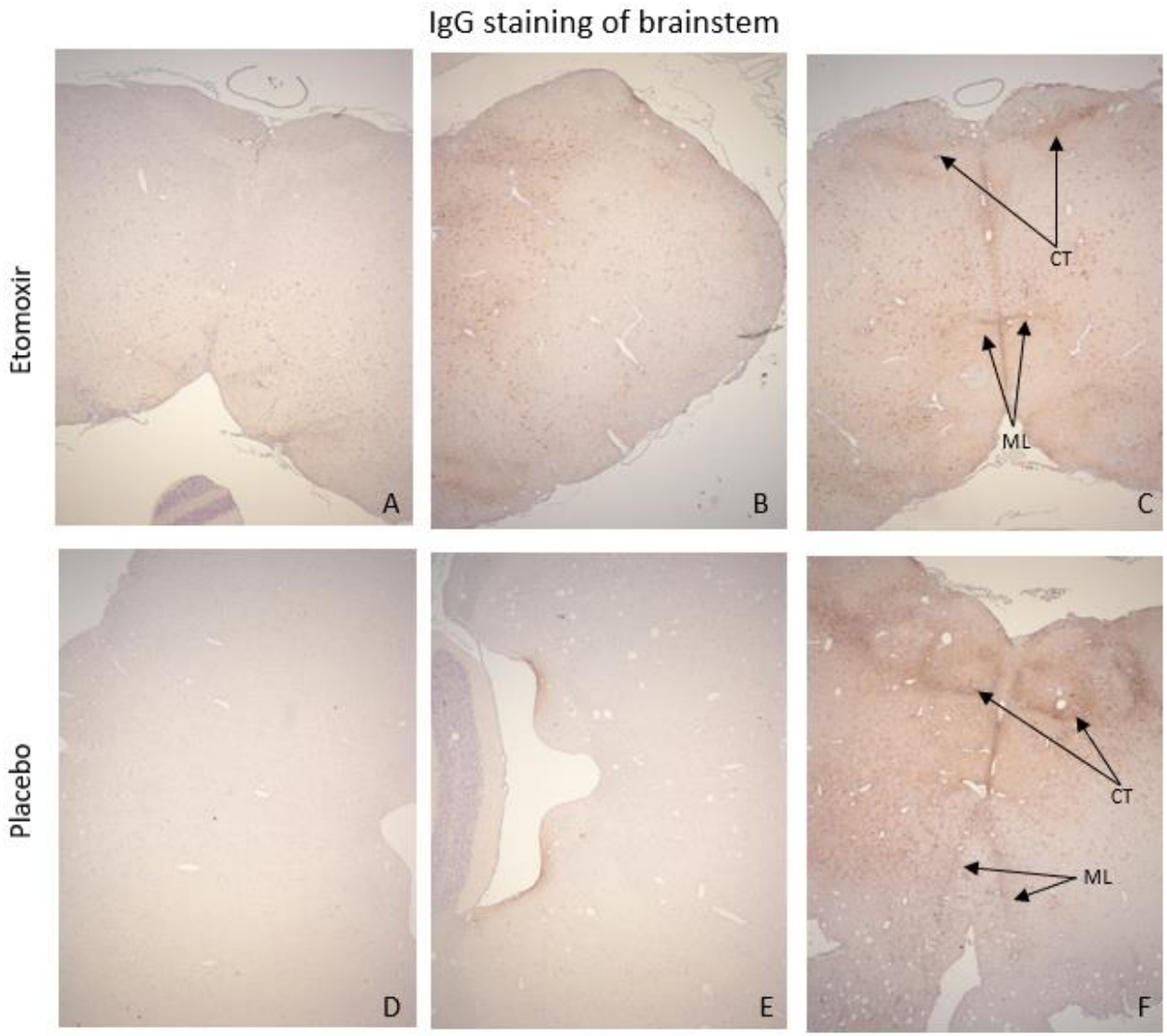

Figure 7 Immunohistochemical staining of IgG in brainstem. In the etomoxir threated rat, the signal intensity varies through-out the rat brainstem. Image A show little to no labelling, whereas image C shows a strong labelling of IgG. However, the strongest label intensity is shown in the placebo group on image F. On both C and F the tractus cirticospinalis (CT) and the medial lemminiscus (ML) is identified and marked.

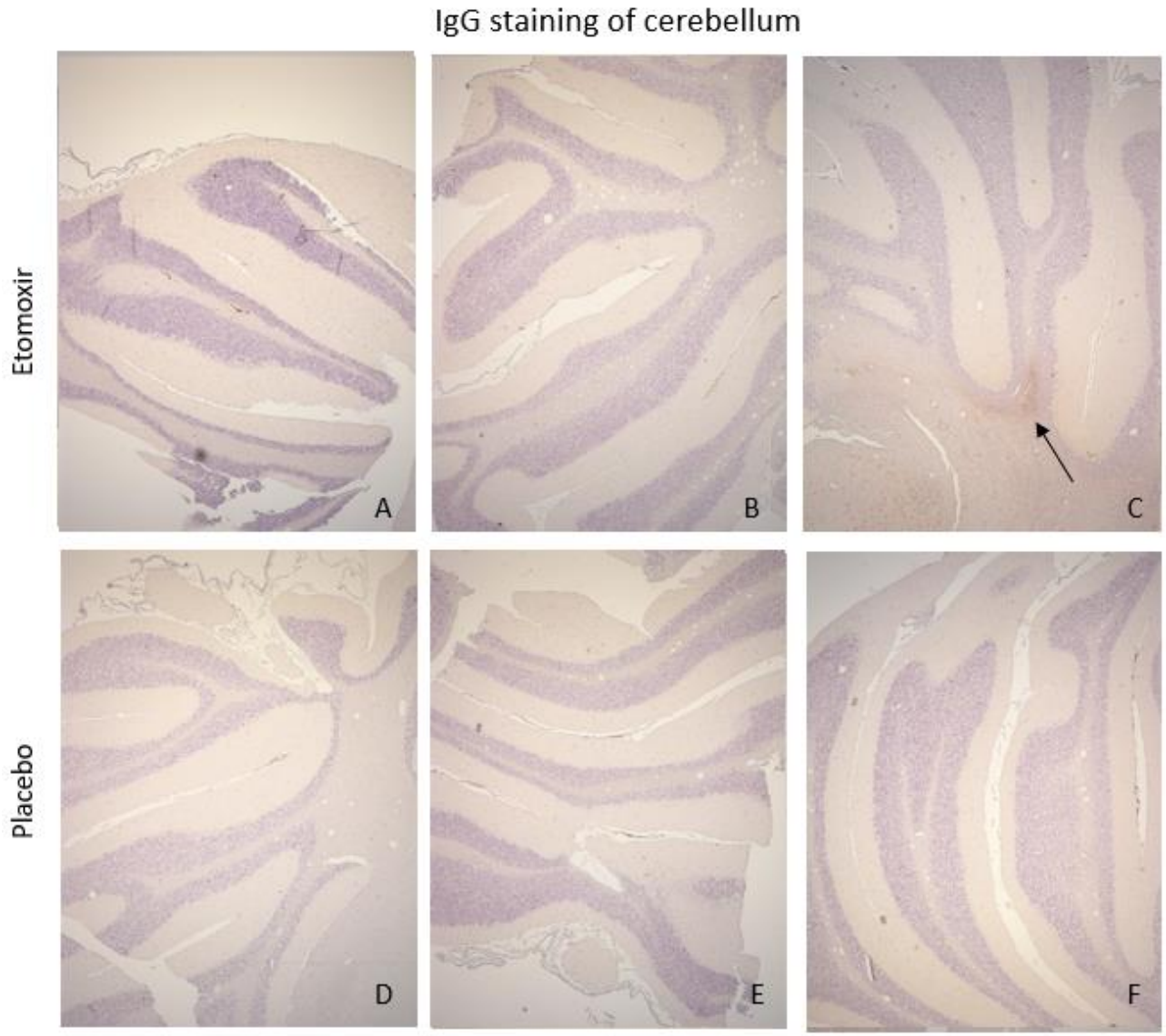

Figure 8 immunohistochemical staining of IgG in cerebellum. little to no signal is observed through-out of the rat cerebellum. C shows a slight signal along the nerves in one of the gyri which is marked with an arrow.

QUANTITATIVE ASSESSMENT

Quantitative assessment of the integrated density in the two anatomical structure; cerebellum and brainstem were performed. A significant difference was found between the groups in cerebellum with a P-value of 0.0073 (Fig. 9A). No significant difference was found between the groups in the brainstem (P-value 0.7859). Indicating that etomoxir significantly is more efficient reverse nerve damage in the cerebellum.

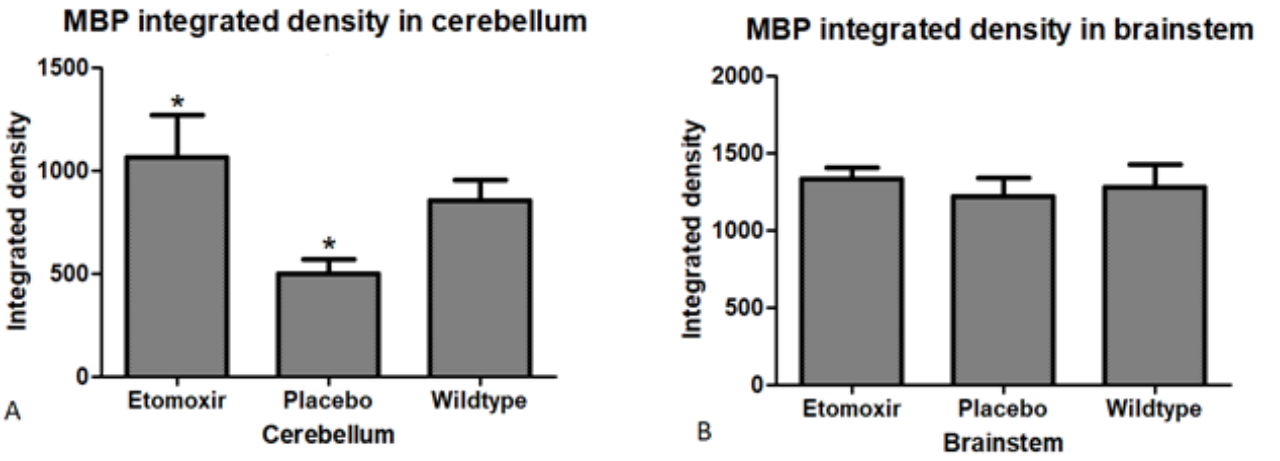

Figure 9 Quantitative assessment of MBP immunofluorescence staining. A total of 196 images were obtained and were first processed in Image J to acquire the integrated density which was used for the statical assessment. A significant difference was found between the density of signal in cerebellum between the treated and placebo animals. No significant difference was found between the group in the brainstem. The asterisk notes the significant difference (p-value 0.0073).

The quantitative assessment of CPT1a showed a significant decrease in this mitochondrial transporter in the etomoxir treated animals both in the cerebellum (P-value <0.0001) and in the brainstem (P-value 0.0001) (Fig. 10). These results indicate the etomoxir have downregulating effect on expression of CPT1a in the cerebellum and the brainstem. Together with the results of the MBP staining in the cerebellum, it is indicating that etomoxir´s nerve regenerative effect is correlated with the lowering of CPT1a expression.

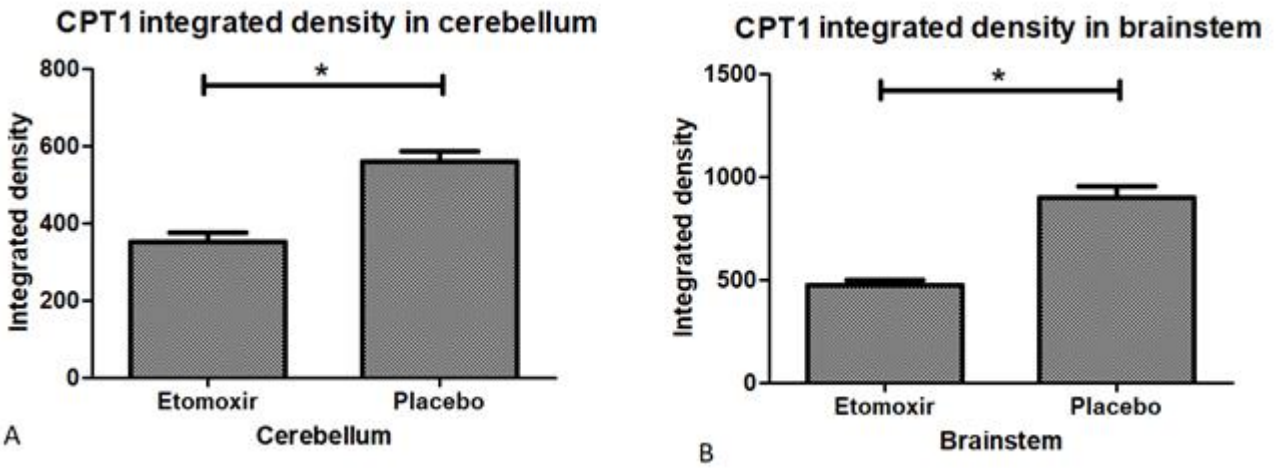

Figure 10 Quantitative assessment of CPT1a immunofluorescence staining. A total of 80 images were obtained and were first processed in Image J to acquire the intergraded density which was used for statistical assessment. A significant difference was found between the placebo and etomoxir treated animals, and in both brain structures. The asterisk notes the significant difference (p-value 0.0001 and <0.0001).

DISCUSSION

The results from this study show an increase of the MBP in the rats treated with etomoxir when compared to placebo and wildtype rats. The immunofluorescent staining was used to investigate the effect of etomoxir on EAE models.

These results showed that etomoxir possesses neurol protective properties and can significantly hinder MBP degeneration. Etomoxir blocks the CPT1a molecule and thereby inhibits the fatty acid transport into the mitochondria, what in turn prohibits the mitochondrial lipid catabolism [5]. Suggesting, that lowering of lipid catabolism with etomoxir´resembles a MS protective mutation in this mutation in this enzyme among the Inuits [5].

Presented results correlate well with the reported literature data. Previously, the effects of etomoxir have shown to provide indirectly neuronal protection and to promote neuronal regrowth [5], [33], [34]. The fluorescent assessments data from sections of brainstem and cerebellum indicated beneficial results of etomoxir treatment. The selection of these particular brain parts was based on literature findings that described neurodegeneration of the brainstem and the cerebellum in MS patients. Neurodegeneration of these CNS areas resulted in autonomic and coordination difficulties [21], [50].

The general purpose of etomoxir treatment was to either stop the MS progression and/or provide neuronal regeneration. The amount of neuronal regrowth provided by etomoxir has not been yet tested. However, this study has shown that etomoxir significantly can increase the concentration of MBP which provides an indirect evidence for rebuilding of myelin. The main goal for this study was to show the neuronal regrowth properties of etomoxir. However, this was appeared to be very difficult due to methodological issues.

The investigation of the effects of etomoxir was performed by immunofluorescent staining together with peroxide immunolabelling. Prior to the described data in this study, several attempts of unsuccessful stainings were performed. The main challenge was the low affinity of primary antibodies and the low emission of signal from the secondary fluorescent antibodies. S100 beta, TU-20, and 3 different ferritin antibodies were used together with MBP. Under the estimation of primary antibody concentration, all these antibodies were disqualified due to low or complete lack of signal except for TU-20 and MBP. Furthermore, two sets of secondary antibodies; Alexa Flour™ 488 chicken anti-rabbit (ThermoFisher Scientific #A-21441), AlexaFlour™ goat anti-rat 555 (ThermoFisher Scientific #A-21434), AlexaFlour™ goat anti-mouse 488 (Thermofisher Scientific #A-28175), and AlexaFlour™ donkey anti-mouse 555 (ThermoFisher Scientific #A-31570) were most probably stored in inappropriate temperature and some of them reached their expiration date. Hence, they were excluded from the project. After working concentration estimation for primary antibodies, TU-20 and MBP were selected for the immunofluorescent staining. The TU-20 antibody was previously used in several stainings. A new TU-20 was ordered from the same company with the same cat number. However, the newly bought TU-20 antibody, showed no or low signal when compared to the previous TU-20 antibody. Thus, finally anti-TU-20 antibody was also excluded from fluorescent staining.

The immunohistochemical stain of IgG showed rather specific labeling restricted to neuronal tracts, whereas the IgE labeling was presence all over the sections mostly in all cell bodies of both neurons and glia. An increased IgE levels were reported in patients with Mycobaterium Tuberculosis, therefore it is possible that the IgE labeling observed I related to the use the same bacteria in our animal model [51]. Experimental allergic encephalomyelitis (EAE) model is very used in MS studies. However, it needs to be taken into account that this model does not represent MS ideally even though it sheers key elements such as demyelination, axonal loss and inflammation. To better recognize and investigate the debut of MS, rats or mice with MS genetic traits should be used. Nonetheless,

these types of animals have not yet been developed and the creation of adequate model is still troublesome due to insufficient knowledge about initial conditions of this disease.

In MS patients, high level of B-cells have been found in the meninges and in the spinal fluid indicating that autoreactive b-cells have a role in MS pathophysiology [26]. Autoreactive B-cells have been also found in other autoimmune diseases such as rheumatoid arthritis [52]. Presence of IgE or/and IgG can tripper B-cells to differentiate into plasma cells and activate further stages of immune response with the secretion of pro-inflammatory cytokines. The autoantibody production in MS was also described in the study by A. S. Mørkholt et. al. (2018) [23]. This together, it seems possible to explain production of IgE as autoantibodies also in our model.

The drug Ocrelizmab, which binds to CD20 receptor that is presence on the premature and newly differentiated B-cells. The results from the phase 3 clinical trial showed a decrease in disability and a lowered volume of T2-lesions in human patients [53]. This emphasized that a lowering the B-cells levels brings beneficial effect for MS patients. However, the exact role of IgE in MS has not been yet fully clarified and requires further investigation.

In the IgG staining, the labeling was more specific and was found mainly around the tractus corticospinalis and at the ML. Nevertheless, the labeling was still mostly restricted to the cell bodies of neurons. The tractus corticospinalis and ML are two of the nerve tracts that are affected by MS. In the early stages of MS, lesions on the tractus corticospinalis have been reported in patients [54]. ML is important for somatosensation from the skin, joints therefore lesion of the medial lemnisci causes an impoairment of vibratory and touch-pressure sense what is connected with the loss of balance and high risk of falling often presence in of multiple sclerosis [55]. Therefore, our experimental IgG staining data found in rat brainstem sections corresponds well with the mentioned mobility impairments and affected structures described in human patients. It should be also noted that investigation of the presence of an immune cell infiltration, a staining with primary antibodies against B- and T-cells was also performed. However, the staining showed little to no results due to the tissue affinity. The staining was examined and deemed as inconclusive and excluded from this study.

It is important to the that difference in the anatomy and histology between rodent and human brain [56]. Especially, while rodent disease models are widely used for investigating unknown pathological background in many diseases. This model is used due to the ethical perspectives where innovative therapeutic approaches are tested and studying human samples in case of neurodegenerative diseases is only possible post mortem.

The sections were preserved in 4% paraformaldehyde for 3 years prior to this experiment. Paraformaldehyde creates cross-linkages in the tissue. These cross-linkages block the antigen binding sites, thus significantly decreasing the chances for recognition of right antigen and appropriate antibody binding capabilities and right recognition. To reverse paraformaldehyde cross-linkage and retrieve binding sites, the collected tissues were extensively washed for a week. However, inability of different tested antibodies to properly bind to targeted antigens can suggest significant loss of affinity in the preserved tissues. Furthermore, the avaliability of especially wild type brains was very limited. An additional important factor was the limited number of brains avabilbe for this study. In many cases damage to important brain structures caused by inaccurate brain extraction were noted. Such extraction related artefacts were unfortunately especially prevallent among wildtype and placebo brains. Moreover, the option of using newer brains together with the stored brain was excluded due to the unknown loss of binding sites and the high risk of compromising finial results. Even tough, different antigen retrieval approaches were tested no considerable improvement was noted in the final result of both immunoperoxide and immunofluorescent staining for the antibodies excluded from the study.

The onset of MS is still unknown but the new metabolic theory which is provided in this study suggest a possible pathway of MS progression [5], [6]. The mitochondrial stress and oxidative stress have shown to be a source of diseases such as cardiovascular; and atherosclerosis, chronic kidney-, and neurodegenerative diseases [38].

A mitochondria with altered morphology and function in consequence of oxidative stress is now a new hallmark of ALS [35]. It is known that NO can in low concentration act as a neurotransmitter. However, larger NO concentration can be dangerous for the cell by inducing oxidative stress. Further investigation of the ROS and NO concentration are needed to clarify the accumulation threshold for the mitochondrial oxidative stress damage to appear [38], [57]. Furthermore, investigation of demyelinating neurons, oligodendrocytes and astrocytes metabolism with single cell transcriptomics or by mass spectrometry could further clarify understanding the background of this pathologic process.

Another factor worth consideration in the case of MS is the impact on the PPAR system on the oxidative stress [58]. The PPAR system regulates various part of the lipid homeostasis [59]. In the presence of oxidative stress, PPARγ levels was decreased in contrast to phosphorylated state of PPARγ which is increased. This phosphorylated PPARγ induced disruption to the mitochondrial homeostatic machinery and was responsible for their persistent cumulative dysfunction[58]. Oxidative stress is known to destabilize the mitochondria and leading to cell stress and apoptosis [58]. PPARα activity has shown to be higher in the presence of oxidative stress through the increased expression of NOX [60]. These interactions can be suspected to be the cause of mitochondrial dysfunction, increased lipid catabolism and higher lipid beta-oxidation in MS.

A newer study by Y. Lages et al (2015) showed that hypoxia can provide an increase in ATP production in neurons [57]. However, was investigated in neuronal progenitor cells, which are more stable than primary neurons. Thus, a noticeable complication in this approach is the fact that neuronal progenitor cells are very adaptable and thereby not the exact representation of neurons (ref). Additionally, it has been reported that increased ATP production can occurs in der apoptotic conditions [61]. In the study from Y. Lages et. al. (2015) they were able to maintain neuronal progenitor cells in apoptotic condition only for 6 days whereas, apoptotic conditions can last longer in the disease affected tissue[57] [62]. Nonetheless, comparison of ATP production measurements described in the of Y. Lages et. al. (2015) and M. Leist et al (1997), indicates that neuronal progenitor cells can indeed increase ATP production in the state of apoptosis [57], [61].

CONCLUSION

Etomoxir effectiveness was clarified by performing immunofluorescent histochemical staining on rat brain. Cerebellum and brainstem were selected for the microscopy, due to the high degeneration of myelin in these two areas in the brain. Furthermore, most patients develop autonomic and coordination symptoms as main symptoms. The sections were stored for 3 years in paraformaldehyde. Prior to stain, the section was washed, embedded and cut.

The brainstem sections of etomoxir-threated rats showed the greater signal intensity when compared both placebo and wildtype rat. The immunofluorescent staining showed insignificant difference between etomoxir-threated, placebo and wildtype rats (p-value 0.0073). No significant differences was found in the cerebellum. CPT1a was found to be downregulated in both the brainstem and cerebellum and was significantly different from placebo (p-value <0.0001).

Based on the observations and the data analysis, etomoxir has shown to upregulate the concentration of MBP in the brainstem. Hence, improving the nerve health and reversing the damage resulting from the induced EAE disease model in the rats.

The immunohistochemical peroxidase staining showed that IgE was presence in both brainstem and cerebellum in both groups; etomoxir- and placebo-treated rats. However, the IgE staining did not show an overall tendency. The IgG staining showed labelling in brainstem, where is signal intensity were strongest in the placebo group. The labelling was no consistent trough-out the section. In the cerebellum, little to no labelling were observed. Due to these inconsistences, no conclusion can be drawn in the matter of the IgE and IgG appearance.

However, further investigation of etomoxir and upregulation of CPT1a is needed.

REFERENCES

[1] G. F. Wu and E. Alvarez, "The immuno-pathophysiology of multiple sclerosis."

[2] M. Krumbholz *et al.*, "B cells and antibodies in multiple sclerosis pathogenesis and therapy," *Nat. Publ. Gr.*, vol. 8, no. 10, 2012.

[3] I. Loma and R. Heyman, "Multiple sclerosis: pathogenesis and treatment.," *Curr. Neuropharmacol.*, vol. 9, no. 3, pp. 409–16, 2011.

[4] C. A. Rush, H. J. Maclean, and M. S. Freedman, "Aggressive multiple sclerosis: proposed definition and treatment algorithm," *Nat. Publ. Gr.*, vol. 11, no. 2, pp. 379–389, 2015.

[5] C. C. Ford *et al.*, "Digging Deeper Into MS Pathology: Is Lipid Metabolism At the Root?," *Sci. MS Manag.*, vol. 7, no. 2, 2017.

[6] A. P. Corthals, "MULTIPLE SCLEROSIS IS NOT A DISEASE OF THE IMMUNE SYSTEM."

[7] H. N. Lemus, A. E. Warrington, and M. Rodriguez, "Multiple Sclerosis Mechanisms of Disease and Strategies for Myelin and Axonal Repair," *Neurol. Clin. NA*, vol. 36, pp. 1–11, 2018.

[8] L. T. Grinberg, U. Rueb, H. Heinsen, and S. F. Cappa, "Brainstem: neglected locus in neurodegenerative diseases," 2011.

[9] J. Voogd, "THE GROSS ANATOMY OF THE CEREBELLUM," *RAT Nerv. Syst.*, pp. 205–242, 2004.

[10] H. J. Groenewegen and M. P. Witter, "Thalamus," *RAT Nerv. Syst.*, pp. 407–453, 2004.

[11] M. Ángeles Fernández-Gil, R. Palacios-Bote, M. Leo-Barahona, and J. P. Mora-Encinas, "Anatomy of the Brainstem: A Gaze Into the Stem of Life," *YSULT*, vol. 31, pp. 196–219.

[12] G. Sengul and C. Watson, "Ascending and Descending Pathways in the Spinal Cord," in *The Rat Nervous System: Fourth Edition*, 2014.

[13] G. J. Augustine *et al.*, *NEOSCIENCE*, 3rd ed. Sinauer Associates, Inc, 2004.

[14] D. Purves *et al.*, "Damage to Descending Motor Pathways: The Upper Motor Neuron Syndrome," in *Neoscience*, Sinauer Associates, 2004, pp. 412–413.

[15] P. Darcy and A. M. Moughty, "Pronator Drift," *N. Engl. J. Med.*, vol. 369, no. 16, p. e20, Oct. 2013.

[16] C. D. Betts, M. T. D, and C. J. Fowler, "Urinary symptoms and the neurological features of bladder dysfunction in multiple sclerosis," *J. Neurol. Neurosurgery, Psychiatry Neurol Neurosurg Psychiatry*, vol. 5656, no. 7, 1993.

[17] J. R. Plemel, W.-Q. Liu, and V. W. Yong, "Remyelination therapies: a new direction and challenge in multiple sclerosis," *Nat. Rev.*, vol. 16, pp. 617–634, 2017.

[18] M. Calabrese *et al.*, "Basal ganglia and frontal/parietal cortical atrophy is associated with fatigue in relapsing-remitting multiple sclerosis."

[19] A. J. Mcdougall and J. G. Mcleod, "Autonomic nervous system function in multiple sclerosis."

[20] S.-M. Choi, "Movement Disorders Following Cerebrovascular Lesions in Cerebellar Circuits," *J Mov Disord*, vol. 9, no. 2, pp. 80–88, 2016.

[21] A. D´Ambrosio *et al.*, "Cerebellar contribution to motor and cognitive performance in multiple sclerosis: An MRI sub-regional volumetric analysis," *Mult. Scler. J.*, vol. 23, no. 9, pp. 1194–1203, 2017.

[22] X. M. Cabezas *et al.*, "Improved Automatic Detection of New T2 Lesions in Multiple Sclerosis Using Deformation Fields," 2016.

[23] A. S. Mørkholt *et al.*, "Identification of brain antigens recognized by autoantibodies in experimental

autoimmune encephalomyelitis-induced animals treated with etomoxir or interferon-β," *Sci. Rep.*, vol. 8, no. 1, p. 7092, Dec. 2018.

[24] K. G. Warren and I. Catz, "Autoantibodies to myelin basic protein within multiple sclerosis central nervous system tissue," *J. Neurol. Sci.*, vol. 115, no. 2, pp. 169–176, Apr. 1993.

[25] W. W. Tourtellotte and B. I. Ma, "Multiple sclerosis: the blood-brain-barrier and the measurement of de novo central nervous system IgG synthesis.," *Neurology*, vol. 28, no. 9 Pt 2, pp. 76–83, Sep. 1978.

[26] B. Serafini, B. Rosicarelli, R. Magliozzi, E. Stigliano, and F. Aloisi, "Detection of Ectopic B-cell Follicles with Germinal Centers in the Meninges of Patients with Secondary Progressive Multiple Sclerosis," *Brain Pathol.*, vol. 14, no. 2, pp. 164–174, Apr. 2004.

[27] G. P. Bienert *et al.*, "Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes," *J. Biol. Chem.*, 2007.

[28] W. E. Paul, R. S. Schwartz, and S. K. Datta, "Autoimmunity and Autoimmune Diseases." Raven Press, pp. 819–866, 1989.

[29] A. G. Solimando, A. Tomasicchio, and S. A. Giovanni, "B-Cell Therapies in Relapsing Remitting and Primary Progressive Multiple Sclerosis: A Short Clinical Review Spectrum of Disease Progression in Multiple Sclerosis," *Biochem Pharmacol (Los Angel)*, vol. 5, p. 218, 2016.

[30] A. Gajofatto and M. D. Benedetti, "Treatment strategies for multiple sclerosis: When to start, when to change, when to stop?," 2015.

[31] B. Gerstl, M. G. Tavaststjerna, R. B. Hayman, L. F. Eng, and J. K. Smith, "Alterations in Myelin Fatty Acids and Plasmalogens in Multiple Sclerosis," *Ann. N. Y. Acad. Sci.*, vol. 122, no. 1, pp. 405–416, 1965.

[32] M. J. Noga *et al.*, "Metabolomics of cerebrospinal fluid reveals changes in the central nervous system metabolism in a rat model of multiple sclerosis."

[33] L. P. Shriver and M. Manchester, "Inhibition of fatty acid metabolism ameliorates disease activity in an animal model of multiple sclerosis," *Sci Rep*, vol. 1, p. 79, 2011.

[34] A. Mørkholt, K. Kastaniegaard, T. M., and et al., "Comparison of eto- moxir, a lipid metabolism blocker, and interferon beta treatment on anti- body recognition of brain proteins in multiple sclerosis.," in *Presented at Con- sortium of Multiple Sclerosis Centers 31st Annual Meeting*.

[35] F. Bozzo, A. Mirra, and M. T. Carrí, "Oxidative stress and mitochondrial damage in the pathogenesis of ALS: New perspectives," *Neurosci. Lett.*, pp. 3–8, 2017.

[36] M. F. Beal, "Mitochondria take center stage in aging and neurodegeneration," *Ann. Neurol.*, vol. 58, no. 4, pp. 495–505, Oct. 2005.

[37] Z. Du *et al.*, "NADPH OXIDASE 2-DEPENDENT OXIDATIVE STRESS, MITOCHONDRIAL DAMAGE AND APOPTOSIS IN THE VENTRAL COCHLEAR NUCLEUS OF D-GALACTOSE-INDUCED AGING RATS," *Neuroscience*, pp. 281–292, 2015.

[38] I. Liguori *et al.*, "Oxidative stress, aging, and diseases.," *Clin. Interv. Aging*, vol. 13, pp. 757–772, 2018.

[39] A. Mirshafiey and M. Mohsenzadegan, "Antioxidant therapy in multiple sclerosis," *Abbas Mirshafiey & Monireh Mohsenzadegan*, vol. 31, no. 1, pp. 13–29, 2009.

[40] J. D. McGarry and N. F. Brown, "The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis.," *Eur. J. Biochem.*, vol. 244, no. 1, pp. 1–14, 1997.

[41] P. R. Devchand, O. Ziouzenkova, and J. Plutzky, "Oxidative Stress and Peroxisome Proliferator-Activated Receptors Reversing the Curse?," 2004.

[42] M. Bristow, W. Stanley, J. Kemer, H. Sabbah, R. Roden, and M. Bristow, "Etomoxir: a new approach to

treatment of chronic heart failure.," *Lancet (London, England)*, vol. 356, no. 9242, pp. 1621–2, Nov. 2000.

[43] A. S. Mørkholt, O. Wiborg, J. G. K. Nieland, S. Nielsen, and J. D. Nieland, "Blocking of carnitine palmitoyl transferase 1 potently reduces stress-induced depression in rat highlighting a pivotal role of lipid metabolism."

[44] J. M. Boggs, "Myelin basic protein: a multifunctional protein," *Cell. Mol. Life Sci.*, vol. 63, no. 17, pp. 1945–1961, Sep. 2006.

[45] P. K. Toshniwal and E. J. Zarling, "Evidence for Increased Lipid Peroxidation in Multiple Sclerosis," 1992.

[46] R. Thavarajah, V. K. Mudimbaimannar, J. Elizabeth, U. K. Rao, and K. Ranganathan, "Chemical and physical basics of routine formaldehyde fixation.," *J. Oral Maxillofac. Pathol.*, vol. 16, no. 3, pp. 400–5, Sep. 2012.

[47] "Anti-Myelin Basic Protein antibody [MBP101] [ab62632) Abcam." .

[48] "Anti-CPT1A antibody [8F6AE9] KO Tested (ab128568) | Abcam." [Online]. Available: https://www.abcam.com/cpt1a-antibody-8f6ae9-ab128568.html. [Accessed: 20-Dec-2018].

[49] A. L. Barsoum, P. G. H~her, and E. K. Kuwert, "Medical Microbiology and Immunology Serum Immunoglobulin E Level and Search for Myelin Basic Protein Specific IgE Antibodies in Patients with Multiple Sclerosis," 1977.

[50] M. K. Skoric, I. Adamac, V. N. Madaric, and M. Habek, "Evaluation of brainstem involvement in multiple sclerosis," *Can J neurol Sci.*, vol. 41, pp. 346–349, 2014.

[51] H. W. Schroeder, L. Cavacini, and L. Cavacini, "Structure and function of immunoglobulins.," *J. Allergy Clin. Immunol.*, vol. 125, no. 2 Suppl 2, pp. S41-52, Feb. 2010.

[52] N. V Giltiay, C. P. Chappell, and E. A. Clark, "B-cell selection and the development of autoantibodies.," *Arthritis Res. Ther.*, vol. 14 Suppl 4, no. Suppl 4, p. S1, 2012.

[53] X. Montalban *et al.*, "Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis," *N. Engl. J. Med.*, vol. 376, no. 3, pp. 209–220, Jan. 2017.

[54] M. Pawlitzki *et al.*, "Loss of corticospinal tract integrity in early MS disease stages.," *Neurol. Neuroimmunol. neuroinflammation*, vol. 4, no. 6, p. e399, Nov. 2017.

[55] B. W. Fling, G. G. Dutta, H. Schlueter, M. H. Cameron, and F. B. Horak, "Associations between Proprioceptive Neural Pathway Structural Connectivity and Balance in People with Multiple Sclerosis.," *Front. Hum. Neurosci.*, vol. 8, p. 814, 2014.

[56] N. A. Oberheim *et al.*, "Uniquely Hominid Features of Adult Human Astrocytes," 2009.

[57] Y. M. Lages, J. M. Nascimento, G. A. Lemos, A. Galina, L. R. Castilho, and S. K. Rehen, "Low oxygen alters mitochondrial function and response to oxidative stress in human neural progenitor cells.," *PeerJ*, vol. 3, p. e1486, 2015.

[58] D. M. Small, C. Morais, J. S. Coombes, N. C. Bennett, D. W. Johnson, and G. C. Gobe, "Oxidative stress-induced alterations in PPAR-γ and associated mitochondrial destabilization contribute to kidney cell apoptosis," *Am. J. Physiol. Physiol.*, vol. 307, no. 7, pp. F814–F822, Oct. 2014.

[59] K. A. Burns and J. P. Vanden Heuvel, "Modulation of PPAR activity via phosphorylation.," *Biochim. Biophys. Acta*, vol. 1771, no. 8, pp. 952–60, Aug. 2007.

[60] T. Kim and Q. Yang, "Peroxisome-proliferator-activated receptors regulate redox signaling in the cardiovascular system.," *World J. Cardiol.*, vol. 5, no. 6, pp. 164–74, Jun. 2013.

[61] M. Leist, B. Single, A. F. Castoldi, S. Kühnle, and P. Nicotera, "Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis.," *J. Exp. Med.*, vol. 185, no. 8, pp. 1481–6, Apr. 1997.

[62] D. R. Green, "Apoptotic Pathways: Ten Minutes to Dead," *Cell*, vol. 121, no. 5, pp. 671–674, Jun. 2005.