Investigating the role of melatonin in multiple sclerosis's pathogenesis and treatment

Thèse

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Résumé

Bien que différents facteurs endogènes et exogènes soient impliqués dans la pathogenèse de la sclérose en plaques (SP), qui est une maladie auto-immune, des résultats contradictoires ont été rapportés concernant les niveaux de synthèse et la fonction de la mélatonine dans la SP. La mélatonine circadienne est une hormone qui est libérée en réponse à la baisse de la luminosité par la glande pinéale.

Dans la présente thèse de recherche, nous avons étudié le rôle de la mélatonine dans la SP à l'aide de différents modèles. Tout d'abord dans le chapitre 1, on découvre que la mélatonine a tendance à augmenter dans le modèle de Cuprizone de la SP. Nous avons constaté que le fait de maintenir les souris dans l'obscurité constante entraîne une augmentation supplémentaire de l'effet de renforcement immunitaire de la mélatonine et l'exacerbation de la démyélinisation et l'infiltration. En revanche, la luminothérapie a considérablement atténué la démyélinisation en inhibant la synthèse de la mélatonine et en augmentant l'effet immunosuppresseur du cortisol. Deuxièmement dans le chapitre 2, nous avons utilisé le modèle EAE de la SP pour traiter les souris avec de la mélatonine exogène. Bien que la mélatonine ait déjà montré une réduction chez le modèle de l'EAE, cette étude a montré que la mélatonine améliore la remyélinisation mais que celle-ci est associée à une inhibition de l'enzyme PDC via PDK4, ce qui inhibe donc la synthèse des acides gras nécessaires à une remyélinisation efficace. Cet effet secondaire est éliminé par la co-administration de mélatonine et de la DADA, un inhibiteur de PDK4. Troisièmement dans le chapitre 3, nous avons proposé une voie mécanistique par laquelle la réduction de la mélatonine par le vieillissement et le changement de mode de vie jouent un rôle critique dans la prolifération incontrôlée des cellules souches neurales dans la SVZ. Il s'agit de l'étape initiale de l'initiation du glioblastome. Cet effet de la mélatonine sur la SVZ a mis en évidence l'importance de la mélatonine dans le processus d'oligodendrogenèse, étant donné que les précurseurs d'oligodendrocytes peuvent provenir de la SVZ dans la SP.

En résumé, nos études suggèrent que la synthèse de la mélatonine pinéale devrait être suivie pour chaque cas afin de déterminer le profil de l'oscillation de la mélatonine du patient en fonction de son régime alimentaire, de son mode de vie, etc. L'administration de mélatonine peut favoriser la SP, en cas de carence en mélatonine, ou l'exacerber, en cas de surdosage en mélatonine. En outre, la luminothérapie peut être une approche efficace pour contrôler la mélatonine du SNC et augmenter le cortisol immunosuppresseur dans la SP.

Abstract

Different endogenous and exogenous factors are involved in multiple sclerosis (MS) pathogenesis, an autoimmune disease. However, conflicting results are reported concerning melatonin synthesis levels, and function in MS. Circadian melatonin is a hormone released in response to the darkness from the pineal gland. In the current research thesis, we investigated the role of melatonin by different models in MS. First, in chapter 1, it is uncovered that melatonin tends to increase in the cuprizone model of MS. We figured out that keeping mice in constant darkness caused further increase in immunoenhancing function of melatonin and exacerbated the demyelination and infiltration. In contrast, light therapy significantly improved demyelination by inhibiting melatonin synthesis and boosting the cortisol immunosuppressant. Second, in chapter 2, we used the EAE model of MS to treat the mice with exogenous melatonin. The current study showed that melatonin improves remyelination; however, is associated with the inhibition of the PDC enzyme via PDK4, which inhibits the fatty acid synthesis required for efficient remyelination. This side effect is eliminated by the co-administration of melatonin and DADA, a PDK4 inhibitor. Third, in chapter 3, we proposed a mechanistic pathway by which melatonin reduction by aging and different lifestyle play a critical role in the uncontrolled proliferation of neural stem cells in the subventricular zone (SVZ). This is the initial step in glioblastoma initiation. This effect of melatonin on SVZ, highlighted the importance of melatonin in the oligodendrogenesis process as oligodendrocyte precursor can originate from SVZ in MS.

To sum up, it suggested that pineal melatonin synthesis should be monitored in each induvial to figure out what is the patient's pattern in melatonin oscillation based on his/her diet, lifestyle, etc. Melatonin administration can improve MS, in case of melatonin deficiency or can exacerbate it, in case of melatonin overdose. Furthermore, light therapy may be an efficient approach for controlling the CNS melatonin and increasing the cortisol immunosuppressant in MS.

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Foreword

This thesis investigates the role of melatonin in the pathogenesis and treatment of multiple sclerosis. This thesis consists of two research projects and two review papers carried out under Dr. Serge Rivest's supervision and in collaboration with Dr. Kazem Zibara and Zahra Farhadi from Lebanon and Iran.

The thesis starts with introduction. To avoid repeating some contents here, which have already been explained within the papers, I provided critical descriptions regarding the MS diseases and factors of interest in this thesis. Furthermore, part of the introduction belongs to two review papers that I've written under the supervision of Dr. Serge Rivest.

The 1st chapter corresponds to the paper that its accepted for publication in journal of Experimental & Molecular Medicine. Briefly, the current work is a follow-up to a study published in Cell (Farez *et al.* 2015), in which melatonin was identified as one factor that influences the severity of MS symptoms in patients and that its administration to an EAE model of MS suppressed disease progression. The authors concluded that "extreme caution should be exercised to evaluate the translational potential of these findings". The current study has been designed to decipher better the role of melatonin, currently under investigation in a clinical trial for MS patients (ClinicalTrials.gov Identifier: NCT03498131). We show that melatonin therapy is a double-edged therapeutic approach that should be considered with extreme caution, as Farez and colleagues warned.

The 2nd chapter corresponds to the paper which is published in Frontiers in Immunology. This work has been carried out under the supervision of Dr. Serge Rivest, at Laval University and in collaboration with Dr. Kazem Zibara and Zahra Farhadi from Lebanese university and Yasuj university of medical sciences. Briefly, this manuscript presents new data regarding the fatty acid metabolism involved in the remyelination process in multiple sclerosis. Indeed, we showed how the PDK4/PDC metabolic axis is affected by demyelination and then showed how melatonin slows down the PDC activity, which is a side effect of melatonin therapy. Eventually, we used DADA in combination with melatonin to eliminate the efficiency of melatonin therapy in the MS model. Furthermore, we presented evidence that oligodendrocytes can use alternative sources of substrate for myelination in case of shortage in the primary source.

In continue, appendix A presents the title and abstract of the review paper that has been published in Expert Reviews in Molecular Medicine. This paper is written based on the recently published paper in Cell (Gengatharan *et al.* 2021), an article to that I had a substantial contribution. This paper explains how melatonin reduces by aging and how it controls the division of neural stem cells in the subventricular zone. Stem cells in this area are involved in the initiation of glioblastoma and the brain's regenerative capacity in remyelination and neurogenesis. The content of this paper is presented as part of the introduction.

Finally, appendix B presents the title and abstract of a review paper that is still under review in the journal PNAS. This paper explains how melatonin and vitamin D are involved in higher MS prevalence in northern countries. The content of this paper is presented as part of the introduction.

The references of introduction and all four chapters have been merged and presented in single bibliography at the end of thesis.

General introduction

1. Multiple Sclerosis

Multiple sclerosis (MS), an inflammatory-mediated demyelinating disease, is on the rise globally, and there is currently no treatment. Current therapeutic options allow to slow down disability progression and improve disease symptoms, partially. However, these medications are only approved for relapsing-remitting MS (RRMS), and there is no FDA-approved treatment for progressive MS. Although the etiology of MS remains unclear, a wide range of risk factors have been proposed, with the immune system, genetics, environment, and infection is the most significant. According to the Atlas of MS in 2020, provided by the Multiple Sclerosis International Federation (MSIF), more than 2.8 million are currently living with MS. Comparing the data from 2013 showed an increase in MS prevalence owing to the rise in the life span of MS patients or the improvement in MS diagnosis. Since the environment is considered one of the most vital risk factors for MS, latitude and the geographical area in which people live is strongly associated with MS prevalence. Vitamin D and melatonin hormones can be affected considerably by latitude and the environment.

Some well-reported studies showed an increase in MS susceptibility by moving from the equator to the poles (Sabel *et al.* 2021; Munk Nielsen *et al.* 2019). As an introduction, we aimed to report the current knowledge regarding the roles of latitude and seasons in MS susceptibility and then discuss how they affect vitamin D, cortisol, and melatonin levels. We will then summarize the studies that reported immunoenhancing and detrimental roles of melatonin in MS. In fact, the beneficial roles of melatonin have been previously reported by several review papers, while negative studies were disregarded. We will support the idea that melatonin is not a universal remedy and that taking it as a supplement does not necessarily improve our health, especially in the absence of studies on the long-term effects of melatonin.

2. Disease Course

Although it is currently impossible to predict the disease progression in each individual, the International Advisory Committee on Clinical Trials in Multiple Sclerosis (Lublin *et al.* 2014) has defined four main MS disease courses, which comprise (A) Clinically isolated

syndrome (CIS) as the first clinical manifestation of disease by characterizing the inflammation and demyelination in the CNS; however, this still does not meet the McDonald MS diagnostic criteria to be considered as MS. In case the patients develop lesions, then the second episode of neurologic symptoms is expected that can lead to relapsing-remitting MS. (B) relapsing-remitting MS (RRMS) is the most common type of MS, and more than 85% of patients are diagnosed with RRMS. In this type of MS, neurologic symptoms can gradually increase following a new attack, termed relapses, or exacerbations, followed by partial or complete recovery or remissions. The latter can last weeks or months. (C) Eventually, some people with RRMS will transit to a secondary progressive MS (SPMS), a phase where the patient accumulates further disability due to progressive exacerbation of the disease. (D) In primary progressive MS (PPMS), neurologic symptoms' severity gradually worsens over time without considerable remission. To date, there is no FDA-approved medication for this category, comprising 10-15% of MS cases. Women are more susceptible to MS than men (3:1); this ratio is 1:1 in PPMS (Lublin *et al.* 2014).

3. Etiology

It is suggested that MS is an immunological convolution between an underlying primary degenerative disorder and the overactivated immune function. Trafficking of leukocytes (including T lymphocytes, monocytes, and other immune cells) to the CNS is of prime importance, which is followed by a series of interactions between some integrin surfaces of leukocytes and ligands cell adhesion molecules (CAM). Subsequently, the inflammatory response is emerged, which could be a double-edged sword as prolonged inflammatory responses not only do not suppress the pathogens or repair the injury but also call for further inflammatory mediators and cause an uncontrolled immune response that leads to host self-destruction in MS cases.

The human body receives input from the outside of the body through our five senses of taste, touch, hearing, smell, and vision and sends this information to the brain as a signal for integration and processing. The brain then sends a signal to the muscle of interest to respond. This signal transmission must be highly rapid and efficient. A lipid-rich membrane known as the myelin sheath is responsible for protecting nerve fibers and facilitating signal

transmission. The myelin is formed by different cells in the peripheral nervous system (PNS) and central nervous system (CNS). Schwann cells and oligodendrocytes are responsible for forming myelin in PNS and CNS, respectively. While each Schwann cell is responsible for forming a single myelin, oligodendrocytes can form several myelinated axons. Despite these differences, the electrophysiological characteristics are essentially the same (Susuki 2010).

Naturally, the immune system serves as your body's protection against illness. In MS patients, the immune system mistakenly attacks the protective myelin sheath around nerve cells, destroying myelin in the brain, spinal cord, and optic nerves. In the normal brain, some immune cells are blocked from coming into the CNS; however, impairment in the integrity of brain barriers like the blood-brain barrier (BBB), and blood-cerebrospinal fluid barrier (BCSFB), some immune cells, including T and B cells to infiltrate the brain and spinal cord. Basically, infiltration of T cell into the brain cause call and activation of more immune cells like B cells. The latter results in the production of antibodies that target myelin and enlist the help of other immune cells (Arneth 2019).

In the absence of a firm understanding of the mechanisms underlying MS, a combination of infectious agents and environmental and genetic factors has been postulated to be involved in disease pathogenesis. Here I discuss the most relevant risk factors in MS in my research thesis, including latitude, sunlight, and vitamin D.

i. Stress

On the other hand, the permeability of endothelial cells of BBB can be facilitated by long-life stressful life or chronic physiological stress (Mu *et al.* 2017; Zheng *et al.* 2013). For instance, restraint Stress sharply increased the permeability of BBB by decreasing the protein expression for tight junctions and damage to the BBB, which is associated with an increased level of IL-1b (Xu *et al.* 2019). Chronic mild stress exacerbates the severity of the animal model of EAE (Gerrard *et al.* 2017). Most importantly, a study on MS patients reported that 85% of MS exacerbations were associated with stressful life events in the preceding six weeks, which shows the importance of stress as a potential trigger of MS (Ackerman *et al.*

2002). Microglial cells have been introduced as a sensor responding to stress (Frank *et al.* 2019).

Furthermore, it is reported by several studies that acute stress affects the gut microbiota function and increases the permeability of endothelial cells of the intestinal tract (Söderholm *et al.* 2001), which is mediated by affecting tight junction proteins like claudin-1 (CLDN1)(Zheng *et al.* 2017). However, a study on ulcerative colitis, an inflammatory bowel disease (IBD) that causes long-lasting inflammation, showed that long-term perceived stress is more important than acute stressful life events (Levenstein *et al.* 2000). Indeed, either physical or psychological stress in rat increased the intestinal epithelial permeability (Saunders *et al.* 2002; Zheng *et al.* 2013)

ii. bacterial infections

Studies on monozygotic twins highlighted the role of environmental factors in susceptibility to MS (Generali *et al.* 2017). Among all the elements, gut microbiota dysbiosis plays a crucial role in the pathogenesis of MS. Although substantial studies investigated the gut microbiota for many years, we are still far away from the exact mechanism by which gut microbiota leads to CNS inflammation and MS. Given that taxonomic variability within the gastrointestinal tract depends on many factors including genetic, physiological, and psychological characteristics of the individual, and environmental factors, the gut microbiota is highly variable (Mohajeri *et al.* 2018). The idea of a leaky gut explains how bacterial components can translocate into the blood stream and disseminate to distal sites from the gut epithelial barrier to enter the CNS through the blood-brain barrier. The intestinal epithelial forms a barrier between the host and environment, but under some circumstances, the leaky gut happens, and the permeability of this wall can be compromised, and pathogens like antigens, toxins, bacteria components, etc. enter the bloodstream (Seguella *et al.* 2020; Obrenovich 2018). While Vandenbroucke's team reported the role of the choroid plexus in sensing peripheral inflammation and transferring this to the cells in the CNS (Balusu *et al.* 2016), they published another paper and reported that the choroid plexus is involved in the communication between the stomach and brain, depends on gastrointestinal leakage which leads to disruption of blood-CSF barrier integrity (Gorlé *et al.* 2018). They further worked

on Helicobacter suis (H. suis), a gram-negative bacterium in the intestine. Most recently, a study on EAE mice suggested that microorganisms should be considered in the pathogenicity of MS. Indeed, they reported that gut microorganisms act together to exacerbate the inflammation in spinal cords of EAE, while the symptoms ameliorated in germ-free mice (Miyauchi *et al.* 2020).

iii. Epstein-Barr Virus

There is growing evidence of the involvement of different infectious agents in MS. Epstein-Barr virus (EBV) infection is reported to increase the chance of MS incidence by 2-fold. However, the mechanistic pathway by which EBV causes MS initiation or development is still unknown; recent studies showed that this virus causes B-cell immortalization and/or transformation, which leads to subsequent inflammatory reactions and MS (Handel *et al.* 2010). However, the mechanism by which EBV plays its role in MS initiation or development is still unknown; it's proposed that likely EBV viral protein sequences mimic myelin proteins which can cause MS (Fujinami et al. 1985). In accordance, a considerable cross-reactivity between a part of amino acids of the viral protein EBV nuclear antigen 1 (EBNA-1) and the human calcium-activated chloride channel Anoctamin 2 (ANO2) has been reported in serum samples of MS patients (Tengvall et al. 2019). An increase in autoantibody reactivity against the ANO2 is observed in MS patients compared to healthy controls (Ayoglu et al. 2016).

Most recently, Bjornevik et al. conducted a Longitudinal analysis of over 10 million young adults on active duty in the U.S. military. They found that 955 of this population had been diagnosed with MS, who were active-duty military personnel. This study which was carried out over a 20-year period, showed that MS risk rises 32-fold after infection with EBV (Bjornevik *et al.* 2022). While EBV infection is positively associated with latitude (Disanto *et al.* 2013), it's reported that Sunlight may improve MS by increasing the number of CD8+ T cells, which has the potential to control EBV infection (Pender 2011).

iv. Genetic and Age

Genome-wide association studies introduced more than 150 single nucleotide polymorphisms associated with MS susceptibility. For instance, it is found that the human leukocyte antigen (HLA) DRB1*1501 is one of the main genetic risks associated with MS. Indeed, Heterozygotes for HLA-DRB1*15:01 have an adjusted odds ratio (OR) estimate of MS>3 and homozygotes >6 (Alcina *et al.* 2012), however, the odds ratio of majority of the other genes is small, around 1.1–1.2.

According to the report of the National Multiple Sclerosis Society (NMSS), most MS cases are diagnosed between the ages of 20 and 50. Age has been considered another involving factor in MS progression. Mansouri and colleges, using collinear determinants of MS relapses, demonstrated that relapses reduce significantly with age advancing (Mansouri *et al.* 2014). Further, Ligouri and colleagues' study in an Italian sporadic MS population of 1463 patients with homogeneous clinical and demographic features showed that clinical disability in MS is influenced by the patient's age, not by the age at onset (Liguori *et al.* 2000).

v. Vitamin D deficiency and low sunlight exposure

Although vitamin D deficiency can result from low consumption of food sources of vitamin D or low skin exposure to sunlight at any latitude, it is well documented that living at higher latitudes, above 37° north and below -37° south, is a risk factor for vitamin D deficiency. Indeed, countries located at high latitudes have very cold autumn, spring, and winter that do not allow people to expose their skin to sunlight (Wacker *et al.* 2013). Furthermore, a cross-sectional survey of 2301 participants with MS showed a direct correlation between moving away from the equator and a reduction of sun exposure and vitamin D synthesis, proposing supplementary vitamin D for improving MS (Jelinek *et al.* 2015). Despite the Farez study in Argentina (38°N) which did not show any significant correlation between UV exposure, Vitamin D, and MS severity (Farez *et al.* 2015); studies in Sweden (60°N) (Bäärnhielm *et al.* 2012; Hedström *et al.* 2020), Germany (51°N) (Ostkamp *et al.* 2021), and Australia (27° to 43° south) (Tremlett *et al.* 2018) showed that

low exposure to sunlight as well as vitamin D insufficiency considerably increase the risk of MS. A study in Denmark (56°N) also showed that lower sunlight exposure in adolescence is associated with younger age at MS onset.(Laursen *et al.* 2016) The importance of sunlight exposure and latitude in MS was also revealed by reviewing the relevant literature and comparing the map of UV index in the United States (i.e. the amount of sunlight received by different regions) in addition to the prevalence of vitamin D deficiency and MS in the north and south of the country (Maeser *et al.* 2018).

Furthermore, a study in the USA investigated the role of residential UV exposure from sunlight in MS patients and reported that a reduction of average lifetime UV exposure in winter is associated with increased MS risk (Gallagher *et al.* 2019). Recently, in their study on the pediatric onset of MS in the USA, Sebastian and colleagues showed that spending half an hour outside in the summer clearly reduced MS development, compared to the most recent summer as control (Sebastian *et al.* 2022).

Although diet and lifestyle should not be ignored, sun exposure is the primary variable for several months after birth. Oral vitamin D and, to a greater extent, sunlight exposure are two primary sources of vitamin D. A systematic review of the relationship between the season of birth and MS revealed that MS prevalence is higher in the spring and lower in the autumn. The authors proposed that the month of labor could be another risk factor for MS and that sunlight exposure may be the most effective agent (Torkildsen *et al.* 2012). In accordance, low levels of vitamin D during pregnancy is reported to be involved in the seasonality of the month of birth of MS cases (Sidhom *et al.* 2015). This idea is supported by a study on dried blood spot samples of 521 patients with MS, which showed that MS patients had significantly lower levels of vitamin D. Indeed, an increase of 25 nmol/L in neonatal vitamin D caused a 30% reduction in MS risk (Nielsen *et al.* 2017). This study confirmed that early life environmental exposure, here sunlight, is involved in MS. Another meta-analysis systematic review found a reduction in MS (11/15 studies) in children born in late autumn in the northern hemisphere. Interestingly, people who migrated from high to low MS risk factor environments experienced a significant decrease in MS incidence only if the migration occurred before the age of 15 (Ismailova *et al.* 2019). In parallel, a study in Newfoundland and Labrador, Canada (53N), investigated 25-hydroxyvitamin D [25-(OH)D] in pregnant women, newborns (umbilical cord blood), and children. Vitamin D insufficiency was found

to be very high since more than 90% of children and 80% of maternal and cord blood showed vitamin D insufficiency in winter; however, this slightly decreased in summer (Newhook *et al.* 2009).

Although the dietary sources of vitamin D are very few, it is estimated that 90% of the vitamin D requirement is efficiently produced by the body after exposure to sunshine. Compared to 100 grams of oily fish salmon as high diet source of vitamin D, which produces 360 IU of vitamin D, only 10-15 minutes of sun exposure in the adult during the spring or summer, with 22% uncovered skin, can make 1,000 IU (Religi *et al.* 2019; Holick 2004). Taking vitamin D supplements is only designed to boost the blood vitamin D level but raising it with sunlight would be a multipurpose strategy. For instance, it is reported that increasing vitamin D levels with supplements cause a significant reduction in cortisol levels (Al-Dujaili *et al.* 2016). This finding was further confirmed by Abu-Samak and colleagues, who reported that people with vitamin D deficiency have higher levels of cortisol (Abu-Samak *et al.* 2019).

In contrast, boosting by light is well reported to increase the cortisol level in human skin *ex-vivo* (Skobowiat *et al.* 2013). Leproult and colleagues demonstrated that the morning transition from dim to bright light leads to an immediate rise in cortisol levels while suppressing melatonin (Leproult *et al.* 2001). However, another study reported that acute exposure to light plays an inhibitory role in cortisol release (Jung *et al.* 2010). Interestingly, another study indicated that summer exposure to natural sunlight not only shortened the nighttime melatonin but also advanced the morning cortisol rise (Vondrasová *et al.* 1997). Investigating the correlation between sunlight exposure and cortisol levels in different seasons in Poland (52°N) indicated significantly increased levels in summer than in winter, with daily sunlight duration of 7.8 hours compared to 2.9 hours; respectively, which was another reason for the increase of cortisol by sunlight (Kanikowska *et al.* 2019). Accumulating evidence demonstrates the beneficial role of cortisol, the primary endogenous glucocorticoid hormone, in MS. Corticosteroids, which are widely used MS medications, are synthetic versions of cortisol (Melief *et al.* 2016).

vi. Calcium Insufficiency

Currently, the mechanistic pathway by which vitamin D affects the immune system function is not well studied. While vitamin D supplement is recommended for boosting the blood levels of vitamin D, a study by Häusler and colleagues in the journal Brain showed that prolonged high doses of vitamin D cause a sharp increase in blood vitamin D, higher than 80 ng/mL, which is associated with facilitating the development of EAE, not its suppression. Indeed, they reported that this could be mediated by the elevation of T-cell excitatory calcium; however, moderate vitamin D doses showed beneficial effects (Häusler *et al.* 2019). This induced hypercalcemia toxicity due to high doses of vitamin D was also reported earlier in the case of MS patients (Marcus *et al.* 2012).

On the other hand, the absorption of intestinal calcium is one of the leading hemostatic roles of vitamin D (Christakos *et al.* 2011; Aloia *et al.* 2014). In fact, calcium is involved in muscle contraction and relaxation in gastrointestinal smooth muscle cells, which use calcium imported from the extracellular fluid (Karaki *et al.* 1984) to mediate the proper movement of the intestine (Somlyo *et al.* 1994). Reduction in this motility, either by a decline in intestinal calcium absorption or any other risk factors, causes a delay in stomach emptying, a deadly condition which is known as Gastric Stasis. Therefore, it will take a longer time to empty the stomach (Schwartz *et al.* 1992). This leads to exaggerated translocation of intestine contents into the blood, especially bacterial toxins which is a well-recognized risk factor for MS known as the gut-brain axis (Camara-Lemarroy *et al.* 2018). It has already been reported that gastric stasis boosts gut permeability and bacterial translocation (Yacyshyn *et al.* 1996; Swank *et al.* 1996; Wolfson *et al.* 2002). This axis can be initiated by the translocation of gut microbiota endotoxin, lipopolysaccharides (LPS), into the blood and its recognition by LPS-binding proteins (LBP). The latter expresses the LPS to the surface of cells such as macrophages and endothelial cells, forming a complex with CD14, a receptor molecule for LPS. The resulting pathway disrupts the integrity of BBB (Braniste *et al.* 2014), and induces neuroinflammation that has been reviewed in our previous study (Ghareghani, Reiter, *et al.* 2018a). In addition to vitamin D deficiency, a study showed that MS patients also suffer from reduced ionized calcium (Kubicka-Baczyk *et al.* 2015). In fact, in the case of vitamin D deficiency, calcium absorption does not reach satisfying levels (Holick 2003). In support of the role of vitamin D and calcium in gastric stasis, El-Maghraby, and colleagues, using gastric

emptying scintigraphy, reported that the gastric emptying rate is slow in MS patients. While the mean half-time of gastric emptying is roughly 41 minutes, this increased more than two folds and reached 97 minutes in MS patients (el-Maghraby *et al.* 2005). Furthermore, a single-center study on 166 MS patients showed that 82% of cases have constipation (Khanna *et al.* 2022). This was further shown in an animal model of MS where EAE induction caused a delay in whole gastrointestinal motility (Spear *et al.* 2018).

vii. Latitude

Several studies have shown that MS prevalence is related to latitude, with people living closer to the equator being at lower risk while those are living at higher latitudes (closer to the north/south poles) being at higher risk. For instance, while only about 15% of the world population lives at latitudes beyond 40th parallels north and south, including Europe, Canada, the north of the USA, and Russia in the northern hemisphere versus New Zealand, Tasmania, and Patagonia in the southern hemisphere, these countries show almost two-fold higher MS prevalence than 85% of the remaining populations living between 40th parallels north and south (Pierrot-Deseilligny *et al.* 2010; Goodin 2009; Simpson *et al.* 2011). An updated meta-analysis published in 2019 found that latitude is significantly related to the prevalence of MS and that moving to higher latitudes increases the susceptibility to MS, possibly due to lower sun exposure (Simpson *et al.* 2019). Another systematic review, covering ten studies, found a clear trend in MS prevalence when moving away from the equator to the south pole, from Panama to Argentina (Risco *et al.* 2011). Another study in Newfoundland, Canada (53°N) showed that while Ultraviolet (UV) exposure flows south to north gradient more at lower latitudes, MS incidence follows north to south gradient more at higher latitudes (Sloka *et al.* 2008). In parallel, a study in north American regions and the continental United States showed that the latitudinal prevalence gradient of MS could be due to UV radiation (Beretich *et al.* 2009). This was further confirmed when a latitude gradient of MS was found by comparing the MS prevalence between the north and south of France (Dalla Costa *et al.* 2018) and New Zealand (Taylor *et al.* 2010).

These studies became even more interesting once Sabel and colleagues, in a recent paper in *Brain*, examined the latitudinal gradient prevalence of MS by taking patient

migration history into account. Indeed, they found that 1587 out of 2127 MS patients in New Zealand were born in New Zealand and that the prevalence latitude gradient was stronger at birth when the birthplace of the patients was considered. This slope of the gradient remained consistent until the age of 12, and then it started decreasing afterward. This study helped clarify the hypothesis that early-life environmental exposure is the leading risk factor for MS and that this exposure can be at birth or even in utero (Sabel *et al.* 2021). In Denmark, a northern country with a high MS prevalence, Munk Nielsen and colleagues also revealed the same idea in another *Brain* paper (Munk Nielsen *et al.* 2019).

4. Melatonin's alternation as a risk factor for MS

i. Melatonin

Melatonin (N-acetyl-5methoxytryptamine) is a neuro-hormone secreted by the pineal gland in response to darkness and regulated by circadian rhythms. The pattern of melatonin oscillation in the vast majority of organisms showed that its synthesis occurs during the night, and the duration of the synthesis is related to the length of the dark period. The suprachiasmatic nucleus (SCN) of the hypothalamus controls the synthesis of circadian melatonin in response to light changes through a sympathetic pathway that is mediated by activation of Arylalkylamine N-acetyltransferase (AANAT), the key regulatory enzyme in melatonin synthesis **(Figure 1).** Melatonin level reaches a peak of its concentration around 1 AM and drops to a minimal level at the beginning of the day (Nakahara *et al.* 2003). Melatonin is currently used by millions of people around the world as a natural supplement and treatment of circadian rhythm sleep disorders. Based on the reported study in the USA, the weighted prevalence of melatonin use, which is sold over the counter, has increased from 0.4 in 2000 to 2.1 in 2018 (Li *et al.* 2022).

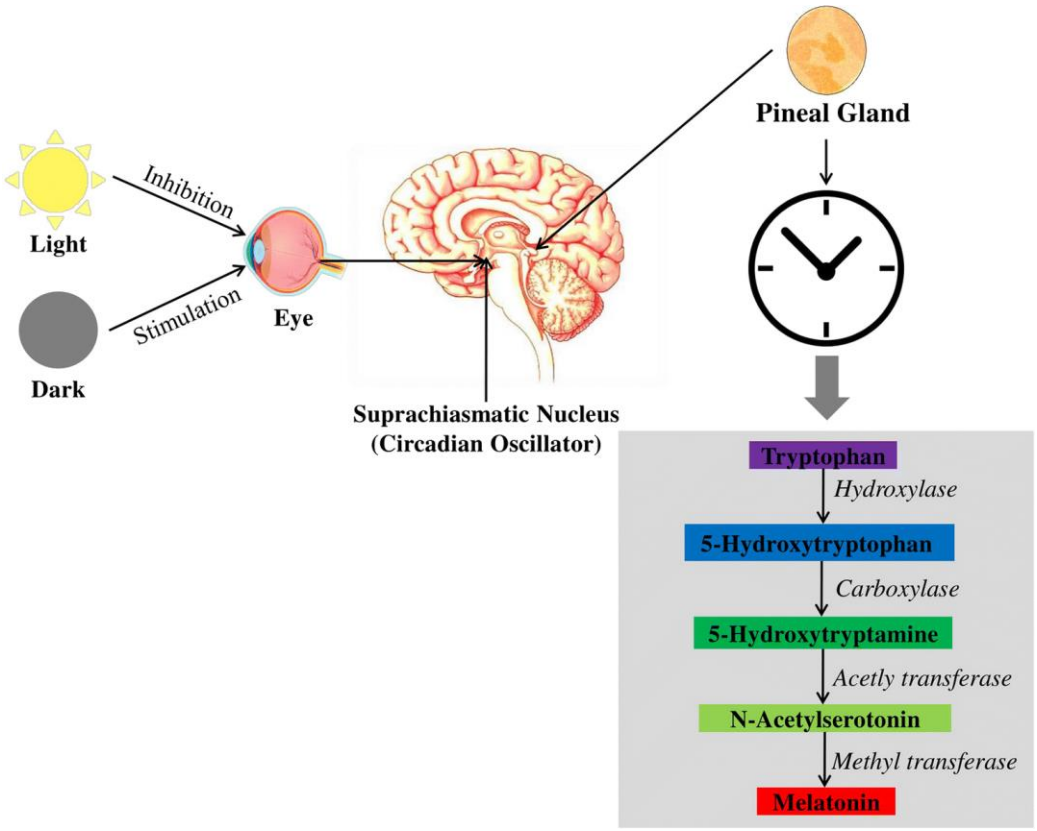

Figure 1: Schematic drawing of pineal melatonin synthesis. Sunlight or any other light, except red light, is perceived by the eye and translated by SCN for the brain to suppress the melatonin synthesis in the pineal gland. In contrast, darkness cause SCN to send a signal to the pineal gland for activation of melatonin synthesis by an almost 100-fold increase in transcription and activity of AANAT. *Adapted from (Hossain et al. 2019)*

ii. Melatonin receptors

Melatonin exerts its effects through binding to its receptors of melatonin receptors MT1A and MT1B, G protein-coupled receptor (GPCR), and activation of them (Reppert *et al.* 1996). Indirectly, melatonin can affect cell physiology as a free-radical scavenger and antioxidant, an effect that is tightly associated with aging and some neurodegenerative diseases. Melatonin receptors, on the other hand, bind individually to melatonin or its antagonist, such

as Luzindole; it has also been observed that these two receptors can form dimers, with each dimer binding to one melatonin. Indeed, the possibility of MT1A/MT1B heteromeric formation is observed in the mouse retina, where they function for the melatonin-dependent increase in light sensitivity at night (Tosini *et al.* 2014).

Both MT1A and MT1B are involved in several signaling pathways, especially Gi/cAMP pathway. MT1A signaling also couple to the Gq/PLC/Ca2+ pathway, but MT1B lack this potential. It is also reported that the heteromers inhibit forskolin-promoted cAMP production more potently compared to homomers. It was suggested that MT1B receptor could be a positive allosteric regulator of MT1A receptors with regard to the activation of the Gq/PLC pathway (Tosini *et al.* 2014; Baba *et al.* 2013)**(Figure 2).**

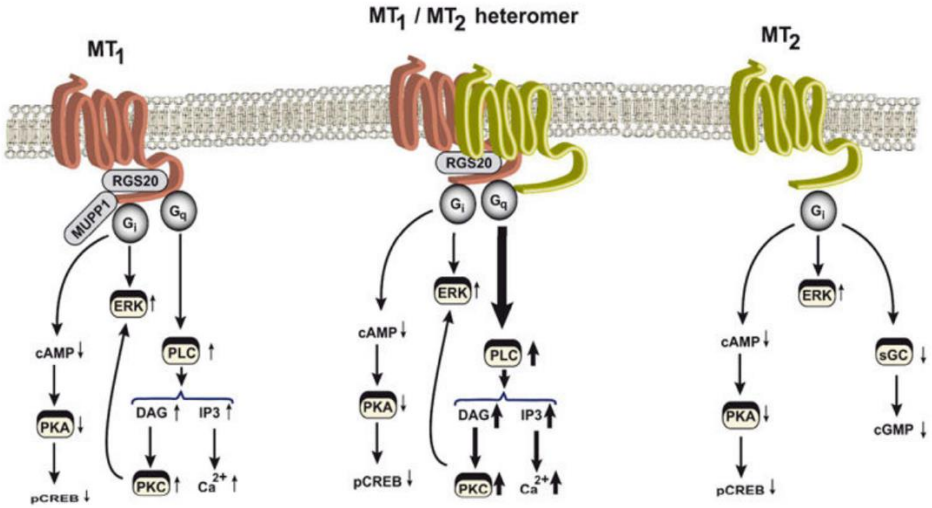

Figure 2: Signaling pathways of melatonin receptors. Melatonin receptors can be in different forms, including MT1 homomers, MT2 homomers, or MT1/MT2 heteromers. *Adapted from (Tosini et al. 2014).*

iii. Sunlight or Vitamin D Supplement May Affect MS via Melatonin-Dependent Pathway

The synthesis pattern of vitamin D and melatonin is opposite to each other. The skin starts to produce vitamin D during daylight and upon exposure to sunshine, whereas the pineal gland starts melatonin synthesis in the absence of any light at night. This means that light (or darkness) will only boost one of these two agents while suppressing the other one. An elegant study by Irving and colleagues in 2019, using vitamin D receptor knockout mice who were also unable to produce vitamin D, showed that UV light improves EAE severity independent of vitamin D and its receptor. This study clearly demonstrated that sunshine might improve MS via a vitamin D-independent mechanism (Irving *et al.* 2019). One of the possible mechanisms by which sunshine or vitamin D affect MS is reported by Golan and colleagues when they treated MS patients with vitamin D. Results showed that after a year of daily treatment of MS patients, high doses of vitamin D significantly suppressed the nighttime melatonin and that there is a negative correlation between vitamin D and melatonin. They suggested that vitamin D mediates its effect via melatonin in MS (Golan *et al.* 2013).

iv. Melatonin Increases at Higher Latitudes by Cold Habitats and Low Exposure to Sunlight

Melatonin levels is found to be higher and to last for longer periods in winter in locations with seasonal variations and significant changes in photoperiod length (Kauppila *et al.* 1987; Wehr *et al.* 2001; Adamsson *et al.* 2016; Stokkan *et al.* 1994; Vondrasová *et al.* 1997). It is widely accepted that bright light exposure is significantly higher in the summer than in the winter, a difference that is more pronounced at higher latitudes (Adamsson *et al.* 2016; Cole *et al.* 1995). Annual variations in melatonin is reported in northern latitude countries, including Sweden (60°N), Estonia (59°N),(Adamsson *et al.* 2016), and Finland (67°N)(Leppäluoto *et al.* 2003), among others. Investigating the melatonin profile in Estonia, a north European country, and Italy (41°N), a southern European country, revealed

significantly higher melatonin in Estonia (Cutolo *et al.* 2005), confirming the trend of melatonin increase from the equator to the poles.

Temperature change is another factor that varies when moving from the equator to the north. Since an increase in pineal gland size is associated with a rise in melatonin synthesis, it has been shown that moving away from the equator causes an increase in pineal gland size (Ralph 1975). In accordance, physicians recommend keeping cold in the bedroom at night to boost melatonin synthesis and thus have better sleep (Fu *et al.* 2017). In addition, the cold temperature was revealed to induce the expression of AANAT, a key enzyme in melatonin synthesis (Xu *et al.* 2018).

v. Early life exposure to low or high melatonin in MS susceptibility

To date, it is shown that melatonin synthesis increases by moving to higher latitudes and by a high melatonin diet. Besides, melatonin synthesis and rhythm are markedly affected by disturbance risk factors such as light at night, LED screens, night shifts, etc. (Ghareghani, Zibara, *et al.* 2022). Pineal melatonin synthesis begins at 4-6 months of age and is produced in a light-dependent circadian rhythm. It reaches its peak during early childhood, between the ages of 5 to 10, and then gradually declines with age (Voiculescu *et al.* 2014; Ghareghani, Zibara, *et al.* 2022). A sharp decline in melatonin levels has been reported during the transition from childhood to puberty (Sandyk 1993). In addition to the endogenous melatonin synthesis, food can greatly increase melatonin, which is abundant in animal foods such as eggs and fish and plant foods such as nuts (Meng *et al.* 2017).

In the absence of research on how melatonin supplements can affect the development of fetal immune and nervous systems, it is best to avoid taking melatonin during pregnancy and gestation unless advised by a physician. Although melatonin receptors are expressed in the embryo and fetus, the fetus and newborn depend on the mother's melatonin via the placenta and milk, respectively. Any change in the mother's melatonin level, either during pregnancy or gestation, quickly affects the development of the fetus or the growth of neonates. In fact, photoperiodic information perceived by the mother is transferred to the fetus (Hsu *et al.* 2020). Melatonin levels naturally increase throughout gestation and decline

right after delivery (Ejaz *et al.* 2020). For instance, while normal non-pregnant women have a melatonin level of 41.7 pmol/l, it increases by two-fold to reach 76.6 pmol/l in the third semester (Voiculescu *et al.* 2014; Kivelä 1991; Ejaz *et al.* 2020).

While some reports showed the beneficial roles of melatonin in fetus development, we focus in this review on the negative studies. A group studied the effect of low to high doses of melatonin in fetus development. Indeed, they administered melatonin daily during the whole gestation period and found that melatonin caused lower maternal weight gain during pregnancy, smaller pups' size, and lower birth weight. Furthermore, the pups mortalities increased in high melatonin-treated rats (Singh *et al.* 2013). Furthermore, using H9c2 embryonic rat cardiac cells, Zhao et al showed that only higher doses of melatonin suppressed cell growth, and induced cell cycle arrest and apoptosis, which was associated with a reduction in gene expression related to heart development (Zhao *et al.* 2021).

It has been reported that taking evening melatonin increases melatonin secretion and its synthesis duration (Grivas *et al.* 2007a). This was also studied for foods containing high levels of melatonin (Sae-Teaw *et al.* 2013; Howatson *et al.* 2012). Considering that melatonin is not a synthetic drug that naturally cannot be found in nature or diet, it is essential to know the patient's lifestyle and diet before prescribing melatonin or melatonin supplements. For instance, a person who consumes melatonin-rich foods in the evening will have an increase in circulating melatonin within 3 hours; therefore, adding a melatonin supplement may result in an unexpected melatonin peak, usually 2 hours after use (Sae-Teaw *et al.* 2013).

How does maternal and fetal melatonin increase by latitude, lifestyle, and diet, can affect the development of the immune and nervous systems? This still remains to be answered. However, based on the current knowledge that melatonin doses usually affect the expression of its receptor by the fetus, we expect that a long-term increase or decrease in melatonin levels can significantly alter the normal development, which may have an impact on the onset of the disease, especially autoimmunity and multiple sclerosis, in the upcoming years of life.

vi. Pathological Roles of Melatonin in Neuroinflammation and Autoimmune Diseases

Pineal melatonin secretion has a seasonal rhythm, increasing in the winter when the nights are longer and decreasing in the summer when the nights are shorter (Arendt 1986). Several investigations on nocturnal and diurnal plasma levels of cytokines clearly showed that most proinflammatory cytokines involved in autoimmune diseases, including IFN-gamma, TNF-alpha, interleukin1 (IL-1) and IL-12, reach the peak of their secretion at night, between midnight and 3-4 AM when melatonin is at its highest level and cortisol at the lowest value. Indeed, administration of cortisone acetate keeps morning cortisol levels at the physiological range and causes a decline in these cytokines (Petrovsky and Harrison 1998; Phillips *et al.* 2006; Gudewill *et al.* 1992; Petrovsky, McNair, *et al.* 1998; Petrovsky *et al.* 2003).

Generally, T helper-1 (Th-1) cells mediate disease development, while Th-2 cells facilitate the recovery phase. Recognition of myelin-specific antigens by CD4+ T helper lymphocytes and macrophage activation is one of the mechanistic pathways by which demyelination happens (Martin *et al.* 1992). Furthermore, interferon-gamma (IFN-γ), released from CD4+ T cells (including Th1), is involved in myelin degradation (Merrill *et al.* 1992; Nagelkerken 1998). The study by Garcia-Mauriño and colleagues' on peripheral blood mononuclear cells (PBMC) showed that melatonin stimulates the release of IL-6, a potent inducer of the acute phase response in MS. This IL-6 was found to be monocyte dependent since CD14+ cells-depleted PBMC failed to affect its release. Interestingly, this stimulatory effect of melatonin was observed only in inactivated or slightly mitogen phytohemagglutinin (PHA) activated PBMC, not in high PHA activated cells (Garcia-Mauriño *et al.* 1997). Furthermore, it has been proposed that monocyte chemoattractant protein-1 [MCP-1, also known as chemokine ligand 2 (CCL2)], plays a key role in MS pathogenesis, most likely by recruiting peripheral monocytes via the blood-brain barrier (BBB) and parenchyma (Mahad *et al.* 2003). Furthermore, investigating the correlation between daytime saliva melatonin and inflammatory cytokines uncovered a positive correlation between increased melatonin levels and MCP-1/CCL2, a finding that highlighted the bidirectional interaction between melatonin and the immune system (Sundberg *et al.* 2020).

On the other hand, research by Hansson et al. on collagen-induced arthritis, as an autoimmune model that mimics rheumatoid arthritis, showed that keeping this mouse in constant darkness caused a worsening in disease severity associated with an increase in anti-type II collagen antibodies and enlarged spleen. This study also proposed that darkness melatonin affects the disease by its impact on neurohumoral compounds via gonadal-independent mechanisms that can enhance T-cell priming (Hansson *et al.* 1990, 1992). Another relevant autoimmune disease is colitis, in which it was shown that acute melatonin therapy reduced its severity; however, long-term melatonin increased its severity in rats (Marquez *et al.* 2006). In addition, they performed a pinealectomy to stop melatonin secretion. They discovered that maintaining pinealectomized mice in complete darkness did not raise melatonin levels; however, it delayed the development and symptoms of induced arthritis. They concluded that increased physiological melatonin levels brought on by darkness stimulate the immune system, aggravating autoimmune diseases (Hansson *et al.* 1993).

In accordance, Cutolo and colleagues reported a correlation between melatonin with another autoimmune disease, rheumatoid arthritis, by considering the latitude. They found that patients from Estonia, northern Europe, have higher levels of night melatonin and an earlier peak than patients from Italy, southern Europe. This was positively correlated with higher levels of the potent pro-inflammatory cytokine TNF-α in Estonian patients (Cutolo *et al.* 2005).

vii. Melatonin's beneficial effects on MS and its models

Given that melatonin is a potent antioxidant, a study for the first time in secondary progressive MS showed that melatonin therapy reduced oxidative stress as measured by analyzing the levels of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxide (GPx) (Miller *et al.* 2013). In parallel, our previous study confirmed this effect in the EAE model of MS. Indeed, we found that melatonin therapy in EAE mice reduces the risk of osteoporosis and reduces oxidative stress (Ghareghani, Scavo, *et al.* 2018a). In accordance, an opposite correlation between the reduction of oxidative stress and the quality of life of MS patients has been reported after taking 5 mg of melatonin

(Adamczyk-Sowa *et al.* 2014). The first beneficial effect of melatonin in EAE mice was reported by Álvarez-Sánchez et al. They showed that treating EAE mice with melatonin improves the severity of the EAE model, which is mediated by decreasing peripheral and central Th1/Th17 responses (Álvarez-Sánchez *et al.* 2015). This is also reported that melatonin improves EAE by boosting the IL-10 expression in regulatory T cells (Chen *et al.* 2016). Given that the kynurenine pathway is the main of tryptophane catabolism, Jand et al. showed that melatonin therapy may improve EAE by modulating the function of this pathway (Jand *et al.* 2022).

viii. Melatonin's adverse effects in MS and its models

A pioneering study by Reuven Sandyk in 1992 investigated the role of melatonin in a patient with chronic-progressive MS (Sandyk 1992b). Since the artificial magnetic field was reported to inhibit the secretion of melatonin by the pineal gland (Welker *et al.* 1983), he used it for the treatment of a 50 years old MS patient. He observed a remarkable and sustained improvement in disease severity. He then treated the patient with 3 mg melatonin after applying the magnetic field and observed, surprisingly, an extreme increase in disease severity within one hour of melatonin administration (Sandyk 1992b).

Janković and colleagues showed, for the first time, that pinealectomy in neonatal rats caused an extreme development in experimental autoimmune encephalomyelitis (EAE), while pinealectomized rats prevented EAE development (Janković *et al.* 1970; Sandyk 1997). They suggested an involvement of the pineal gland in the function of Blood Brain Barrier (BBB) and that there is an age-dependent function of melatonin. We tested this hypothesis and found that EAE development is exacerbated following melatonin therapy in younger rats (Ghareghani, Dokoohaki, *et al.* 2017a). This negative effect of melatonin in EAE was also reported earlier by Constantinescu and colleagues, who showed that functional inhibition of melatonin receptors by Luzindole, an antagonist, prevented EAE development in mice; hence, they concluded that melatonin is an immunoenhancing agent for autoimmune demyelination (Constantinescu *et al.* 1997).

ix. Melatonin Global Usage in Developed Countries

A recently published study by Bliddal and colleagues analyzed melatonin use from 2012 to 2019 in Denmark. They reported a sharp increase in the consummation rate from 2.4 to 3.9 in every 1000 people. Among those, 31% of long-term consumers are 5 to 13 years old. Indeed, 75% of melatonin consumers had registered complaints about psychopathological problems within 2-year of their first melatonin prescription. Anxiety disorder was reported as the most common psychiatric diagnosis, especially among 14-17 years old (Bliddal *et al.* 2022). This high use among children was also reported in Stockholm, Sweden (Tedroff *et al.* 2022). Besides, after receiving 90 cases of adverse effects following the intake of these supplements, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) filed an internal request to conduct an expert appraisal on the risks associated with the use of melatonin-enriched foods. ANSES recommended limiting the use of melatonin supplements to occasional use due to the lack of studies on the long-term effects of melatonin supplements on human health. In addition, they prohibited the prescription of melatonin supplements to pregnant and breastfeeding women.

Furthermore, an interview survey in the USA showed that melatonin consumption among children aged 4-17 years increased from 0.1% in 2007 to 0.7% in 2012. Melatonin is reported as the second most used natural product among American children, after oil fish, and fourth among adults (Black *et al.* 2015). Recently, the trend in consumption of melatonin supplements among 55,021 people in the USA showed a roughly 5-fold increase from 0.4% in 1999 to 2.1% in 2018. This rise in the use of melatonin in the absence of enough evidence of its safety is highly worrying. Consistent with the expert's recommendations in taking doses below 5 mg/day as a safe dose, the use of melatonin above this dose was not reported until 2006 (0.08%) in the USA, while higher doses increased by 3-fold (0.28%) in 2018 (Li *et al.* 2022). In Canada, Erland and Saxena analyzed the melatonin content in 31 supplements. Surprisingly, they found that the actual melatonin in each melatonin supplement showed a huge variability compared to the labeled doses. Indeed, some doses were 83% below or 478% above the labeled dosage. For instance, among the different types of supplements, chewable tablets labeled as 1.5 mg, used mainly by children, showed an actual content of 9 mg (Erland *et al.* 2017).

It is worth noting that some food products are enriched with melatonin in European countries. These countries limited melatonin supplements to low doses of usually 1 mg, while doses of 5 mg are made available only by prescription. Although some countries, such as Denmark and the Czech Republic, even prohibited melatonin as food supplements, there is roughly a 2-fold increase in the number of users of prescribed melatonin in Denmark from 2012 to 2017, according to Statista, a well-known German company specialized in the market and consumer data. However, USA and Canada are selling melatonin in different forms over the counter, at doses even as high as 10 mg. Based on Statista, the total global melatonin supplements market size has increased from 851 million USD in 2016 to 1.5 billion USD in 2021 worldwide.

5. Cells involved in MS pathogenesis

i. Biological brain barriers

Cerebrospinal fluid (CSF) is the liquid found in the brain and spinal cord and plays an essential role in maintaining the homeostasis of the CNS. CSF represents not only mechanical brain protection and facilitates communication between the CNS and peripheral nervous system, vascular, lymphatic, and immune systems, but also it is a rich source of signaling factors. The choroid plexus is reported to be the primary source of CSF production and control of the release of CSF into the ventricular brain system. Structurally, it consists of a mono layer of epithelial cells located on a basement membrane, connective tissue, and fenestrated capillaries (Hofman *et al.* 2016) **(Figure 3)**

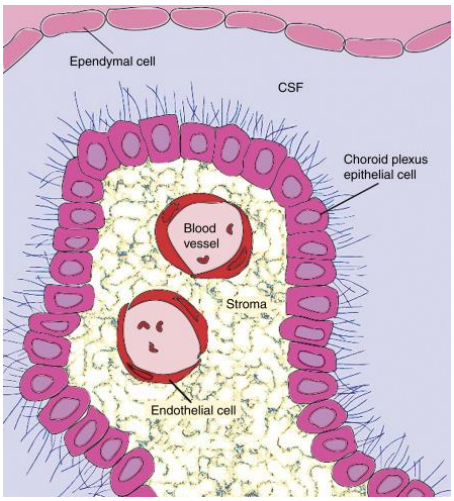

Figure 3: Schematic overview of the choroid plexus. The primary site of CSF production is the choroid plexus located in the lateral, third, and fourth ventricles of the brain. The choroid plexus is an epithelio-endothelial convolute, which is made of vascularized stroma with connective tissue, and epithelial cells. The stroma comprises fenestrated vessels. *Adapted from (Hofman and Chen 2016).*

The CNS is tightly protected from the changeable milieu of blood by two barriers: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). The BBB plays a critical role in protecting the brain parenchyma against circulating toxins or pathogens and providing allowing vital nutrients to reach the brain. Anatomically, it is observed that BBB is localized at the level of the endothelial cells within CNS microvessels, while the BCSFB is structured by the epithelial cells of the choroid plexus; however, BCSFB is among the least studied structures of the CNS. The barrier between the blood and the ventricular CSF is located at the choroid plexus (Engelhardt and Sorokin 2009). In addition to BBB and BCSFB, the avascular arachnoid epithelium is laid under the dura and completely encases the brain, which is thought to be the third brain barrier. This barrier's exchange rate between blood and the brain is limited (Kadry *et al.* 2020) **(Figure 4).**

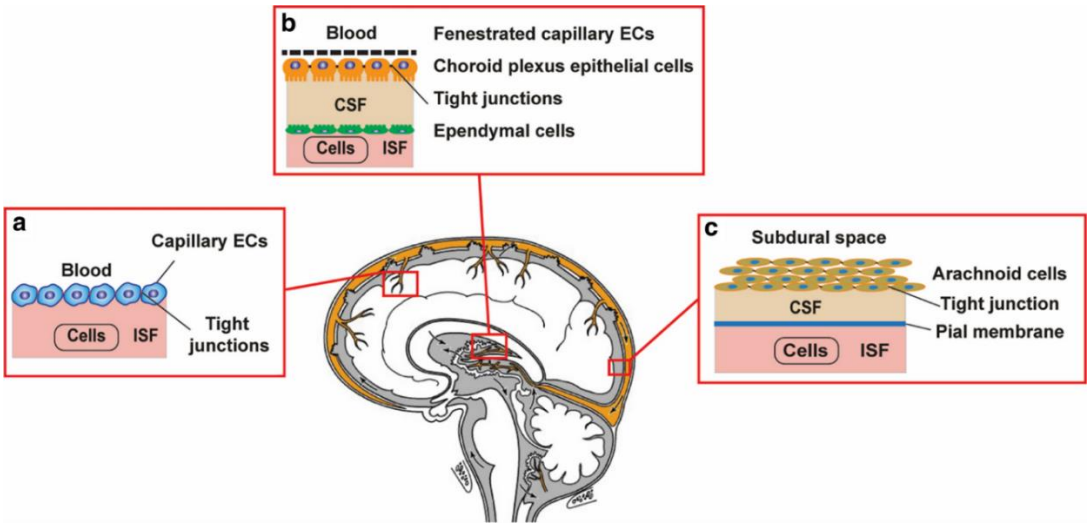

Figure 4: Schematic overview of the biological barriers protecting the brain. a,blood–brain barrier (BBB); b blood–CSF barrier (BCSFB); c the arachnoid barrier. These biological barriers are established by different cells and are important for CNS normal functioning and protecting the CNS environment form invading factors. *Adapted from (Kadry et al. 2020).*

ii. Microglia and macrophages

Within the CNS, microglia, the resident immune cell, provide routine maintenance for the CNS and immune surveillance. It is known that blood-derived immune cells represent an additional critical cellular component in mediating CNS inflammation. Microglia, as part of innate immune cells in the CNS, in addition to monocytes, macrophages, and dendritic cells- play a crucial role during healthy CNS development and support CNS homeostasis throughout adult life by monitoring the CNS to respond quickly to pathogens and injury. Microglia are a unique lineage of brain macrophages that are derived from primitive macrophages present in the yolk sac, not myeloid cells, enter the brain early in embryonic development and prior to BBB closure, and these cells are not replaced by hematopoietic precursors throughout life. These brain resident macrophages are classified into three categories based on where they reside: perivascular macrophages (PVM), meningeal macrophages (MGM), and choroid plexus macrophages (CHM). Non-parenchymal perivascular and meningeal CNS resident macrophages arise from embryonic populations.

However, choroid plexus macrophages can be partially replaced by bone marrow-derived monocytes (Goldmann *et al.* 2016).

Perivascular macrophages are located within the perivascular space surrounding arterioles and venules and exert immune functions such as phagocytosis and antigen presentation, providing nutrients to endothelial cells, maintaining BBB integrity and lymphatic drainage, clearing debris, etc. It increases the anastomoses and the repair of vasculature upon exposure to CNS injury (Yang *et al.* 2019) (**Figure 5).** Meningeal macrophages have a high similarity to perivascular macrophages and can be found in association with the meninges. Both macrophages express similar markers like CD163 and CD206, which can be used to discriminate these cells from monocytes and quiescent microglia. Choroid plexus macrophages are divided into stromal macrophages, which are stellate in the stroma and are similar to PVM and MGM, and Kolmer macrophages, which are ameboid shape and associated with the apical surface of choroid plexus epithelium.

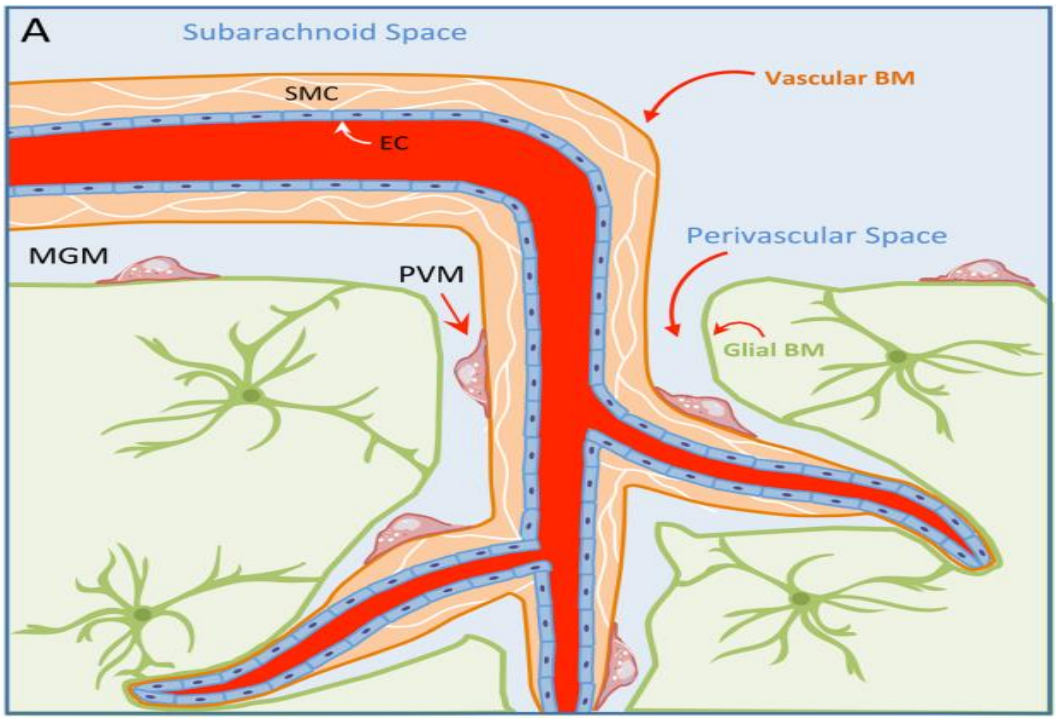

Figure 5. Anatomical localization and distribution of Perivascular Macrophages (PVM) and Meningeal Macrophages (MGM). PVM are located in the perivascular space surrounding arteries and veins as they

penetrate deeply into the brain tissue, whereas MGM can be found in association with the meninges. *Adapted from (Faraco et al. 2017)*

iii. Monocytes

It is revealed that macrophages are critical for successful remyelination (Rawji *et al.* 2020). The macrophages in MS brains is comprised of brain-resident macrophages, formed by activated microglia, and periphery-derived macrophages, which are differentiated from infiltrated monocytes (Masuda *et al.* 2019). However, it is not revealed whether macrophages in MS suppress the demyelination progression or induce it; Lampron and colleagues reported that resident macrophages play a vital role in the removal of debris in MS, and impairment of this function causes failure in remyelinating processes (Lampron *et al.* 2015). Difficulty in distinguishing the bone from brain-derived macrophages in both in vitro and in vivo studies could be considered as one of the reasons that restricted our understanding of the role of the macrophage in MS. Hematopoietic stem and progenitor cells in the bone marrow are considered as the primary source of monocytes. They migrate into the blood in the circulation and then infiltrate into the inflamed tissue for other functions, either for tissue homeostasis or further exacerbation of the inflammation (Swirski *et al.* 2009).

Circulating peripheral blood monocytes exist as different subsets. Monocyte in mouse is classified as inflammatory phenotype, classical phenotype, and patrolling phenotype, non-classical monocyte. Inflammatory monocytes express scavenger receptors and Toll-like receptors (TLRs) and recognize pathogen-associated molecular patterns (PAMPs) and eliminate the microorganisms, dying cells, etc., through phagocytosis. This subtype generates effector molecules, including cytokines, myeloperoxidase, and superoxide, and contributes to local and systemic inflammation (Yasaka *et al.* 1981). On the other hand, patrolling monocytes patrol the microvascular lumen to monitor PAMPs and become tissue-resident macrophages and take action for the removal of debris (Thomas *et al.* 2015). Monocytes' subtypes can be distinguished according to their expression of specific markers. For instance, classical monocyte ($CCR2^{high}$ $Ly6C^{high}$ in mice and $CCR2^{high}$ $CD14^{+}CD16^{-}$ in

humans) are distinct from patrolling monocyte ($CX3CR1^{high}$ $Ly6C^{low}$in mouse and $CX3CR1^{high}$ $CD14^{dim}CD16^{+}$ in humans). In addition to classical and patrolling monocytes, the third subset is considered intermediate, which may represent a transition between inflammatory to patrolling monocytes; however, the current knowledge regarding this is limited (Thomas *et al.* 2015).

iv. Oligodendrocytes and myelin

Oligodendrocytes, the last brain cells to be generated during development, are one of the major glial cells in the CNS. These cells produce a lipid-rich membrane called myelin. The majority of oligodendrocyte cells are generated during embryogenesis and early postnatal life. However, the adult brain preserves the oligodendrocyte generation but in a limited capacity to restore insulation of demyelinated axons, a process which is called remyelination, in response to pathological state causing damage to the oligodendrocytes (Gonzalez-Perez 2014) **(Figure 6)**. In the mammalian CNS, oligodendrocyte precursor cells (OPCs), characterized by the expression of the platelet-derived growth factor receptor α (PDGFRα) and the neuron-glial antigen 2 (NG2) proteoglycan, represent a fourth major glial cell population throughout adulthood that proliferate and generate myelinating cells and comprise 3-8% of the total number of cells. To date, there is no single marker for identifying OPCs; a combination of markers is suggested as they express the O4 antigen and the transcription factors Olig1, Olig2, and Nkx2. Some markers can be expressed in other cells (Polito *et al.* 2005).

Myelination in the developing brain is a complex process in which OPCs are first generated in the germinal zones, where they will proliferate, migrate to the site, and differentiate to make mature oligodendrocytes. Oligodendrocyte is associated with the expression of mature oligodendrocyte markers such as proteolipid protein (PLP) and myelin basic protein (MBP) etc. (Fernandez-Castaneda *et al.* 2016). Upon Oligodendrocytes maturation, they synthesis a large amount of plasma membrane and begin to wrap around neuronal axons to form the myelin sheath. Each oligodendrocyte enwraps up to 50-60 axonal segments.

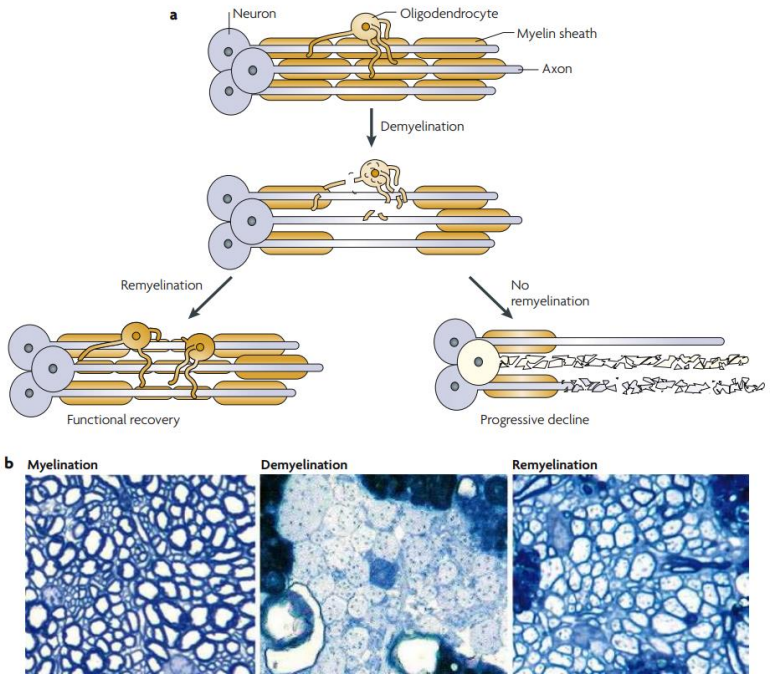

Figure 6: oligodendrocyte and demyelination. Following damage to myelin in the CNS, a demyelinated axon can be recovered, at least in most experimental models, by generating new oligodendrocytes. Spontaneous remyelination is not observed in MS patients because of inflammatory attacks to the myelin and the low capacity of adult OPCs for remyelination. *Adapted from (Franklin et al. 2008)*

v. Neural stem cells and subventricular zone

Oligodendrogenesis actively takes place during CNS development, but it is limited during adulthood. Oligodendrogenesis in adults is triggered and increased in response to any damage to myelin. To date, it is reported that the brain parenchymal is rich in OPCs that can proliferate, migrate, and differentiate to mature oligodendrocytes for remyelination. In addition, precursors in the subventricular zone have been uncovered as another pool of OPCs. Post-mortem analysis of MS patients demonstrated high activation of cells in the subventricular zone (SVZ), indicating the involvement of SVZ in myelin recovery (Nait-Oumesmar *et al.* 2007).

Endogenous neural stem cells (NSCs) reside in two major germinal neurogenic niches in the adult brain; the sub-granular zone (SGZ) of the hippocampus and the SVZ of the lateral ventricles (Morales *et al.* 2019). SVZ is the main neurogenic niche in the adult brain located below the ependymal lining of the lateral ventricles. SVZ comprises several cell types; **Ependymal cells:** The innermost layer contains a monolayer of ependymal cells lining the ventricle, which separates the NSCs of SVZ from the lateral ventricles and contributes to CSF flow through ciliary beating (Quiñones-Hinojosa *et al.* 2007; Mirzadeh *et al.* 2008). **Type B NSCs, is** a type of slow-dividing cells that shows many features of astrocytes, like expressing astroglial markers glia fibrillary acidic protein (GFAP), and some contain a primary cilium with an apical surface that is exposed to signals in the CSF of lateral ventricles (Mirzadeh *et al.* 2008) as well as basal processes contact blood vessels (Doetsch *et al.* 1999). Type B NSCs can give rise to transit-amplifying precursors **(type C cells)** cells upon activation, then subsequently turns into neuroblasts **(type A cells)** that finally migrate to the OB (Doetsch *et al.* 1997).

NSCs in the SVZ receive a huge number of signals from CSF, and following integration and decoding, the signals decide to start the cell proliferation or stay in a quiescent state (Bjornsson *et al.* 2015). Recently, it was found that less than 10% of adult NSCs are active, and the most significant portion is in a quiescent state in the physiological condition that leaves a slim hope for CNS self-regeneration. Although these NSCs can increase in response to pathological conditions to generate new neurons and glial cells, including astrocytes and oligodendrocytes, only a limited portion of these cells survive or become mature oligodendrocytes or neurons (Doetsch *et al.* 1999). SVZ-derived oligodendrocytes undergo all the oligodendrocyte cell lineage stages to produce mature oligodendrocytes and complete the remyelination step. Although the successful therapeutic strategy reported so far is to increase the NSCs activation with an external stimulus, we are still lacking a detailed understanding of the endogenous factors that sustain the aNSCs pool while ensuring life-long neurogenesis (Nakatomi *et al.* 2002) **(Figure 7).**

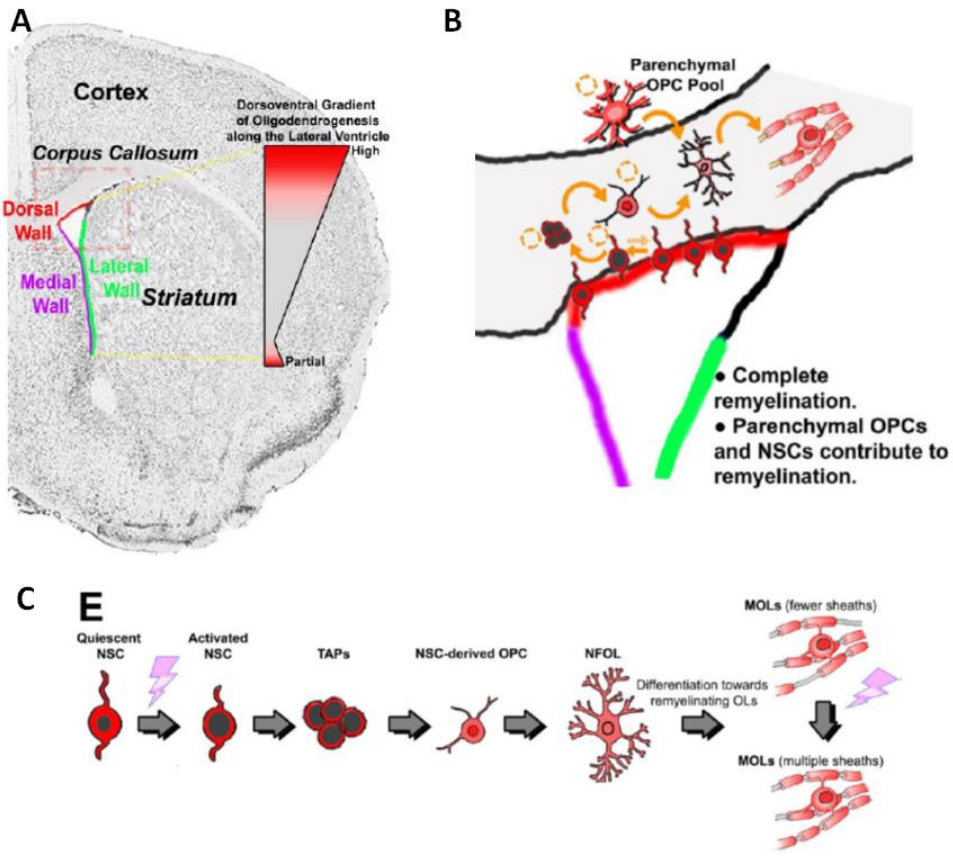

Figure 7: A schematic diagram depicting OPCs sources in oligodendrogenesis. OPCs are located in the brain parenchyma and also are generated from NSCs in the SVZ. NSCs undergo mitosis and can generate transiently amplifying progenitors (TAPs), considered as pre-OPC stage. This migrates to the site of injury and becomes a mature oligodendrocyte (OPC) to produce newly formed (NF)OLs, which have a complex process-bearing morphology and are non-proliferative. NFOLs differentiate into mature myelinating (M)OLs. *Adapted with some modifications from (Butt et al. 2022).*

vi. Melatonin and neural stem cells proliferation

While some studies reported proliferative activities of melatonin, including studies on cultured cells from mice SVZ, C6 glioma, uterine leiomyoma, preadipocyte (Sotthibundhu *et al.* 2010; Sotthibundhu *et al.* 2016; Qu *et al.* 2013; Lin *et al.* 2020; Zwirska-Korczala *et*

al. 2005), some other reported its anti-proliferative activity including studies on various cancers like prostate, breast, ovary (Mao *et al.* 2005; Tamarindo *et al.* 2019; Menendez-Menendez *et al.* 2018; Codenotti *et al.* 2015; Blask *et al.* 2002). More interestingly, inhibition of pineal melatonin secretion led to accelerated growth and proliferation of the tumors (Turgut *et al.* 2005; Bartsch *et al.* 2000). Recently, it is uncovered that melatonin controls the proliferation of NSCs via intracellular Ca2+-dependent pathway. Indeed, this study showed that luzindole as an antagonist of the melatonin receptor causes a decrease in the frequency of Ca2+ events and an increase in the cytosolic level of Ca2+, which leads the cell to an actively proliferating state.

We have recently proposed how melatonin affects the neural stem cell division in SVZ, where distribution in melatonin synthesis causes uncontrolled proliferation of neural stem cells and glioblastoma initiation (Ghareghani, Zibara, *et al.* 2022).

Glioblastoma is an aggressive malignant primary brain tumor that grows extremely fast and invades adjacent tissues. The self-renewing capacity and invasive nature of these cells have devastating consequences. Aging contributes significantly to glioblastoma initiation and progression (Ladomersky *et al.* 2019; Kim *et al.* 2021). On a different front, two primary sources of neural stem cells (NSC) exist in the adult brain; these include the sub-granular zone of the hippocampus (SGZ) and the subventricular zone (SVZ) of the lateral ventricle. A recent pioneering study by Lee and colleagues (Lee *et al.* 2018) on human isocitrate dehydrogenase (IDH)-wildtype glioblastoma, which accounts for 90% of the cases and with frequent occurrence in older individuals, reported that glioblastoma originates from NSC located in SVZ. These cells contain somatic mutations that are implicated in gliomagenesis.

The physiology of adult NSC is under the control of a complex repertoire of signals residing in the SVZ niche. SVZ-located NSC, in contrast to those of SGZ, are in direct contact with soluble factors of the cerebrospinal fluid (CSF) via NSC processes that penetrate between the overlying ciliated ependyma. Thus, factors in the CSF influence the proliferation rate of NSCs (Codega *et al.* 2014). Following the integration and decoding of the numerous CSF signals, NSC either remains in the quiescent state, undergoes self-renewal, or differentiates. Self-renewal allows NSC to retain their number, whereas differentiation

promotes the generation of new cell types, including astrocytes, neurons, and oligodendrocytes.

Uncontrolled proliferation of NSC may increase their susceptibility to malignant transformation and trigger glioblastoma initiation. The current knowledge regarding the physiology of NSC is poorly understood. Recently, Gengatharan and colleagues (Gengatharan *et al.* 2021) estimated the cell proliferation rate of adult NSC in freely-behaving mice using the most advanced *in* vivo imaging techniques. This study reported that NSC primarily exists in the quiescent state, a mitotic-dormant state, with less than 10% regularly undergoing mitosis. The proliferation rate of these cells is under the control of melatonin, a master circadian hormone. In response to darkness, pineal melatonin is synthesized and released from the pineal gland into both the blood and the third ventricular CSF. The authors report a significant difference in NSC division rate throughout a 24-hour period, with 70% of the mitoses occurring during the day. In addition to *in situ* hybridizations of melatonin receptors (MT1 and MT2) on NSC in the SVZ, pharmacological and genetic manipulations of melatonin receptors confirmed their role in melatonin regulation of cell division. Activation of melatonin receptors by this ligand decreased NSC division. Conversely, blockade of the receptors with luzindole or CRISPR technology to selectively repress melatonin receptor expression caused an increase in cell division. Furthermore, boosting endogenous melatonin synthesis by keeping mice in constant darkness for 3 to 7 days induced a significant reduction in NSC division. In contrast, suppressing melatonin synthesis by retaining the mice in continuous light for 3 or 7 days resulted in a marked rise in NSC division.

Regarding the role of melatonin in NSC physiology, the study by Gengatharan et al. is the most comprehensive study to date. This relates to the use of the most advanced *in vivo* imaging approach in freely behaving mice, where the NSC population was labeled with fluorescent tags and with an NSC-specific promoter. The inhibitory effect of melatonin on SVZ-NSC proliferation *in vivo,* and the mounting evidence that glioblastoma originates from SVZ, bolsters the correlation between melatonin and glioblastoma. In line with these observations, *in vitro* studies reported a therapeutic role of melatonin in reducing tumor cell proliferation, self-renewal, and clonogenic ability, especially in glioblastoma cells (Fernandez-Gil *et al.* 2021). Furthermore, an *in vivo* study showed that constant light

exposure for five weeks, a strategy that inhibits circadian-dependent melatonin synthesis, enhanced glioma tumor growth in rats (Guerrero-Vargas *et al.* 2017).

Nestin is one of the markers expressed by neural stem/progenitor cells (NSPCs) of SVZ. Wu and colleagues carried out a systematic meta-analysis and proposed a positive correlation between nestin expression and the overall survival in glioma patients, especially at the early stage of the disease (Wu *et al.* 2015). Interestingly, Jang et al, using a rat model of chemical glioma, showed that the over-expression of nestin cells is considered an early stage of the neoplastic process. Furthermore, they found a cluster of nestin cells in the wall of SVZ whose size grew markedly by age (Jang *et al.* 2004). Since there is a significant decline in melatonin synthesis in rats, similar to humans, we suggest that the expansion in the size of nestin cluster cells in SVZ might be caused by a reduction of melatonin associated with aging.

Since the introduction of artificial electrical light sources, human lifestyles have changed dramatically with marked changes in sleeping habits, which impacts human health. The exposure of humans to blue light wavelengths inhibits the synthesis and release of melatonin from the pineal gland and shortens the duration of nocturnal melatonin secretion (Gooley *et al.* 2011a; Gooley *et al.* 2011b). This occurs during night shift work, indoor lightning when blue-enriched light emitting diodes are used, and viewing screens of electrical devices such as smartphones, TVs, or tablets before sleeping. Moreover, excessive exposure to light-at-night reportedly increases the incidence of a variety of different cancers (Garcia-Saenz *et al.* 2018). We hypothesize that melatonin suppression brought on by this lifestyle, which leads to a decrease in overall melatonin synthesis, may either increase cancer incidence by itself or synergizes with other environmental factors such as ingestion or inhalation of toxins.

Increased age is a significant factor in decreasing endogenous melatonin levels and in altering the expression of melatonin receptors. Melatonin levels are higher in children than in adults. After adulthood, endogenous melatonin levels fade such that a nocturnal rise in blood melatonin levels may be barely discernible in elderly individuals (Grivas *et al.* 2007b). The rapidity with which melatonin levels drop during aging varies widely among individuals and may relate to their general health (Karasek 2004). Gengatharan et al did not quantify the expression levels of MT1 and MT2 receptors; however, *in situ* hybridization showed a stronger expression for MT2 in the SVZ. When they used an *in vivo* treatment strategy with

luzindole, a non-selective receptor antagonist to block both MT2 and MT1 melatonin receptors, this was associated with a significant promotion in cell division. Consistent with this finding, a recent *in vitro* study by Kinker et al demonstrated that MT1 activation reduced the proliferation of glioma and medulloblastoma cell lines, while MT2 had the opposite effects (Kinker *et al.* 2021).

It is noteworthy that there is a sex-specific difference in glioblastoma incidence, as it is 60% higher in men than women (Carrano *et al.* 2021). Interestingly, a slightly higher amplitude of melatonin rhythm has been reported in women compared to that in men (Cain *et al.* 2010). The higher melatonin in women and the lower glioblastoma incidence might justify considering melatonin as a significant factor in glioblastoma inhibition.

In conclusion, the recent study by Gengatharan et al. not only presented a better view of SVZ NSC division *in vivo*, it also identified a day/night difference in the mitotic activity that is mediated by melatonin and its receptors. This clearly introduces melatonin as a key regulator of neural mitosis since higher levels of melatonin, and increased function of melatonin receptors are associated with lower NSC division. Other studies have reported that endogenous melatonin levels and its receptors drop significantly during aging, which highlights the possibility that initiation or progression of glioblastomas, originating from SVZ-NSC, may accelerate as a result of the age-associated loss of melatonin. Moreover, a greater understanding of the potential negative consequences of risk factors arising from modern lifestyles, including artificial light-at-night, night shift work, sleep disturbances, and insomnia, is required. This information may lead to the definition of a mechanistic pathway allowing for the design of new therapeutic strategies to reduce/treat glioblastoma. **Figure 8** depicts how these variables come together to initiate glioblastoma over time.

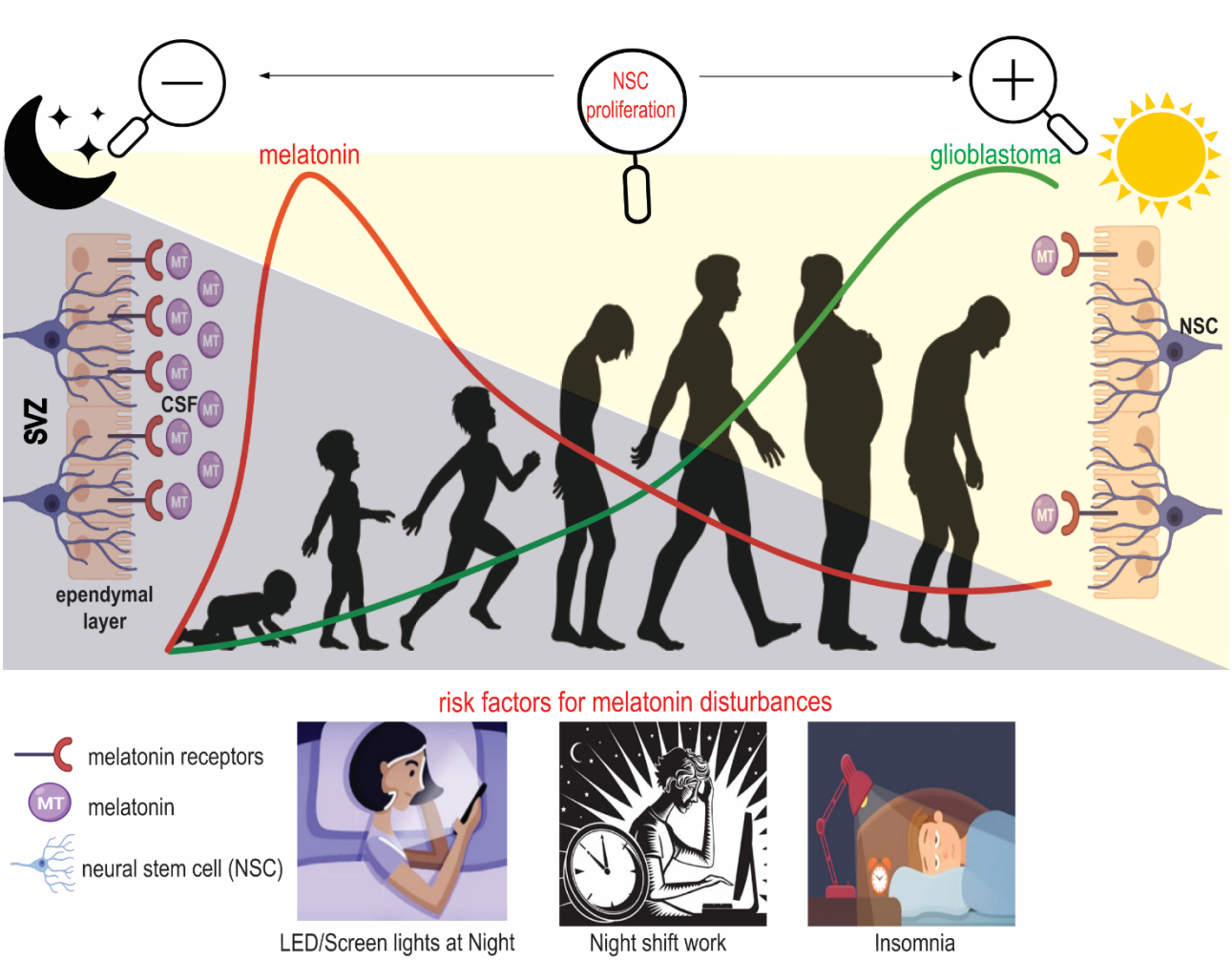

Figure 8: The proposed association of melatonin with the incidence of glioblastoma. In human newborns, pineal melatonin synthesis begins at 4-6 months of age and thereafter melatonin is produced in a light-dependent circadian rhythm. Maximum levels of melatonin are attained during early childhood between 5-10 years old and after adulthood they exhibit a gradual decline into old age. Circadian melatonin is released directly into the third cerebral ventricle from where it circulates throughout the cerebrospinal fluid (CSF). Cilia of the ependymal cells and processes of NSC in the SVZ are in direct contact with circadian and non-circadian CSF melatonin to decode and integrate the signals in order to determine the fate of NSC. Among other factors, the mitotic activity of NSC is under the control of physiological melatonin levels which seem to fine-tune cell division of NSC. Elevated melatonin levels during darkness and longer night periods cause a reduction of NSC mitotic activity, while light exposure or longer day length reduces the regulatory effects of melatonin on NSC and causes an increased NSC division. Melatonin synthesis perturbations due especially to exposure of blue wavelengths during the normal period of darkness depresses the normal circadian melatonin rhythm and may stimulate glioblastoma incidence; likewise, this also occurs in aged individuals where the circadian melatonin rhythm is severely dampened.

vii. Effects of melatonin on oligodendrocyte differentiation and maturation

NSCs in the SVZ can differentiate into different cells, including astrocytes, neurons, and oligodendrocytes. Previous studies showed the potential of melatonin in NSCs' differentiation and survival of undifferentiated neural precursor cells (Moriya *et al.* 2007). However, little evidence is available on its effects on oligodendrogenesis; our previous study on cultured neural stem cells showed that melatonin dose-dependently increases the NSCs differentiation toward oligodendrocytes as quantified by the expression of MBP (Ghareghani, Sadeghi, *et al.* 2017a). A study on injured white matter in neonatal rats showed that melatonin failed to increase the number of immature oligodendrocytes, but it increased the maturation of oligodendrocytes (Olivier *et al.* 2009). Furthermore, a study on neonatal stroke reported that melatonin might improve myelination by reducing inflammation (Villapol *et al.* 2011). This was further studied by Kashani et al. by IP injection of melatonin to the cuprizone model of MS. They found that melatonin managed to inhibit the increase in the number of mitochondria and their size, which was induced by the cuprizone diet; however, no mechanistic pathway was proposed (Kashani *et al.* 2014).

viii. Fatty acid synthesis in remyelination

Metabolic disturbances have been implicated in demyelinating diseases, including MS. Although the leading causes of MS are still unknown, numerous factors have been proposed to affect disease progression. Among these, oxidative stress, mitochondrial dysfunction, and abnormality in fatty acid (FA) beta-oxidation are involved in the MS disease state (Pastural et al. 2009; Senanayake et al. 2015; Jellinger 2010). FA synthesis within oligodendrocytes plays a key role in myelination and remyelination (Dimas et al. 2019a). Among metabolic pathways, lipid synthesis is a crucial process during remyelination. In fact, lipids and cholesterol are critical components of myelin, and their increased synthesis has been linked to the therapeutic mechanism of MS medications (Sedel *et al.* 2016). FA synthesis and/or lipid availability are considered to be rate-

limiting steps for myelin synthesis since the inhibition of cholesterol synthesis caused hypo- and delayed- myelination in mice (Saher *et al.* 2005). Furthermore, FA synthesis is considerably involved in the immune function of cells.

Analysis of the dried myelin showed that FA and cholesterol make up over 70% of the myelin sheath, and 20-30% are proteins. As it's mentioned earlier, oligodendrocytes generate the myelin sheaths in the CNS, and maintenance of myelin structure and function requires constant FA, cholesterol, and protein synthesis; however, this is significantly increased in the case of the remyelination process.

According to the current knowledge, it has been suggested that oligodendrocytes can work as a lactate shuttle, importing lactate from different sources and exporting it to periaxonal space to be imported later by axon (Ichihara *et al.* 2017). In addition, oligodendrocytes can import glucose, lactate, and ketone bodies from vessels through monocarboxylate transporter 1 (MCT1) or Glut1 transporter. Moreover, they can also import lactate from astrocytes via MCT1, and this lactate would have already been transferred from astrocytes to the matrix by MCT4. Furthermore, oligodendrocytes can use gap junctions to import astrocytic glucose and lactate directly. Following the synthesis of pyruvate in oligodendrocytes, it can undergo a series of reactions, in the matrix of the mitochondria, in the Kreb's cycle (TCA: tricarboxylic acid cycle) to be converted into acetyl-CoA, a primary source of FA synthesis and the most efficient ATP-producing pathway, accompanied with the production of CO2, NADH, and FADH2. While the diet can also supply FA, its substrate acetyl-CoA can only be provided by pyruvate, converted from lactate, or by conversion of N-acetyl aspartate (NAA), imported from axons, or ketone bodies (Tepavčević 2021). While the main pathway of acetyl-CoA production in oligodendrocytes was thought to be mediated by pyruvate dehydrogenase complex (PDC), whose function is under the control of pyruvate dehydrogenase kinase-4 (PDK-4), interestingly, a study using impairment of PDC activity in oligodendrocytes showed that inhibition of acetyl-CoA production through the PDC pathway is not necessary for myelin maintenance (Della-Flora Nunes *et al.* 2017). As a pitfall of the study, the authors did not check PDC in pathological demyelination conditions to investigate PDC's role when cells need more acetyl-CoA for the synthesis of new myelin sheets. Furthermore, the source of acetyl-CoA that oligodendrocytes used for the

maintenance of myelin was not explored. In fact, high amounts of fatty acids could be provided by oligodendrocytes or dietary sources in the deficiency of fluxed fatty acids from astrocytes (Camargo *et al.* 2017).

ix. Melatonin effect on mitochondrial reactions involved in FA synthesis

Melatonin as a protector of mitochondria has been reported by Mansouri et al. they showed that melatonin significantly decreases the severity of hepatic mitochondrial DNA depletion induced by ethanol (Mansouri *et al.* 1999). Indeed, they proposed that this could be due antioxidant role of melatonin. Another study showed the potential of melatonin to prevent the inhibition of mitochondrial complexes I and IV following induction by ruthenium red (Martín *et al.* 2000). It is reported that melatonin can easily be transported into the mitochondria and accumulate against a concentration gradient which is caused by having active transportation via mitochondrial melatonin transporter (Tan *et al.* 2016). This highlights the importance of alternation in melatonin levels and its effects on reactions within the mitochondria.

The beneficial effects of melatonin in animal models of MS suggested that melatonin could be a potential candidate for clinical investigation on MS patients. While the role of melatonin on cuprizone and experimental autoimmune encephalomyelitis (EAE) animal models of MS is controversial, our previous studies showed both beneficial and detrimental roles of melatonin in EAE (Ghareghani, Scavo, *et al.* 2018b; Ghareghani, Zibara, *et al.* 2018; Ghareghani, Dokoohaki, *et al.* 2017b). Later, we found that melatonin improved the severity of EAE, associated with a side effect which is to suppress the function of PDC by PDK4, critical for fatty acid synthesis during the remyelination process. However, we found that cells use an alternative source for fatty acid synthesis in myelination (Ghareghani *et al.* 2019a). This observation has been summarized in **Figure 9.** PDK4 is responsible for the switch from mitochondrial oxidation to cytoplasmic glycolysis. We showed that melatonin inhibits PDC, the main pathway involved in the synthesis of acetyl-CoA, the latter being used by the TCA cycle to produce citrate and export it to the cytoplasm for further steps of FA synthesis. This PDC inhibition is suggested to be a side effect of melatonin therapy.

On the other hand, we observed that melatonin improved oligodendrogenesis and modulated the immune system function. Therefore, we hypothesized that oligodendrocytes use an alternative pathway to prepare enough substrate for FA synthesis during remyelination. On the other hand, melatonin increased the levels of NAA. Axonal NAAs are known to get transferred into oligodendrocytes, are converted to acetate, and then acetyl-CoA, a substrate for FA and cholesterol synthesis. This seems to be the alternative pathway potentiated following decreased PDC activity. This alternative pathway appeared slower than the main FA synthesis pathway that PDC regulates. This data has recently been published in Frontiers in immunology (Ghareghani *et al.* 2019a).

On the other hand, a study on the cuprizone model of MS by Vakilzadeh and colleagues showed that melatonin caused a significant increase in nuclear factor kappa B (NFκB), directly correlated with oligodendrocytes death, and failed to improve remyelination (Vakilzadeh *et al.* 2016).

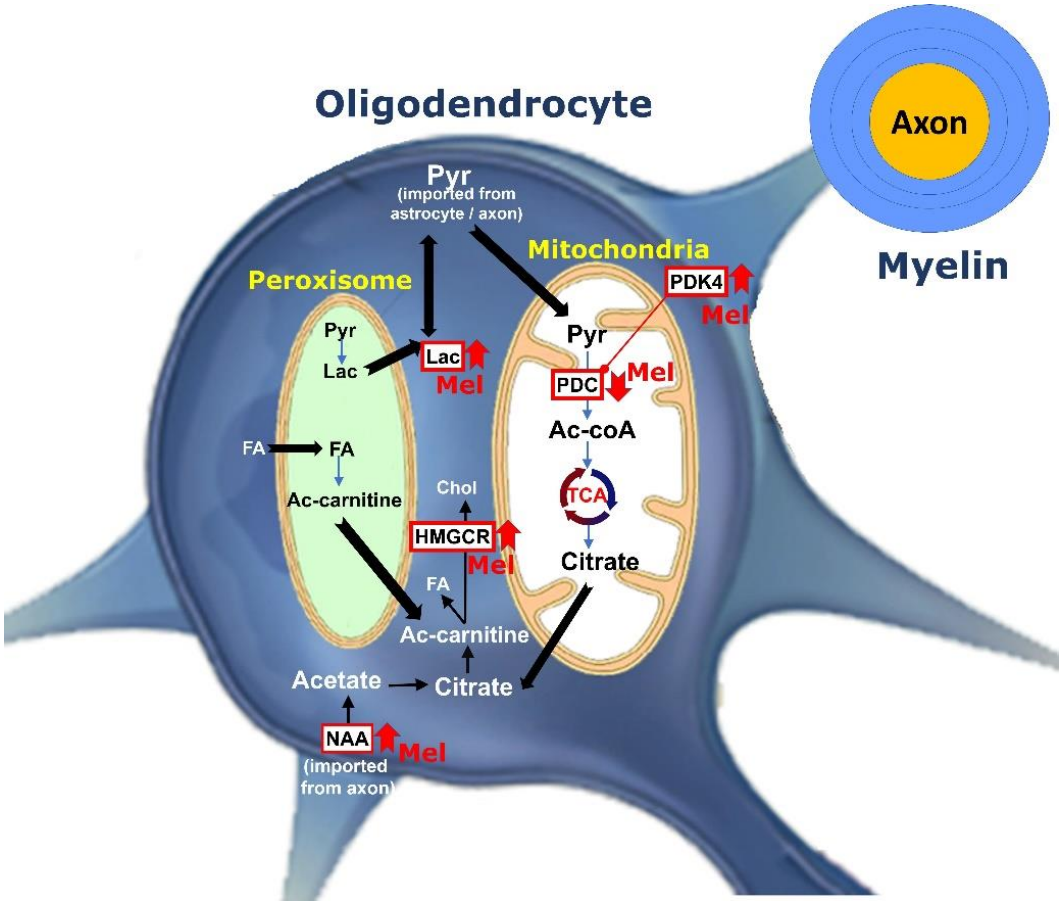

Figure 9. Schematic representation for the role of melatonin on oligodendrocyte metabolism during remyelination. Imported Pyr into the oligodendrocyte is converted to Ac-CoA by PDC, which is under the control of PDK4. This pathway produces citrate and then cholesterol for FA synthesis in new myelin. Melatonin therapy increases the PDK4 expression and subsequently inhibits the PDC and suppresses the pathway. Since the remyelination still is ongoing, it was suggested that oligodendrocytes use an alternative source for FA synthesis that possibly is imported by NAA. (PDC, Pyruvate dehydrogenase complex; PDK4, pyruvate dehydrogenase kinases 4; Ac-CoA, Acetyl-CoA; Ac-carnitine, Acetyl-carnitine; HMGCR, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; TCA, tricarboxylic acid cycle; Chol, Cholesterol; Pyr, Pyruvate; NAA, N-acetylaspartate, Lac, lactate, FA, Fatty acid, Mel, melatonin).

x. Fatty acid synthesis pathway in function of the immune system

Monocytes, macrophages, and microglia play a key role in the pathogenesis of MS. Monocytes differentiate into two main types of macrophages; "M1" or inflammatory phenotype, which is distinguished from $Ly6C^{high}$ monocytes, and "M2" or anti-inflammatory phenotype which are differentiated from $Ly6C^{low}$ monocytes and promote resolution and repair of inflammation. Although the importance of cerebral metabolism in macrophage polarization (M1/M2) is well documented (Van den Bossche *et al.* 2017), however, our understanding to the role of specific enzymes and metabolites of metabolic pathways, especially the checkpoint for switching between inflammatory and anti-inflammatory states, is very limited. It is already well known that the FA synthesis pathway plays a crucial role in macrophage polarization toward the inflammatory M1 phenotype, and induction of FA synthesis is an essential requirement for phagocytosis (Ecker *et al.* 2010). Given that PDC mediates the oxidative decarboxylation of pyruvate conversation to acetyl-CoA, which can be used in the TCA cycle or for fatty acid and cholesterol synthesis, it was reported that inhibition of FA synthesis prevents phagocytosis of macrophages, a critical process required for the clearance of myelin

debris ahead of replacing the damaged cells with newly generated ones (Zhang *et al.* 2014; Lampron *et al.* 2015). It is recently shown that the combined inhibition of PDK2 and PDK4 prevented macrophage polarization to the M1 phenotype in response to lipopolysaccharide (LPS) and IFN-γ inflammatory stimuli (Min *et al.* 2019). However, the role of this inhibition in MS models is not studied yet; it has been reported that LPS induces PDK4 expression (Park *et al.* 2016). Furthermore, activated macrophages, by LPS-released IFN-γ, goes through glycolysis as the main metabolic pathway, whereas IL-4/IL-13-induced macrophages primarily use oxidative phosphorylation (Groh *et al.* 2018). This suggests that PDKs is more expressed/activated following an inflammatory condition in order to conserve the glucose conversion to acetyl-CoA, while it is reduced in an anti-inflammatory state to ensure the activation of the pyruvate conservation into citrate or acetyl-CoA by oxidative phosphorylation and tri-carboxylic acid (TCA) cycle. Inhibition or knockdown of PDKs selectively increased IL-10 expression in macrophages (Na *et al.* 2020). A study SParated monocytes from healthy donors and differentiated to macrophages in vitro and showed that this process is associated with a strong decrease in PDK4 expression (Wallner *et al.* 2014).

6. Animal models in MS studies

The human body is incredibly complicated, and the interrelations between different cells and alternation in cell function at molecular levels is not fully uncovered. On the other hand, the unknown causes of MS initiation and the mechanism of demyelination make it even more complicated to replicate the human disease using in vivo models entirely. But currently, there are different models of MS that mimic certain stages of the disease course. The two most characterized animal models of MS are EAE and toxin-induced demyelination.

i. Experimental autoimmune encephalomyelitis (EAE)

EAE is one of the most studied animal models in MS research. This model has been widely used for the development of a number of first-line treatments in MS that target the inflammatory phase. There are different types of EAE, each displaying some aspects of MS.

To induce active EAE (**Figure 10)**, the myelin-related antigen is emulsified in Complete Freund's Adjuvant (CFA), a suspension of heat-inactivated mycobacteria in mineral oil. CFA basically increases peripheral immune response and increases BBB permeability. Furthermore, it causes a Th1 immune response or Th2 response in case of adjuvant uses without Mycobacterium. Mice, rats, and guinea pigs are some strains susceptible to EAE induction. Myelin-related antigens can be myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), proteolipid protein (PLP), or spinal cord homogenate which causes the development of activated myelin-specific T cells. However, the exact mechanism by which EAE is induced is not fully understood. Injection of Pertussis toxin, a significant virulence factor of Bordetella pertussis, on the day of emulsion injection and 48 hours post-injection is critical to facilitate immune cell entry to the CNS. Furthermore, it can increase T cells' proliferation and cytokine production (Bjelobaba *et al.* 2018). Subsequently, it increases both the incidence and severity of the disease. This model can provide fast results in screening the effects of drugs on CNS inflammation involved in MS. To induce passive EAE, one set of mice is directly immunized to generate myelin-specific T cells, which are then cultured and transferred into donor mice without treatment with CFA or pertussis toxin. The advantage of this model is its rapid onset and increased severity which permits the separation of the effector phase from induction. In this model, differentiated Th1 or Th17 populations can be studied well. Furthermore, the donor T cells can be tracked in the recipient mice in vivo.

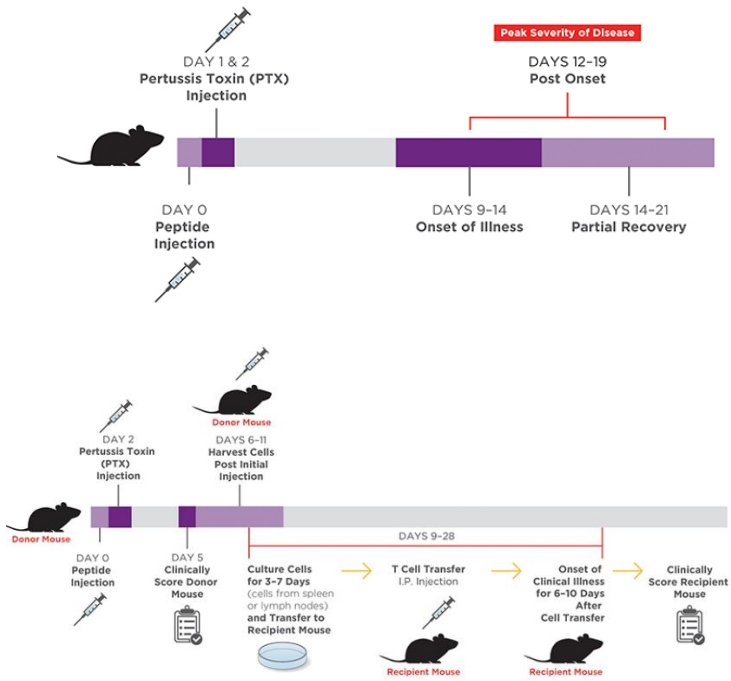

Figure 10: Different EAE models. The upper panel shows the key step for adaptive EAE, and the lower panel for passive EAE. *Adapted* from *Taconic Biosciences' website.*

ii. Cuprizone

While few agents can be injected directly into the white matter, such as ethidium bromide and lysolecithin, to induce the demyelination, systemic administration of toxins such as cuprizone, (oxalic acid bis [cyclohexylidene hydrazide]), is widely used model for studying demyelination. The cuprizone model is a model for toxic demyelination. In this model, young mice are fed with cuprizone, a copper-chelating agent, causing oligodendrocyte apoptosis and leading to reversible demyelination. Mice chow enriched with cuprizone and withdrawal of cuprizone from the diet is associated with spontaneous remyelination 4-5 days later (Torkildsen *et al.* 2008). Most recently, it was found that BBB is disrupted in this model, and brain-intrinsic inflammatory responses can be studied. Furthermore, this model is followed by the activation of the innate immune cells in the brain in which astrocytes and microglia are involved (Berghoff *et al.* 2017; Shelestak *et al.* 2020). This model mimics several characteristics of progressive MS.

While the Cuprizone model mimics several characteristics of progressive MS, it mainly causes CNS toxicity and chelates the brain's copper. Its already shown that copper plays a regulatory role in the function of the key enzymes in melatonin synthesis, as copper administration to organ cultures of rat pineal glands caused a reduction in the activity of pathways involved in melatonin synthesis (Parmar *et al.* 2001).

7. Hypothesis

Melatonin has been recognized as a neurohormone that controls the function of cells in different tissues and organs. To date, studies reported both beneficial and detrimental roles of melatonin in autoimmune disease, especially MS, including our previous 8 published studies on melatonin and MS, which are already discussed in detail in the introduction (Dokoohaki *et al.* 2017; Ghareghani, Reiter, *et al.* 2018b; Ghareghani, Sadeghi, *et al.* 2017a; Ghareghani *et al.* 2019a; Ghareghani, Scavo, *et al.* 2018a; Ghareghani *et al.* 2016; Ghareghani, Farhadi, *et al.* 2022; Ghareghani, Zibara, *et al.* 2018; Ghareghani, Dokoohaki, *et al.* 2017a). On the other hand, it's reported that light improves MS independent of the vitamin D pathway. In search of other possible mechanistic pathways for light-mediated improvement in MS, we found that melatonin and cortisol both are two hormones that are highly affected by light in a circadian-dependent manner. Parallel to MS prevalence which increases by moving from the equator to the poles, circadian melatonin also increases.

How would melatonin be a universal remedy and be beneficial for MS while northern countries, with long-term natural higher endogenous melatonin because of the climate and increased usage of melatonin, are among the high-risk MS countries? To answer this question, we hypothesized that living in higher latitude keeps the melatonin level in value more heightened than the normal physiological range for the long term while cortisol is reduced. This long-term high melatonin can increase melatonin's immunoenhancing function, which is considered a risk factor for MS and may explain why northern countries have higher MS prevalence.

8. Objectives

To test our hypothesis, we first used the cuprizone model, whose melatonin we predicted to be high due to cuprizone's putative effects on melatonin production pathways, mimicking the high level of endogenous melatonin in northern nations.

Then, given that we already found that melatonin therapy improves EAE severity due to recovering the dropped melatonin by EAE induction, we aimed to increase the efficiency of melatonin therapy in EAE.

CHAPTER 1 : Inhibiting Night's Melatonin and Boosting Cortisol Raise Patrolling Monocytes, Phagocytosis, and Myelination in a Murine Model of Multiple Sclerosis

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Running title: Circadian Melatonin and Cortisol in MS

Résumé

Des résultats divergents ont été rapportés concernant la synthèse de la mélatonine dans la sclérose en plaques (SP) en raison des variations entre les modes de vie des patients, qui n'ont pas été prises en compte lors de la supplémentation en mélatonine. Étant donné que la mélatonine agit par l'intermédiaire de ses récepteurs, nous avons identifié des récepteurs de mélatonine dans les oligodendrocytes (OLs) du corps calleux, où se produit la démyélinisation, dans la zone sous-ventriculaire, où se trouvent les cellules souches/progénitrices neurales (NSPC), et dans le plexus choroïde, où elle exerce une fonction de barrière sang-fluide céphalorachidien. En outre, en utilisant des souris chimériques, on a constaté que les macrophages résidents expriment les récepteurs de la mélatonine, alors que les macrophages dérivés de la moelle osseuse perdent cette expression dans les cerveaux démyélinisés. Ensuite, nous avons montré que les souris nourries avec de la cuprizone, un modèle de SP, ont tendance à augmenter leurs niveaux de mélatonine. Bien que nous ayons utilisé différentes approches pour modifier le rythme circadien de la mélatonine et du cortisol, seule l'exposition de façon constante à la lumière a permis d'augmenter chacun de ces paramètres: La prolifération et la différenciation des NSPC en précurseurs d'oligodendrocytes (OPCs), leur maturation en OLs et leur recrutement au site de démyélinisation, le nombre de monocytes patrouilleurs et la phagocytose. En revanche, l'obscurité constante et la mélatonine exogène ont exacerbé tous ces événements et amplifié l'infiltration des monocytes. Par conséquent, la mélatonine ne doit pas être considérée comme un remède universel, comme on le prétend actuellement. Nos données soulignent l'importance de surveiller l'oscillation mélatonine/cortisol chez chaque patient atteint de SP en tenant compte de son régime alimentaire et de son mode de vie, afin d'éviter un surdosage de mélatonine.

Abstract

Conflicting results are reported concerning melatonin synthesis in multiple sclerosis (MS) due to variabilities between patients' lifestyles, not considered when supplementing melatonin. Since melatonin acts through its receptors, we identified melatonin receptors in: oligodendrocytes (OLs) of the corpus callosum, where demyelination happens; subventricular zone, where neural stem/progenitor cells (NSPCs) are located, and choroid plexus, where it functions as a blood-cerebrospinal fluid barrier. Moreover, using chimeric mice, residential macrophages were found to express melatonin receptors, whereas bone-marrow derived macrophages lose this expression in the demyelinated brain. Next, we showed that cuprizone-fed mice, a MS model, tend to increase melatonin levels. While we employed different approaches to alter circadian rhythm of melatonin and cortisol, only the constant light approach increased each of: NSPC's proliferation and differentiation to oligodendrocyte precursor cells (OPCs), their maturation to OLs and recruitment to the site of demyelination, the number of patrolling monocytes, and phagocytosis. In contrast, constant darkness and exogenous melatonin exacerbated all these events and amplified monocytes infiltration. Therefore, melatonin should not be considered as a universal remedy, as currently claimed. Our data emphasize the importance of monitoring melatonin/cortisol oscillation in each MS patient by considering their diet and lifestyle, to avoid melatonin's overdose.

1. Introduction

Since the discovery of melatonin (N-acetyl-5-methoxytryptamine) in 1958 by Aaron B. Lerner and co-workers, a substantial number of investigations have been carried out to elucidate its source, pattern of synthesis, physiological and pathophysiological functions. Melatonin is mainly synthesized by the pineal gland in a circadian-dependent manner. Indeed, its secretion starts gradually by onset of the dark phase at night, and is increased to a maximum value at midnight, then falls to a lower level by sunrise. However, any dim to bright light at night significantly affects the secretion pattern by delaying the peak secretion or shortening the secretion period (Arendt 1988). Moreover, melatonin is released by other sources in circadian-independent manner such as the gastrointestinal tract. Several studies investigated the role of melatonin in the pathophysiology of multiple sclerosis (MS), a chronic disease of the central nervous system (CNS). In fact, MS is suspected to result from an autoimmune attack leading to demyelination of nerve fibers and progressive neurodegeneration. One of the pioneering studies on melatonin role in MS was carried out by Constantinescu et al. (Constantinescu *et al.* 1997) in 1997, whose findings implicated the deleterious role of melatonin in experimental autoimmune encephalomyelitis (EAE). Indeed, EAE mice treated with luzindole, an antagonist of melatonin receptors, did not develop EAE; hence, the authors concluded that inhibition of melatonin could be used as a therapeutic strategy. However, other later studies reported conflicting results. For instance, Kang et al, in 2001 showed that the inhibitory effect of melatonin in EAE mice was mediated by suppression of intercellular adhesion molecule-1 (ICAM-1) (Kang *et al.* 2001). On the other hand, Álvarez-Sánchez' et al. (Álvarez-Sánchez *et al.* 2015), and Chen et al. (Chen *et al.* 2016), reported the beneficial role of melatonin by altering the T effector/regulatory balance and enhancing interleukin-10 (IL10) expression and chemotaxis suppression. These neuroprotective effects of melatonin were further demonstrated by Wen et al. (Wen *et al.* 2016) in 2016 and Long et al. (Long *et al.* 2018) in 2018.

Our previous results showed that melatonin is reduced in EAE mice and that melatonin therapy improved the severity of the disease, as observed by a reduction in inflammation and increase in oligodendrogenesis, but not in younger mice (Ghareghani, Dokoohaki, *et al.* 2017a; Ghareghani, Scavo, *et al.* 2018a; Ghareghani, Zibara, *et al.* 2018). In addition, we showed that melatonin therapy reduces the risk of osteoporosis and normalizes bone

formation in EAE mice (Ghareghani, Scavo, *et al.* 2018a). Moreover, administration of a muscle relaxant before melatonin therapy improved the efficiency of melatonin in EAE model since melatonin peaks are concomitant with the time at which the muscles are resting and the body is exerting its antioxidant activity (Ghareghani, Zibara, *et al.* 2018). Furthermore, amelioration of EAE by corticosteroid therapy was shown to be associated with a reduction in endogenous melatonin levels (Dokoohaki *et al.* 2017). At the metabolic level, melatonin therapy in EAE was linked to the inhibition of Pyruvate dehydrogenase complex (PDC) activity by Pyruvate dehydrogenase kinase 4 (PDK4), a key enzyme in fatty acid synthesis during the remyelination process(Ghareghani *et al.* 2019a). Overall, these studies highlighted those previous conflicting results were in part due to the difference in age, sex, dose and timing of melatonin administration in the various MS models. In addition to EAE, the cuprizone (CPZ) demyelination model also showed contradictory results. In fact, Labunets and Rodnichenko revealed that melatonin therapy improved neurogenesis in CPZ mice (Labunets *et al.* 2019). In contrast, Vakilzadeh et al, in 2015 reported a significant increase in NFκB in CPZ-fed mice, directly correlated with oligodendrocytes (OLs) death, which was further amplified by melatonin therapy and without any remyelination improvement (Vakilzadeh *et al.* 2016).

On the other hand, melatonin is released into the third ventricle from the pineal gland, interacting with ependymal cilia of the subventricular zone (SVZ), where neural stem/progenitor cells (NSPCs) are located. Although NSPC differentiation and maturation leads to OLs formation; however, their function in the adult is poorly understood. In mice, NSPCs were shown to migrate to the olfactory bulb to produce new neurons, but also to the site of injury to produce new glia cells, including astrocytes and OLs (David-Bercholz *et al.* 2021).

Overall, due to the conflicting results and the lack of a mechanistic pathway by which melatonin affects MS, we aimed to investigate the effect of manipulating both the endogenous and exogenous melatonin levels on the activation or inhibition of melatonin receptors and on NSPCs activation and differentiation to OLs. We also aimed to study the expression of melatonin receptors in different regions of the brain and to investigate their role on monocytes phenotypes, infiltration, and phagocytosis.

2. Material and Methods

i. Antibodies and products

Specifications of antibodies used in this study for tissue immunofluorescent staining and for Fluorescence-Activated Cell Sorting (FACS) analysis are listed in **Table 1**. All other products are listed in **Table 2.**

ii. Mice

Adult 12-weeks old male C57BL/6J wild type mice were used for the of 3-weeks treatment protocol. We also used adult male C57BL/6J wild type and sex-matched CX3CR1-GFP mice to generate chimeric mice for the 1-week treatment protocol. Experimental animal protocols complied with all relevant ethical regulations, in accordance with the Canadian Council on Animal Care guidelines, administered by the Laval University Animal Welfare Committee. All animals were acclimatised to standard laboratory conditions (12 h light, 10 h dark cycle; lights ON at 07:00 and OFF at 19:00 h) with free access to rodent chow and water.

iii. Demyelination model

To induce demyelination, cuprizone (0.2% wt/wt) was incorporated into standard irradiated ground rodent chow. Wild type or chimeric mice were fed with normal chow, as control groups, or cuprizone-supplemented ground chow over the course of 5 weeks. The chow container was checked every 2 days and uneaten chow was removed and replenished with fresh chow. Mouse weight was monitored once per week and loss of more than 15% of weight was considered as the exclusion criteria in the experiment.

iv. Experimental groups

Mice were randomly allocated to 6 groups, as follows: **(A)** Control mice fed with normal chow (Ctrl); **(B)** Cuprizone mice treated with vehicle DMSO/Oil (**Vhl**); **(C)** Cuprizone mice maintained in constant light (CL); **(D)** Cuprizone mice maintained in constant darkness (CD); **(E)** Cuprizone mice treated with 30 mg/kg Luzindole (LUZ); and **(F)** Cuprizone mice treated

with 80 mg/kg melatonin (MLT). All treatments (LUZ and MLT) and light manipulations were initiated at the beginning of the 3rd or 5th week, for 3 weeks in wild type mice or 1 week in chimeric mice, respectively.

The dose of 30 mg/kg luzindole was selected based on its effect in supressing the severity of EAE mice, as previously described (Constantinescu *et al.* 1997). Luzindole was administrated intraperitoneally at 6 PM to inhibit the melatonin receptors at night. The dose of 80 mg/kg of melatonin was selected based on its efficacy in ameliorating the severity of EAE, without any toxicity (Álvarez-Sánchez *et al.* 2015). Melatonin was administered intraperitoneally at 9-10 AM when melatonin is at its lowest level since melatonin is normally high at night (Schuster *et al.* 2001; Rosenstein *et al.* 1991). In fact, this protocol is designed to have high melatonin during the day. Both luzindole and melatonin were freshly prepared by dissolving in 5% dimethyl sulfoxide (DMSO) and diluting them in corn oil before each injection. Vehicle (untreated CPZ mice) and experimental groups received the same percentage of 5% DMSO and corn oil. On the other hand, to manipulate the light and darkness period, mice were transferred into "Red LEDDY Cage", a fully programmable IVC lighting control system for circadian rhythms studies (Tecniplast, Italy). Mice were exposed to constant light (24 h/day bright light) at an intensity of 80 lux, or constant darkness (24 h/day darkness) for 3 days, followed by a resting day of normal 12/12 light/night. Briefly, mice were kept for 3 days under constant light or constant darkness, to suppress or increase; respectively, the pineal-dependent melatonin synthesis, as 3 days was the optimal time to reach this aim. This was followed by a day of recovery to allow mice to re-establish a normal day of 12h light, 12h night. to minimize the pathological situation that may arise by keeping mice in non-stop light or darkness environment. This cycle was then repeated for 5 times. The experimental procedures and the various groups are summarized in a schematic representation in **Figure 11A**.

v. Chimeric mice

Bone marrows (BM) were extracted from sex-matched CX3CR1-GFP donors and transplanted into 8-week-old recipient male C57BL/6J wild type mice, who would have been ablated earlier. Briefly, antibiotic therapy (SPtra, 100 mg/ml drinking water) was administered to recipient mice for 7 days, followed by Busulfan chemotherapy (10 mg/kg; two IP injections with 12 hours interval) for 4 consecutive days and then IP injections of Cyclophosphamide (100 mg/kg; once per day) for two days. At 48 hours after the last Cyclophosphamide injection, BM cells of donor mice were extracted and approximately 8×10^6 cells were intravenously transferred to each recipient mice. To validate the chimerism, peripheral blood samples were assessed by FACS to quantify the number of donor cells in recipient mice at 8 weeks post-transplantation. Mice with more than 94% circulating donor cells were considered chimeric. The protocol is summarized in **Figure 17A**.

vi. BrdU incorporation and detection

For detection of regeneration of oligodendrocyte precursor cells (OPCs) and mature OLs, the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) was dissolved in sterile 0.9% NaCl solution and added to drinking water at a concentration of 1 mg/ml. BrdU was administered at the beginning of all therapies (LUZ and MLT) and light manipulations (CL and CD), at week 3 of cuprizone diet, and was changed every two days with freshly prepared solution. The volume of consumed BrdU-supplemented water was checked every two days to ensure that all mice received the same amount of BrdU.

vii. Sacrifice

All mice were deeply anesthetized with Ketamine/Xylazine (75 mg/kg versus 8 mg/kg, ip) prior to transcardial perfusion with 0.9% saline and 4% paraformaldehyde (PFA; pH 7.4). Brain tissues were collected and postfixed by immersion in the same fixative at 4°C overnight and then transferred to and stored in 20% sucrose supplemented PFA (pH 7.4) for 12 hours. Brains were then cut into 25 µm-thick sections with freezing microtome (Leica Microsystems), serially collected in anti-freeze solution and stored at -20°C until further

processing. For brain ELISA, brains of another set of experiments were collected quickly after transcardial perfusion with 0.9% saline and stored at -80°C until further processing.

viii. Detection of brain's cortisol and melatonin

Though the synthesis of cortisol in mice is debated and it was thought that mice do not produce appreciable cortisol, Gong et al, using high performance liquid chromatography (HPLC), electrospray ionization mass spectrometry (ESIMS), and ELISA methods, showed that dynamics of secretion of cortisol and corticosterone in mice is closely correlated in response to pathological conditions, including stress (Gong *et al.* 2015). This is further confirmed by following studies (Sierra-Ramírez *et al.* 2022; Seo *et al.* 2016; Huang *et al.* 2022). Furthermore, Reiter et al, studied the cortisol level in C57BL/6J mice in collaboration with Charles River research animal diagnostic services (Reiter *et al.* 2017). Here we used a mouse ELISA kit which has high sensitivity and excellent specificity for detection of mouse cortisol, by which no cross-reactivity or interference between mouse cortisol and analogues has been observed.

Briefly, mice were anesthetized, and brains quickly extracted without perfusion with fixation (all the mice within 1 hour) and stored at -80 for further studies. Thereafter, brain samples were halogenated and the concentration of protein in each sample calculated by colorimetric protein measurement: BCA (bicinchoninic acid assay). Cortisol and melatonin concentrations were measured according to the kit protocol and values reported in mg of brain (**Table 2**).

ix. Fluorescence-Activated Cell Sorting (FACS) Analysis

Blood was obtained from the submandibular vein before perfusing the mice and samples were collected in heparin-coated tubes. Since the interval between sample preparation and analysis is a critical parameter for confounding factors in functional immunological assays, we carried out FACS analysis within 1 hour. Briefly, erythrocytes were lysed using ACK lysis solution, and then leukocytes were washed twice by centrifugation at 500× g. Cells were

then incubated for 15 min on ice with purified rat anti–mouse CD16/CD32 antibody and washed by centrifugation at 500× g. Next, cells were incubated with the cocktail of fluorescently conjugated antibodies CD45, CD11b, Ly6C, Ly6G and Live Dead Blue fluorescent dye for 30 minutes. Then, cells were washed and resuspended in PBS containing 123count eBeads and FACS analysis was performed. Results were analyzed by FlowJo software (v10.0.7).

x. Immunostaining

Brain sections were washed in KPBS, and then pretreated with blocking solution containing 1% (v/v) bovine serum albumin (BSA), 4% appropriate serum, and 0.4% (v/v) Triton X-100 in KPBS for 2 hours. Thereafter, primary antibodies (Olig2, 1/1000; APC, 1/1000; CD68, 1/1000; Caspase-3, 1:500; SOX2, 1/1000; Ki67, 1/1000; BrdU, 1/1000; MT1A, 1/200; MT1B, 1/200) were added to the sections in 1:2 diluted blocking buffer with KPBS and incubated overnight at 4°C, except for MT1A and MT1B which were incubated for 48 h. Next, sections were washed and incubated with corresponding secondary antibodies for 2 hours at room temperature. Sections were counterstained with DAPI (Sigma-Aldrich) and mounted onto MicroSlides Superforst® and cover slipped with Fluoromount-G. HCl treatment (2 N HCl, 30 min at 37 °C) and subsequent naturalization with butyric acid for 10 min were performed ahead of blocking for BrdU staining. Twenty min antigen retrieval with 95-degree Celsius Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) was performed for SOX2 and Caspase-3 antibodies ahead of blocking. Antibodies information are listed in **Table 1**.

xi. Luxol Fast Blue (LFB) staining

A series of sections were stained with 1% Luxol Fast Blue (LFB) in 95% ethanol, sealed overnight at 60°C, washed with distilled water, and placed in 95% alcohol for 10 min. Differentiation was performed with 0.05% lithium carbonate, for 10 s and 70% alcohol solution for 20 s. These last two steps were repeated until the grey and white matter were

clearly observed under light microscope. Thereafter, slides were washed and counterstained with pre-heated cresyl violet solution at 55°C for 1 minute. Sections were then dehydrated and sealed with neutral gum.

xii. Image Acquisition

Sections were examined and photographed under a Zeiss LSM800 confocal microscope supported by the Zen software (2.6 system). Confocal images were then processed using Fiji (ImageJ Version 2.0.0-rc-43/1.51n). All image analysis were performed blinded to avoid bias of analysis. For analyses and bright field image acquisition of LFB staining, 8-bit polychromatic TIFF images of the regions of interest were taken in a single setting for all slides with a Qimaging camera (Qcapture program, version 2.9.10) attached to Nikon microscope (C-80) with the same gain/exposure settings for every image. Quantification of CD68+ and GFP+ particle perimeter was performed using threshold/analyze particle features in Fiji. Background was subtracted from the images and intensities were equally adjusted within each set of experiments.

xiii. Data analysis

All groups were blinded to the experimenters for all quantifications. Results are expressed as mean ± SEM (standard error of the mean). Data distribution was analyzed by the Shapiro–Wilk normality test whereas Brown–Forsythe was used to check the homogeneity of variance for ANOVA test analyses. All data presented in the manuscript passed both tests and were analyzed as normally distributed and with equal variances. Descriptive and inferential statistics was applied to the data using GraphPad Prism version 8.01 (San Diego, CA, United States). P values less than 0.05 ($p<0.05$) were considered to be statistically significant. Significance is indicated by $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, and$****p < 0.0001$. All panels were assembled using Adobe Photoshop CC 2018 (version 19.1.0) and Adobe Illustrator CC 2018 (version 23.0.1).

3. Results

i. Brain levels of melatonin and cortisol are affected by light manipulation

In order to manipulate endogenous circadian melatonin and cortisol, mice were kept with constant light (CL) or constant darkness (CD) for 3 days followed by a day of recovery using normal day:night cycle, a protocol which was repeated for 5 cycles for a total of 20 days **(Figure 11A)**. On the other hand, exogenous melatonin or luzindole were used as an agonist or antagonist of melatonin receptors, respectively **(Figure 11A)**, for the same study period. Results showed that feeding mice with CPZ for 5 weeks caused a 23% increase ($p>0.05$) in brain levels of melatonin in vehicle, compared to control healthy mice (1652 ± 60 vs 2031 ± 87 ng/mg/brain; **Figure 12B**), whereas cortisol levels did not change compared to controls (7.2 ± 0.3 vs 7.6 ± 0.4 pg/mg/brain; $p>0.05$; **Figure 12C**). On the other hand, manipulation of circadian rhythm in the constant light (CL) group caused 33% reduction ($p>0.05$) in melatonin (1341 ± 88 vs 2031 ± 87 ng/mg/brain;) and a significant (****$p<0.0001$) 82% increase in cortisol (13.8 ± 1.7 vs 7.6 ± 0.4 pg/mg/brain) levels, compared to vehicle. However, constant darkness (CD) caused an opposite trend, a significant (****$p<0.0001$) increase in melatonin (3344 ± 329 vs 2031 ± 87 ng/mg/brain;) and an inhibitory effect (*$p<0.05$) on cortisol secretion (3.6 ± 0.2 vs 7.6 ± 0.4 pg/mg/brain), in comparison to vehicle mice. In contrast, no significant changes in the levels of melatonin and cortisol were observed when melatonin receptors were either stimulated using exogenous melatonin as an agonist or inhibited using luzindole as an antagonist (**Figure 12B and. 12C**).

ii. Melatonin receptors are expressed by residential macrophages, oligodendrocytes, and NPCs

To study melatonin receptors 1A (MT1A) and 1B (MT1B) expression by brain resident and BM-derived (peripheral) macrophages in the CNS, these two cell types were

discriminated by the use of chimeric mice. Since BM hematopoietic cells are replaced by GFP-expressing cells, any observed GFP cell in the adult CNS would correspond to an infiltrated cell. Activated microglia were labeled with CD68, a marker that can be expressed by both monocytes and embryo-derived brain resident macrophages (Walker *et al.* 2015). Therefore, the CD68-positive macrophages are considered as residential macrophages if they do not colocalize with GFP; however, they are considered as BM-derived peripheral macrophages if they colocalized with GFP. Results showed that residential macrophages ($CD68^+/GFP^-$) express both MT receptors (**Figure 12A**; short yellow arrows) whereas peripheral macrophages ($CD68^+/GFP^+$) lack these expressions (**Figure 12A**; long yellow arrows). In fact, detection of infiltrated GFP in normal mice is not possible because of the absence of infiltration; therefore, cuprizone mice were used whose expression of macrophages (CD68) is high in the corpus callosum. CD68 is localized in cellular, lysosomal, and endosomal membranes. Given that both melatonin receptors are expressed by these brain-resident macrophages, we detected a huge dispersion of MT1A and MT1B which correspond to dispersion of CD68.

In fact, CD68 cells can be either monocytes or macrophages while GFP-expressing circulating monocytes could differentiate into macrophages and dendritic cells upon infiltration into the brain. Therefore, it would be expected that some CD68/GFP cells might be undifferentiated peripheral monocytes, or at the beginning of their differentiation to macrophages, which may still express the MT1A and MT1B receptors. To examine this, we quantified the percentage of MT expressing $CD68^+$ cells among GFP cells in the CC over the course of demyelination. Results showed that 22% of CD68/GFP cells do express MT1A while 19% express MT1B receptors, two weeks after feeding with cuprizone (**Fig. 12A**; low chart). However, expression of MT1A and MT1B receptors decreased to 9 and 12 percent; respectively, after 5 weeks of cuprizone feeding. This result suggests that circulating monocytes in the CNS may still express MT receptors as long as they have not finished their differentiation to macrophages.

We further studied the expression of melatonin receptors in 3 regions: the corpus callosum, where OLs are damaged in the CPZ model; the choroid plexus, where immune cells could traffic into the CNS; and the SVZ region, where a pool of NSPCs are located. The immunofluorescent staining showed that both MT1A and MT1B receptors were found on

APC-expressing OLs in the corpus callosum (**Figure 12B;** upper panel**)** as well as in the choroid plexus **(Figure 12B;** middle panel**)** and SVZ (**Figure 12B;** lower panel**)** regions. In addition to the monocytes/macrophages, we have examined the expression of MT receptors over the course of demyelination before cuprizone diet, 2 and 5 weeks after cuprizone. Results showed that the expression of MT1A and MT1B in APC oligodendrocytes are not affected by demyelination and that all APC cells express these receptors.

iii. Constant light and inhibition of endogenous melatonin promote oligodendrogenesis in the corpus callosum, mainly due to maturation of OPCs rather than proliferation

Olig2, a helix-loop-helix transcription factor, is a marker that is expressed by both immature oligodendrocyte precursor cells (OPCs) and mature OLs. These cells were discriminated by double staining with antibodies against Olig2 and adenomatous polyposis coli (APC), a marker of mature OLs. In addition, since we started the BrdU administration at the initiation of our light and treatment conditions (week 3 of CPZ diet), we only counted newly generated OPCs and OLs which express BrdU (OPC: $BrdU^+/Olig2^+/APC^-$ versus OL: $BrdU^+/Olig2^+/APC^+$ cells). In fact, NSPCs from SVZ can migrate to both medial and lateral areas of the corpus callosum; however, all our quantifications were performed in the middle corpus callosum along the rostrocaudal axis since it is highly vulnerable to CPZ-induced demyelination, whereas the lateral area is more resistant (Brousse *et al.* 2021).

Results showed a significant (*$p<0.05$) increase in newly generated oligodendroglia cells ($BrdU^+/Olig2^+$ cells), which includes both OPCs and OLs, in vehicle mice (369 ± 23) compared to controls (78 ± 8). Discrimination between the two cell types, among these newly generated Olig2 cells, demonstrated that this increase occurred mainly in OPCs **(Figure 13A)**. Indeed, the OPC population ($BrdU^+/Olig2^+/APC^-$ cells) was significantly (**$p<0.05$) higher in vehicle mice (264 ± 48), compared to controls (37 ± 10), while the OLs population (BrdU+/Olig2+/APC+ cells) was higher in vehicle (105 ± 27), compared to controls (37 ± 5), without reaching statistical significance **(Figure 13B-D)**.

In addition, Olig2+ cells showed a further significant increase due to constant light (737 ± 106) or luzindole (636 ± 74), in comparison to vehicle mice (369 ± 23) (**p<0.01 vs *p<0.05, respectively) **(Figure 13B)**; however, this raise was mainly in mature OLs, not immature OPCs. In fact, immature OPCs did not increase by constant light (233± 22) or luzindole (282 ± 80) in comparison to the vehicle group **(**264 ± 48) **(Figure 13C)**; however, the maturation of OPCs into OLs increased significantly by constant light (505 ± 100) or luzindole (353 ± 64,) **(Figure 13D)**, in comparison to the vehicle group (105 ± 27) (***p<0.001 vs *p<0.05, respectively) (**Figure 13B-D**).

In contrast, both constant darkness (107 ± 12) and melatonin (285 ± 65) caused an insignificant reduction in total Olig2+ oligodendroglia, compared to the vehicle group (367 ± 105), with constant darkness being very close to significance (p=0.051). However, this reduction was mainly in immature OPCs, not mature OLs. Indeed, Immature OPCs were significantly (**p<0.01) reduced by constant darkness (29 ± 8), but not by melatonin (112 ± 27), in comparison to vehicle (264 ± 48 vs 105 ± 27, respectively). In contrast, mature OLs did not significantly change by constant darkness (78 ± 4), or melatonin (139 ± 54). It seems that constant darkness, and less efficiently exogenous melatonin, reduce OPCs proliferation (**Figure 13B-D**).

iv. Increased Oligodendrogenesis is not due to reduced apoptosis

To investigate whether increased OLs by constant light or luzindole is due to increased maturation of OPCs or to reduced apoptosis, the number of apoptotic OLs were quantified using Caspase-3, which was colocalized with the marker of OLs APC (**Figure 13E**). Results showed that apoptotic OLs (Caspase-3+/APC+ cells) were decreased in the constant light group only (39 ± 14) in comparison to vehicle mice (115 ± 27). However, this decreased apoptosis did not reach statistical significance possibly because of ongoing apoptosis due to the CPZ diet. On the other hand, none of luzindole (84 ± 24), constant darkness (144 ± 22) or melatonin (162 ± 43) groups showed a significant change in apoptosis, compared to the vehicle group (115 ± 27) (**Figure 13F**).

v. Constant darkness and exogenous melatonin therapy suppress NSPCs proliferation

Given that NSPCs in the SVZ region are among the oligodendroglia pools in adults and since melatonin receptors are expressed by SVZ cells, we investigated the effect of light and melatonin manipulations on NSPCs response to CPZ-induced demyelination. Results showed that proliferation of NSPCs ($SOX2^+/Ki67^+$ cells) in the SVZ region significantly (*$p<0.05$) increased in the vehicle CPZ group (2219 ± 289), in comparison to controls (1109 ± 112) **(Figure 14A and 14B)**. This increase in NSPCs proliferation was not altered by constant light (2344 ± 191) or luzindole (2164 ± 339) therapy; however, constant darkness (625 ± 62, ***$p<0.001$) and melatonin (1117 ± 214, *$p<0.05$) significantly inhibited this proliferation **(Figure 14B)**.

Given that uncontrolled proliferation can lead to depletion of the NSPC pool, SOX2 expressing NSPCs were counted in the SVZ region **(Figure 14C)**. Results showed that SOX2 cells were increased by CPZ in the vehicle group (7148 ± 559), compared to control mice (6180 ± 596), without reaching statistical significance. Moreover, constant light (7320 ± 673) or luzindole (7672 ± 298), did not affect the number of SOX2 expressing NSPCs. In contrast, constant darkness (4344 ± 182; **$p<0.01$) or melatonin (4305 ± 534; **$p<0.01$) significantly reduced the number of SOX2 expressing NSPC cells, compared to vehicle mice (7148 ± 559) **(Figure 14C)**. Therefore, it is demonstrated that increased proliferation of SOX2 did not lead to a depletion of the NSPC pool.

vi. Constant light and luzindole promote the recruitment of OPCs to the site of demyelination and the restoration of myelin sheaths

It is already reported that SOX2 is expressed by both OPCs and astrocytes in the CPZ model, with a different ratio, probably due to the time or site of assessment following induction of demyelination(Zhao *et al.* 2015). They also showed that an increase of SOX2 is crucial for the differentiation and recruitment of new OPCs to the site of injury. Here, we investigated whether increasing SOX2 proliferation in the SVZ was associated with an increase in the number of OPCs in the corpus callosum, which would lead to differentiation

and recruitment of OPCs to the site of demyelination **(Figure 15A)**. Double immunostaining with SOX2 (nucleus) and Olig2 showed that the percentage of SOX2 expressing Olig2 cells increased more than 2-fold in vehicle mice (11.7 ± 0.9) compared to controls (4.2 ± 0.6), without reaching statistical significance (p=0.16). This was further increased by constant light (25.5 ± 2.9, **p<0.01) or luzindole (17.0 ± 3.8, p>0.05) groups. However, both constant darkness (5.6 ± 1.1, p>0.05) and melatonin (7.3 ± 1.1, p>0.05) did not cause a significant decrease in the percentage of Olig2+/SOX2+ cells in the corpus callosum **(Figure 15B)**.

This increase in recruitment of Olig2 cells to the demyelination site was further examined by measuring the restoration of myelin sheaths by LFB staining **(Figure 15C)**. It is expected to observe myelin regeneration and less demyelinated area if Olig2 cells were recruited to the demyelination site and maturation of OLs was well established. Indeed, results showed that the demyelinated area was significantly reduced by constant light (43.4 ± 0.7 %; ****p<0.0001) and luzindole (48.0 ± 1.4 %; **p<0.01), in comparison to vehicle mice (53.3 ± 0.8 %). In contrast, melatonin caused a significant increase (56.7 ± 0.6 %; **p<0.01) in demyelination area, but not constant darkness (54.4 ± 1.0 %; p>0.05) **(Figure 15D)**.

vii. Constant light increases patrolling monocytes, phagocytosis, and clearance of myelin debris

Considering that classical monocytes ($Ly6C^{high}$) play a proinflammatory role whereas nonclassical monocytes ($Ly6C^{low}$) can patrol the vasculature for phagocytosis and to prepare the ground for remyelination (Pons *et al.* 2022), these monocytes were explored in the circulating blood using FACS **(Figure 16A)**. Results showed that feeding mice with CPZ did not affect the population of classical $Ly6C^{high}$ monocytes (59.8 ± 2.2), in comparison to controls (55.1 ± 1.6) **(Figure 16B)**. However, constant light (36.9 ± 2.4) caused a significant (****p<0.0001) decrease in these monocytes, compared to vehicle (59.8 ± 2.2). On the other hand, constant darkness (60.1 ± 2.6), luzindole, (53.9 ± 2.6), or melatonin (61.2 ± 2.4 %) failed to affect the percentage of $Ly6C^{high}$ cell population **(Figure 16B)**.

Given that classical Ly6C^{high} monocytes can switch to patrolling Ly6C^{low} monocytes (Pons and Rivest 2022), we further quantified this population **(Figure 16C)**. Data showed that Ly6C^{low} monocytes significantly (*p<0.05) decreased in vehicle mice (12.0 ± 0.8), in comparison to controls (19.7 ± 1.8). Importantly, this reduction was recovered by constant light (26.8 ± 2.9), which caused a significant (****p<0.0001) increase in the level of Ly6C^{low}, in comparison to vehicle, highlighting that light could switch the classical monocytes into patrol ones. However, neither luzindole (16.6 ± 1.4), nor constant darkness (12.8 ± 1.5), or melatonin (12.6 ± 0.9) could affect the percentage of Ly6C^{low} monocytes, compared to vehicle mice **(Figure 16C)**.

Since an increase in patrolling monocytes is accompanied with an increase in phagocytosis, we examined CD68 expression, a marker of phagocytosis, in the corpus callosum **(Figure 16D)**. Results showed that phagocytosis was significantly (****p<0.0001) increased in vehicle mice (212 ± 27), in comparison to controls (15 ± 4), which was significantly (***p<0.001) accentuated by constant light (357 ± 17) therapy. However, none of luzindole (262 ± 23), constant darkness (198 ± 17) or melatonin (215 ± 14) showed any significant changes, compared to vehicle mice **(Figure 16E)**.

viii. Immune cell infiltration into the CNS is exacerbated by constant darkness

In addition to the blood brain barrier (BBB), formed by brain endothelial cells (ECs), the blood-cerebrospinal fluid barrier (BCSFB), formed by epithelial cells of the choroid plexus (CP), plays a crucial role in infiltration into the CNS (Solár *et al.* 2020). Despite the very limited studies on immune cells infiltration into the CNS in the CPZ demyelination model, Shelestak et al showed that the BBB loses its integrity 3 days after starting the CPZ diet, when there is no demyelination yet. (Shelestak *et al.* 2020) In addition, it has been reported that melatonin can protect BBB integrity (Alluri *et al.* 2016). However, our results showed that melatonin receptors are expressed in the choroid plexus where the BCSFB is established. In light of the above, we speculated that altering the endogenous/exogenous levels and function of melatonin receptors by our manipulations may affect the infiltration rate. To this end, we exploited BM-chimeric GFP mice (protocol summarized in **Figure 17 A and 17B**) and found that 5 weeks of CPZ diet resulted into a massive infiltration of

systemic immune cells (31.3 ± 6.3), compared to control mice, where no GFP is detected. It is worth noting that the treatment was started at the beginning of week 5, when disruption of immune cell barrier in the brain and demyelination are at their peaks, which allows us to better analyze the potency of our treatments in ameliorating or exacerbating the infiltration. Our results showed that constant darkness (116.8 ± 26.3) caused a significant (**$p<0.01$) exacerbation of immune cell infiltration; however, melatonin (56.4 ± 13.2; $p>0.05$), constant light (39.4 ± 9.6; $p>0.05$) or luzindole (30.9 ± 4.7; $p>0.05$) failed to reduce the GFP levels, in comparison to the vehicle group **(Figure 17C and 17D)**.

4. Discussion

The present study revealed that induction of demyelination by cuprizone is followed by a slight increase in melatonin levels in the brain when melatonin is assessed at the end of the protocol. In order to rule out any bias, we believe that melatonin measurements are needed during the various time periods of the experimental procedure to uncover any increase in melatonin over the course of the disease. In addition, constant light conserved brain's melatonin at a low level, while constant darkness substantially increased it. In contrast, administration of exogenous melatonin or luzindole, had no effect on melatonin levels. These findings demonstrated the capability of our protocol to chronically reduce or increase melatonin by the pineal gland, which is the only light dependent source of melatonin synthesis.

Although there is no report regarding the effect of cuprizone as a copper chelating agent on melatonin synthesis, Parmar and Daya showed that treating pineal organ culture by copper causes an inhibition of the key enzyme in melatonin synthesis (Parmar and Daya 2001). We believe that chelating copper by cuprizone eliminates the regulatory effects of the enzyme leading to an increase in melatonin synthesis. On the other hand, constant light was associated with a sharp increase in brain cortisol levels while constant darkness did the opposite. Previous studies reported that continuous light at night in mice considerably increased the level of corticosterone(Ishida *et al.* 2005). Glucocorticoids, such as cortisol, can inhibit the recruitment of immune cells to the site of injury or act as immunosuppressants (Barnes 2006). In MS, it has been shown that rats that were chronically exposed to stress for 3 weeks had a reduction in EAE incidence and myelin loss and lower leukocyte infiltration into the CNS (Pérez-Nievas *et al.* 2010). In fact, corticosteroid therapy, designed to mimic the anti-inflammatory effect of the naturally released cortisol, is widely used for MS therapy. Therefore, we speculated that the simultaneous increase of cortisol (a powerful immunosuppressive hormone) and the chronic inhibition of melatonin peak secretion at night can keep melatonin at sufficient levels to apply its beneficial effects as immunoregulator rather than an immunoenhancer in MS. This could be considered as an invasive strategy preparing the ground for either suppressing the deleterious effects of immune cell attack to myelin or increasing the oligodendrogenesis process, required for recovering the lost myelin. In fact, melatonin showed a potential in reducing cortisol levels (Campino *et al.* 2008), which

may further explain why a reduction of melatonin in the constant light group was associated with a sharp increase in cortisol levels.

On the other hand, most studies on MS administrated melatonin in the evening before the night starts, a protocol which causes a disequilibrium of the circadian rhythm (Deacon *et al.* 1995). Nocturnal rise in melatonin cause more than 40% decrease in circadian core body temperature (Cagnacci *et al.* 1995). However, evening administration of melatonin leads to longer and earlier reduction in core body temperature which suppresses the immune system and play an anti-inflammatory role (Polderman 2012). In fact, evening melatonin therapy plays a beneficial role in MS indirectly, but studies are needed to know its effect when melatonin levels are over boosted. In this study, treatment was performed during the day, when melatonin is at its lowest level, to boost the diurnal melatonin, in addition to its effect at night. Based on our previous study (Farhadi *et al.* 2016), placing mice in constant light for 10 days failed to diminish serum melatonin levels, possibly due to melatonin synthesis by compensatory mechanisms of pineal-independent sources which occur after the first days of darkness. In accordance, Hongas et al. (Hong *et al.* 2019), employed constant light or darkness therapy at the day of induction of Spinal Cord Injury (SCI) and showed that melatonin levels in the cerebrospinal fluid (CSF) increased at day 3 post SCI in the group of constant darkness; however, this elevation started to drop off after 3 days and approached the control levels at day 7 of darkness. This further emphasized that long-term light manipulation could not be profitable to keep melatonin at low or high levels. Our new protocol of light manipulation in this study aimed to minimize the negative feedback of the pineal gland or other organs in response to the continuous elevation of melatonin in the darkness group, and ii) the compensatory mechanisms of other sources of melatonin synthesis in response to the continuous decreased melatonin levels in constant light.

Since melatonin affects the cell physiology through its receptors, we investigated the expression of melatonin receptors 1A and 1B (MT1A and MT1B) in the corpus callosum. We detected both receptors in OLs of the corpus callosum. Although feeding mice with cuprizone causes demyelination, the number of immature OLs, but not mature OLs, increases as a self-regeneration mechanism in mice. Our data showed that constant darkness markedly suppressed OPC proliferation compared to untreated CPZ mice. In contrast, both constant light and luzindole therapy did not boost OPC proliferation. It seems that the CPZ-induced

pathological condition already exerted its maximum stimulatory effects on OPC proliferation; hence, constant light and luzindole failed to stimulate the proliferation further, which is expected to lead to NSPC pool depletion should it happens. Interestingly, although the increased number of immature OPCs in untreated CPZ mice failed to become mature OLs; however, constant light and luzindole significantly protected the maturation of OPCs to OLs. This was further confirmed by LFB staining and showed that constant light and luzindole not only increased the maturation of OPCs, but also completed the remyelination process and myelin sheath reconstruction.

Since we revealed that MT1A and MT1B receptors were expressed in the SVZ region and considering that previous reports showed that OPCs in the corpus callosum can originate from SVZ under demyelination conditions (Menn *et al.* 2006; Butti *et al.* 2019), we observed that CPZ induces a sharp increase in the proliferation of SVZ NSPCs, most probably because of SVZ's response to demyelination and inflammation to recover the damaged OLs. However, neither constant light therapy nor luzindole could increase SOX2 activation, which seems to be at its highest level. Since SOX2 cells can migrate and differentiate to neurons, astrocytes, and OLs in demyelination sites, we quantified Olig2/SOX2 expression in the corpus callosum, as SOX2 is expressed by new OPCs in order to contribute to their recruitment at the demyelination site (Zhao *et al.* 2015; Zhang *et al.* 2018). Although all groups of vehicles, constant light and luzindole had similar levels of SOX2 activation in SVZ, only constant light increases Olig2-expressing SOX2 cells in the corpus callosum, which implies that a significant portion of SOX2 cells had differentiated to OPCs.

From an immunological perspective, using chimeric mice, we observed that BM-derived microglia/macrophages ($CD68^+/GFP^+$ cells) in the CNS do not express MT1A and MT1B; however, brain-resident microglia/macrophages ($CD68^+/GFP^-$ cells) express both receptors. In support of this finding, an early *in vitro* study in 1998 using binding experiments of 2-[^{125}I]iodomelatonin, showed that fresh and 1-day cultured human monocytes express high levels of melatonin receptors, which is lost after 3 days and 15 days of culture because of maturation or differentiation of monocytes, respectively. The authors also showed that U937 monocytic cell line does not express melatonin receptors (Barjavel *et al.* 1998). In contrast, another group used the same binding technique on purified U937cell nuclei and membranes and reported that these cells express MT1 receptors only (García-Mauriño *et al.* 2000).

However, the findings regarding melatonin receptor expression in macrophages, not monocytes, are still controversial. It seems that while monocytes express melatonin receptors in the circulation, they lose this expression upon differentiation to macrophages in the CNS of demyelinated mice. The current knowledge and our findings regarding melatonin receptor expression in macrophages is illustrated in (**Figure 1.8).**

The involvement of monocytes in the CPZ model is rarely studied; therefore, we further investigated the response of monocytes to our various manipulations. Our data revealed that while CPZ diet significantly decreased the classical anti-inflammatory monocytes ($Ly6C^{low}$), constant light and luzindole recovered this expression, unlike constant darkness or melatonin. This further highlights the existence of melatonin receptors in monocytes; however, more experiments are needed to elucidate the exact expression of the various melatonin receptors in different types of monocytes. Our data revealed that both melatonin receptors MT1A and MT1B are expressed by the choroid plexus, which is one of the barriers for immune cell infiltration into the CNS. Using chimeric mice treated for 1 week, at week 5 of CPZ diet, we demonstrated that monocytes infiltration into the CNS is exacerbated by constant darkness and melatonin, but not constant light and luzindole. Since even one week of treatment plays a deleterious role in infiltration, this experiment should be performed at the time of CPZ diet initiation, or even before, as well as with chronic treatment conditions. Furthermore, characterization of the function of macrophages, either peripheral or residential, in the corpus callosum revealed that both constant light and luzindole increased the phagocytotic activity of macrophages. Since BM-derived macrophages in the CNS possibly do not express melatonin receptors, it implies that these effects on the phagocytotic activity of macrophages are mediated by brain resident microglia/macrophages. Furthermore, a study on murine macrophage cell line, RAW264.7, showed that melatonin causes inhibition of phagocytosis as visualized and quantified by CFSE Staining of E. coli(Kadena *et al.* 2017).

While the beneficial effect of melatonin has been reported in a wide range of diseases, it should be noted that this molecule has pleiotropic effects. In fact, its immunoenhancing or immunosuppressive roles in autoimmune disease are still controversial, with some studies reporting a deleterious role in MS models. In addition, case studies reported that using picoTesla magnetic fields improved the disability in MS patients, which is accompanied by a reduction of melatonin levels, possibly by downregulation of its immunoenhancing effects

(Sandyk 1992a). This deleterious role of melatonin in MS was also proposed by Kuklina et al. (Kuklina 2016), and Constantinescu et al. (Constantinescu *et al.* 1997) Moreover, in cultured human monocytes, melatonin showed a high immunoenhancing effect by activating these monocytes and increasing their IL-1α and IL-1β(Barjavel *et al.* 1998).Furthermore, another study showed the potential of melatonin in increasing IFN-γ, IL-2, and IL-6 via human circulating CD4+ cells through melatonin nuclear receptor (Garcia-Mauriño *et al.* 1997). Finally, investigation in Parkinson's Disease also showed that melatonin therapy exacerbates the motor function and associated behavioural impairment while an improvement of these abnormalities is achieved by suppression of endogenous melatonin synthesis by pinealectomy or constant light (Willis *et al.* 1999).

Although current medications ameliorate or control relapsing-remitting MS (RRMS), there is still no FDA approved medication for primary-progressive MS (PPMS) or secondary-progressive MS (SPMS). Patients from the latter categories do not respond to medications prescribed to RRMS. Therefore, instead of using the EAE model which corresponds to RRMS, we took advantage of the cuprizone model which mimics several characteristics of progressive MS. This allowed to demonstrate that suppressing natural melatonin and increasing the immunosuppressant property of cortisol can be a new approach for improving the disease course in progressive MS.

Overall, this study highlights the importance of inspecting melatonin levels in MS patients. Our constant light manipulation only affects the surge of melatonin at night in cuprizone mice, an animal model with a tendency to increase melatonin levels. However, other light-independent sources of melatonin synthesis such as the BM, thymus, lymphocytes, and gastrointestinal tract provide considerable melatonin levels that seems to be enough for mediating its normal physiological activity. Current reports regarding melatonin did not consider that MS patients have different diet habits which could be high or low in melatonin, and different lifestyles such as light at night, night shift work, or looking at LED screens at night, all of which interrupt the circadian rhythm of melatonin. In fact, melatonin administration must be prescribed based on its circadian rhythm in each patient in order to avoid melatonin's overdose, due to high endogenous as well as exogenous levels. Therefore, melatonin seems to have its optimal beneficial effect when it is at its physiological

concentration, or when its immunoenhancing effect resulting from its peak secretion at night is inhibited. Finally, one limitation to this study was the need to measure melatonin levels in patients before and during exogenous melatonin therapy. It would be of great importance that the findings of this study be translated to a clinical study by examining the MT receptors expression in circulating monocytes, or in microglia/macrophages of post-mortem MS patients.

5. Declarations

Abbreviations

Multiple sclerosis (MS); central nervous system (CNS); experimental autoimmune encephalomyelitis (EAE); Pyruvate dehydrogenase complex (PDC); diisopropylamine dichloroacetate (DADA); cuprizone (CPZ); oligodendrocytes (OLs); oligodendrocyte precursor cells (OPCs); neural stem/progenitor cells (NSPCs; subventricular zone (SVZ); Fluorescence-Activated Cell Sorting (FACS); constant light (CL); constant darkness (CD); melatonin (MLT); Luzindole (LUZ); dimethyl sulfoxide (DMSO); Bone marrows (BM); 5-bromo-2'-deoxyuridine (BrdU); paraformaldehyde (PFA); BCA (bicinchoninic acid assay); bovine serum albumin (BSA); Luxol Fast Blue (LFB); green fluorescent protein (GFP); spinal Cord Injury (SCI); melatonin receptors 1A (MT1A); melatonin receptors 1B (MT1B); relapsing-remitting MS (RRMS); primary-progressive MS (PPMS); secondary-progressive MS (SPMS).

Ethical Approval and Consent to participate

Experimental animal protocols complied with all relevant ethical regulations, in accordance with the Canadian Council on Animal Care guidelines, administered by the Laval University Animal Welfare Committee.

Availability of supporting data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

M.G. devised the project, performed experiments, collection, data analysis, and wrote the paper; V.P. participated in data collection; V.P and N.L project management; K.Z

participated in designing the project, data analysis, critically revised the article; S.R. Funding acquisition, supervision of project and revising the manuscript. All authors read and approved the final manuscript.

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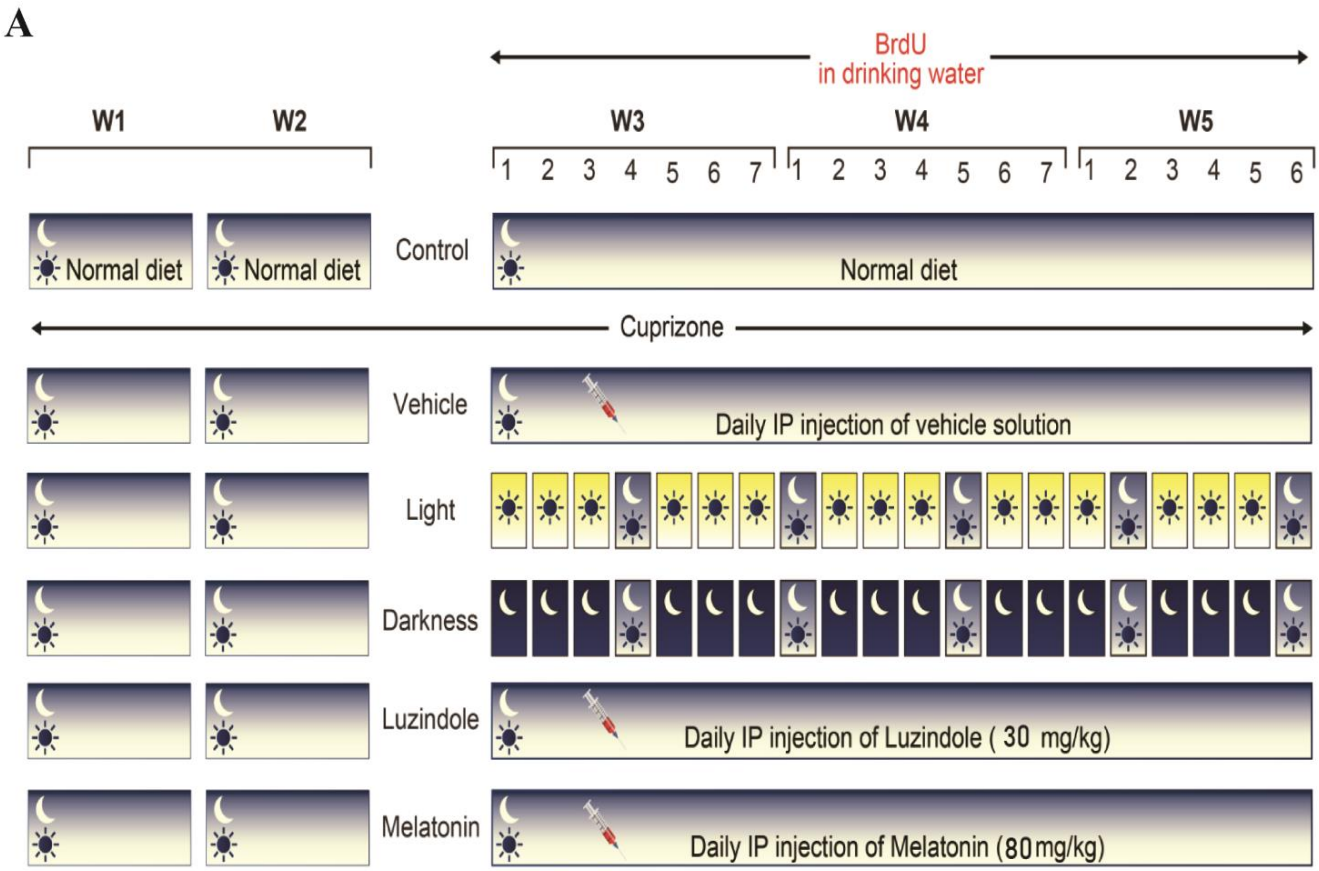
Not applicable

Table 1: List of antibodies used in this study for tissue immunofluorescent staining and Fluorescence-Activated Cell Sorting (FACS) analysis.

Num	Product	CAS No.	Vendor
1	Rabbit Melatonin Receptor 1A	Orb221456	Boorbyt
2	Rabbit Melatonin Receptor 1B	NLS932	Novus Biologicals
3	Rabbit Anti-Olig2	AB9610	Millipore
4	Mouse Anti-APC (CC1)	OP80	Calbiochem
5	Rat Anti-BrdU	AB6326	Abcam
6	Rat Anti-SOX2	14-9811-82	Invitrogen
7	Rabbit Anti-Ki67	NB110-57147	Novus Biologicals
7	Rabbit Anti-Caspase-3	9664S	Cell Signaling
8	Rat anti Mouse CD68	MCA1957	BIORAD
9	DAPI	D3571	Invitrogen
10	Mouse CD11b, AF700	56-0112-82	Invitrogen
11	Mouse Ly-6C, V450	560594	BDbioscience
12	Mouse Ly6G, PE	551461	BDbioscience
13	Mouse CD45, V500	561487	BDbioscience
14	CD16/CD32 (Mouse BD Fc Block™)	553142	BDbioscience
15	LIVE/DEAD™ Dead Cell Stain Kit	L23105	Invitrogen

Table 2: List of chemical products and drugs used in the study.

Num	Product	CAS No.	Vendor
1	Melatonin	sc-207848B	Santa Cruz
2	Luzindole	sc-202700B	Santa Cruz
3	5-Bromo-2′-deoxyuridine (BrdU)	B5002	Millipore
4	Solvent Blue 38 practical grade (LFB)	S3382	Sigma-Aldrich
5	Bis(cyclohexanone)oxaldihydrazone (Cuprizone)	14690	Sigma-Aldrich
6	Mouse Melatonin (MT) ELISA Kit	MBS263465	MyBioSource
7	Mouse Cortisol ELISA Kit	MBS704879	MyBioSource


A
BrdU
in drinking water
W1
W2
W3
W4
W5
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6
Normal diet
Normal diet
Control
Normal diet
Cuprizone
Vehicle
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Light
Darkness
Luzindole
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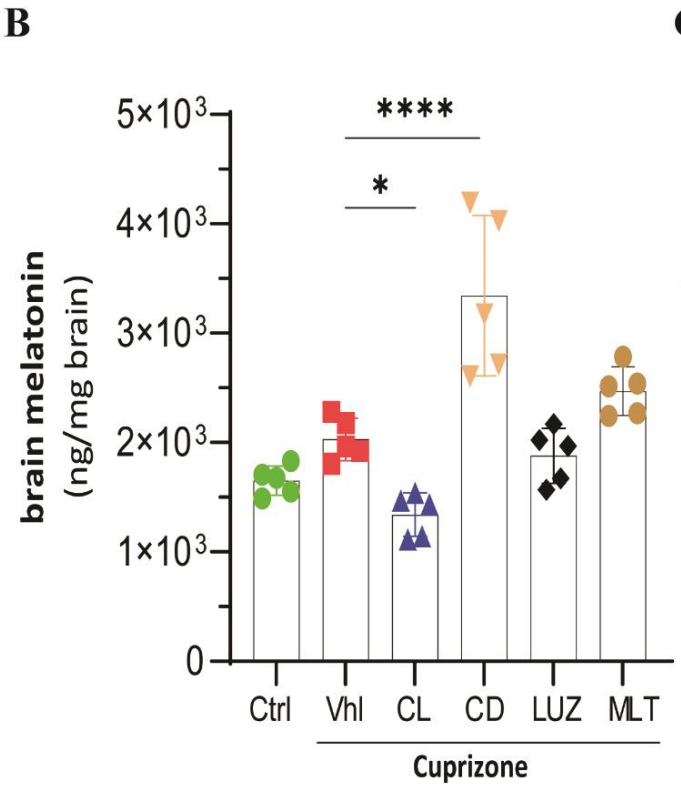
Melatonin
Daily IP injection of Melatonin (80mg/kg)


B
brain melatonin
(ng/mg brain)
5×10³
4×10³
3×10³
2×10³
1×10³
0
****
*
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone

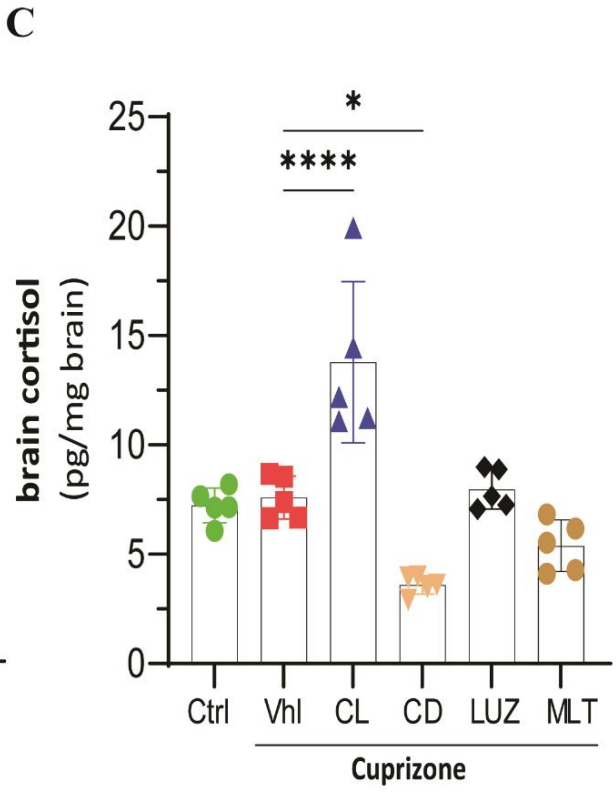

C
brain cortisol
(pg/mg brain)
25
20
15
10
5
0
*
****
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone

Figure 11: Schematic representation of the treatment protocols. (A) Briefly, mice were fed with CPZ for 2 weeks under normal light/night cycle. Thereafter, treatments were initiated at the beginning of weeks 3, in presence of CPZ. Both the Ctrl group and the vehicle (untreated CPZ) mice were kept in normal light/night cycle. Melatonin (80 mg/kg) and Luzindole (30 mg/kg) groups received these drugs IP, daily at 9 AM and 6 PM, respectively; and both kept in normal light/night cycle. Light was set ON or OFF for 3 days in constant light or constant darkness groups, respectively, followed by a day of normal light/night. This cycle was repeated 5 times. BrdU in drinking water started at the beginning of manipulations at week 3**. (B)** Brain levels of melatonin and **(C)** Cortisol were analyzed using ELISA at the end of study (**n**=5 mice/group). Significance is indicated by $*p < 0.05$; $**p < 0.01$; and $****p < 0.0001$.

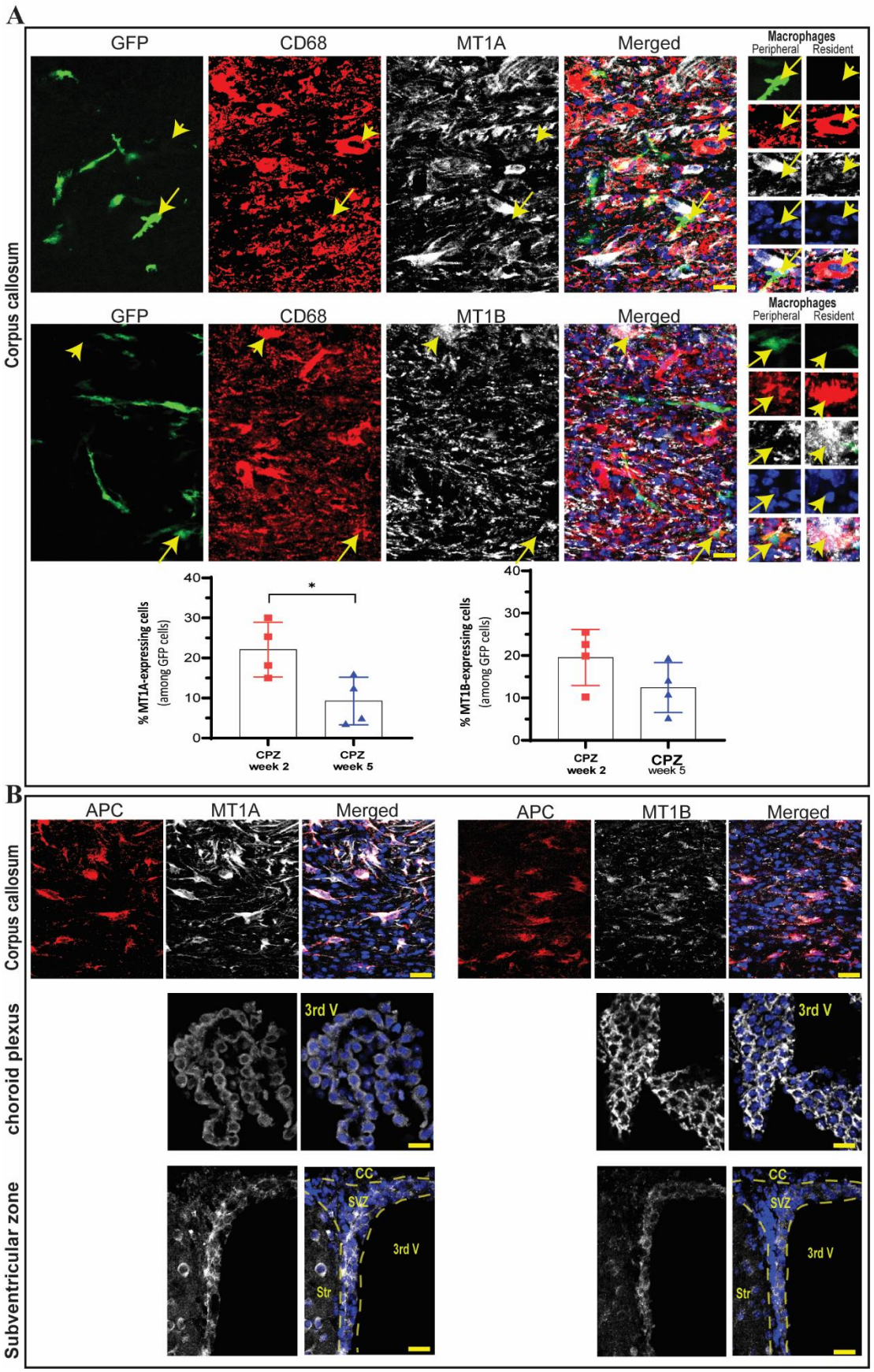

A
GFP
CD68
MT1A
Merged
Macrophages
Peripheral
Resident
Corpus callosum
GFP
CD68
MT1B
Merged
Macrophages
Peripheral
Resident
% MT1A-expressing cells
(among GFP cells)
40
30
20
10
0
*
CPZ
week 2
CPZ
week 5
% MT1B-expressing cells
(among GFP cells)
40
30
20
10
0
CPZ
week 2
CPZ
week 5
B
APC
MT1A
Merged
APC
MT1B
Merged
Corpus callosum
choroid plexus
3rd V
3rd V
Subventricular zone
CC
SVZ
3rd V
Str
CC
SVZ
3rd V
Str

Figure 12: Fluorescent staining for detection of melatonin receptors MT1A and MT1b. (A) Chimeric mice, whose BM-derived monocytes were GFP-tagged, were used to detect the colocalization between GFP (BM-derived cells) and CD68 (monocyte/macrophages). Lack of GFP expression in CD68 positive cells indicate that these cells were brain-resident macrophages (long yellow arrow). However, expression of GFP demonstrated infiltered cells as BM-derived macrophages (short yellow arrow) in the corpus callosum. MT1A and MT1B were SParately added to GFP/CD68 panel for triple staining. **(B)** Expressing of MT1A and MT1B in oligodendrocytes (APC cells) of the corpus callosum (upper panel); choroid plexus (middle panel) and subventricular zone (lower panel). Scale bars, 20 μm. MT1A and MT1B expressing GFP cells (bone-derived monocytes/macrophages) were quantified 2 and 5 weeks after cuprizone diet. (n=4 mice/group). Significance is indicated by $*p < 0.05$ using unpaired t-Test.

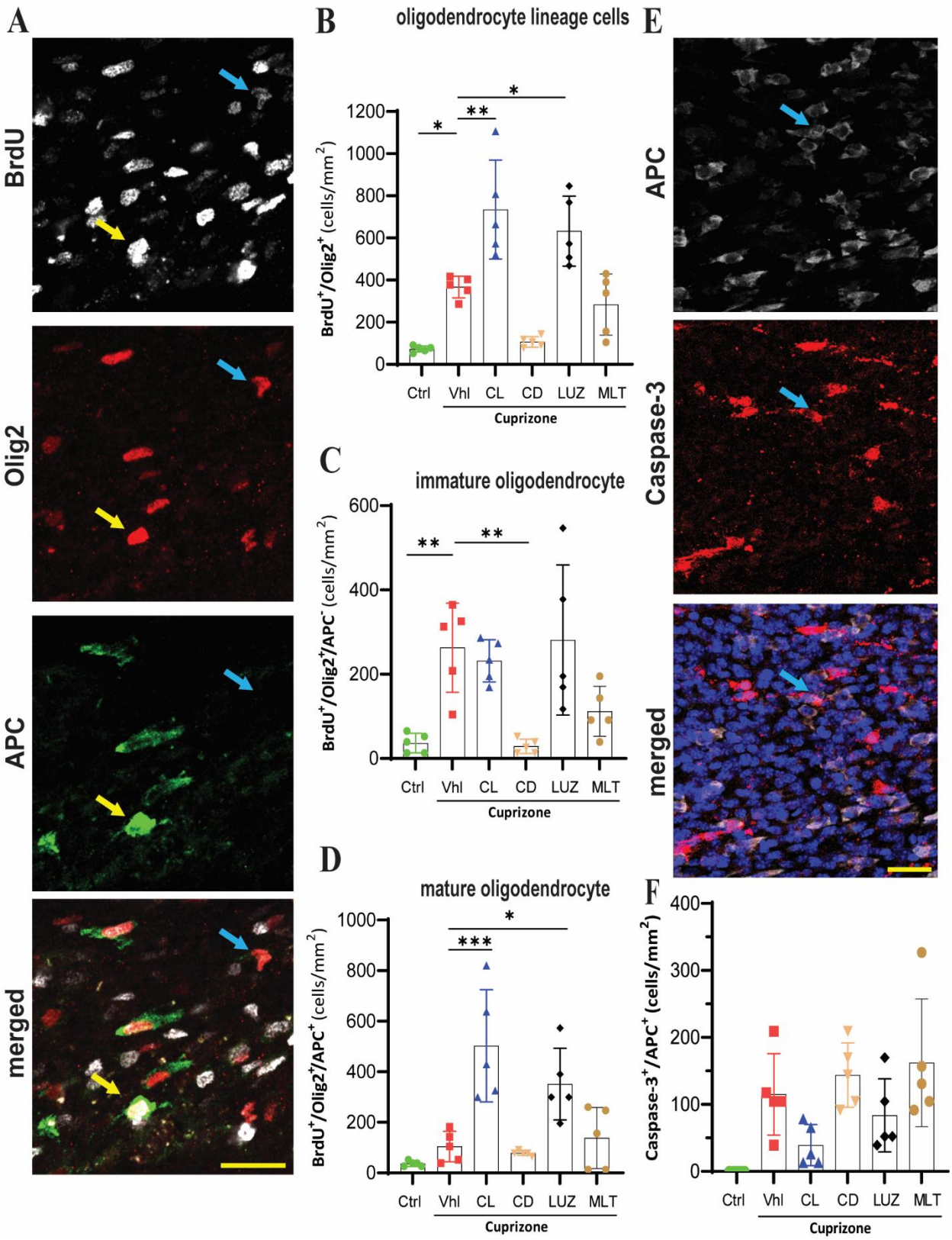

A
BrdU
Olig2
APC
merged
B
oligodendrocyte lineage cells
BrdU+/Olig2+ (cells/mm2)
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone
C
immature oligodendrocyte
BrdU+/Olig2+/APC- (cells/mm2)
D
mature oligodendrocyte
BrdU+/Olig2+/APC+ (cells/mm2)
E
APC
Caspase-3
merged
F
Caspase-3+/APC+ (cells/mm2)

Figure 13: Quantification of oligodendroglia response to demyelination and treatments in the corpus callosum. (A) Fluorescent staining of the newly generated BrdU+ cells; Olig2 is a marker of the oligodendroglial lineage; APC is a marker of mature oligodendrocytes. DAPI stains the nucleus. Left panel, blue arrow shows immature OLs, and yellow arrow shows mature OLs. **(B)** Quantification of oligodendroglia lineage (BrdU+/Olig2+ cells), comprising both immature and mature OLs. **(C)** Quantification of immature OLs (OPCs; BrdU+/Olig2+/APC- cells). **(D)** Quantification of mature OLs (BrdU+/Olig2+/APC+ cells). **(E)** Co-staining of apoptosis marker, Caspase-3, with oligodendrocyte marker, APC. Right panel: arrow indicates the Caspase-3 colocalization with APC. **(F)** Quantification of the mean number of Caspase-3+/APC+ cells. All quantifications were performed in the middle corpus callosum along the rostrocaudal axis. (n=5 mice/group; 3 slides per mouse). Significance is indicated by $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$. Scale bars, 20 μm.

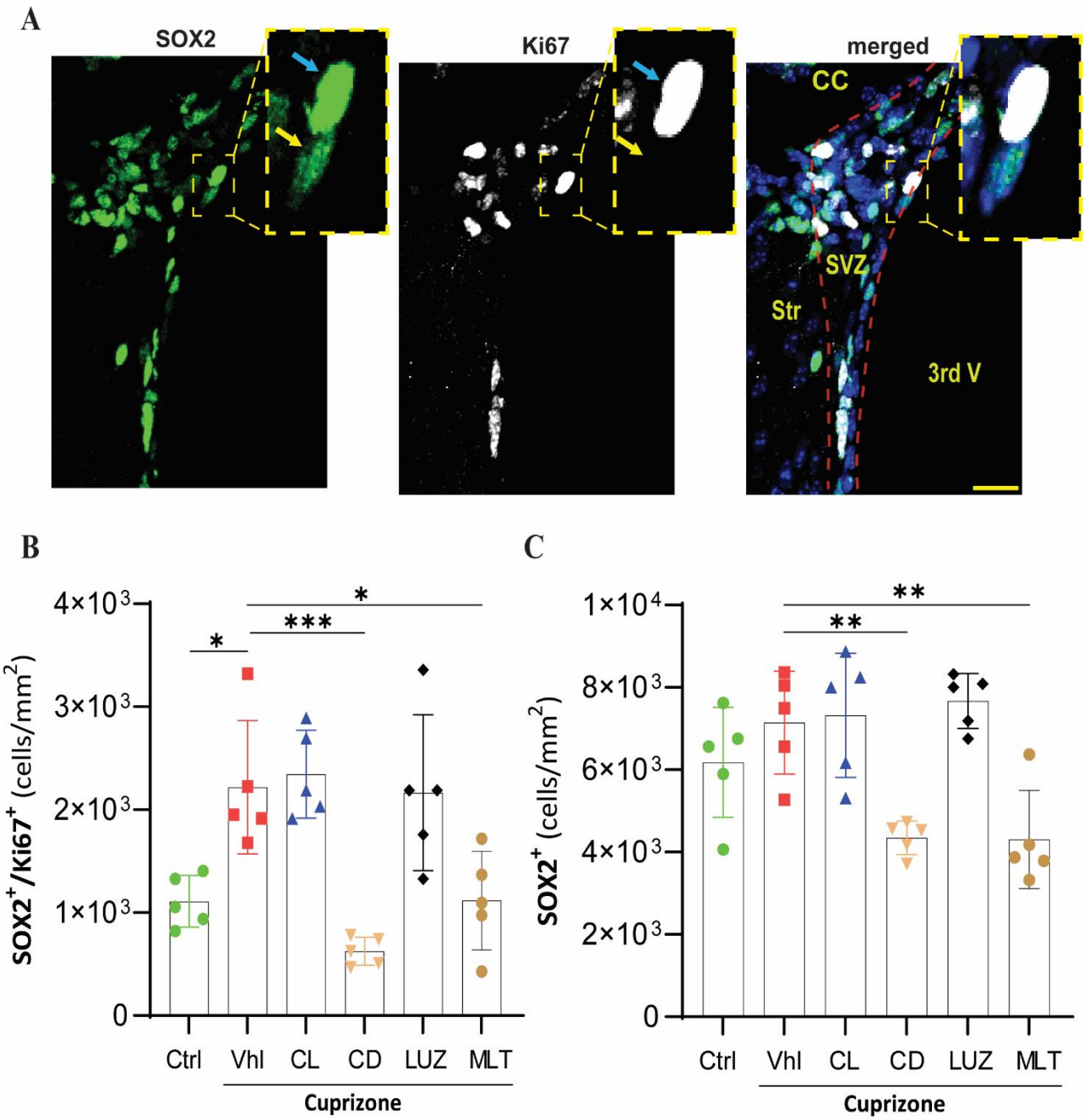

A
SOX2
Ki67
merged
CC
SVZ
Str
3rd V
B
SOX2+/Ki67+ (cells/mm2)
4×10^3
3×10^3
2×10^3
1×10^3
0
*
***
*
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone
C
SOX2+ (cells/mm2)
1×10^4
8×10^3
6×10^3
4×10^3
2×10^3
0
**
**
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone

Figure 14: Quantification of NSPCs response to demyelination and treatments in the SVZ. (A) Fluorescent staining of NSPCs marker, SOX2, proliferation marker, Ki67 along with DAPI. Blue arrow indicates the proliferative SOX2 and yellow shows quiescent NSPCs. **(B)** Quantification of the number of proliferative NSPCs cells (SOX2+/Ki67+). **(C)** Quantification of the number of NSPCs cells (SOX2+), which included both quiescent and proliferative NSPCs. All quantifications were performed in the SVZ region. (n=5 mice/group; 3 slides per mouse). Significance is indicated by $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$. Scale bars, 20 μm.

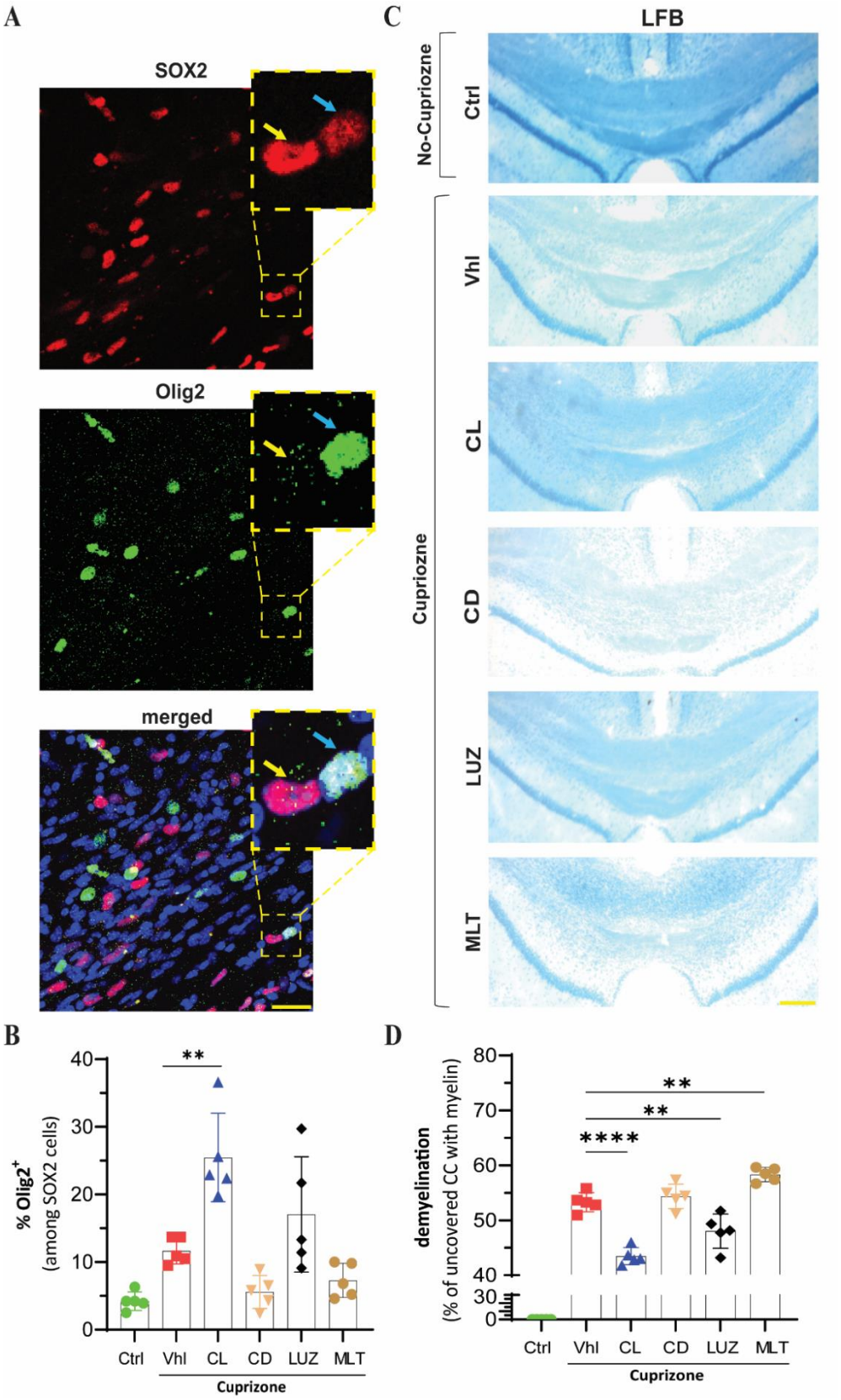
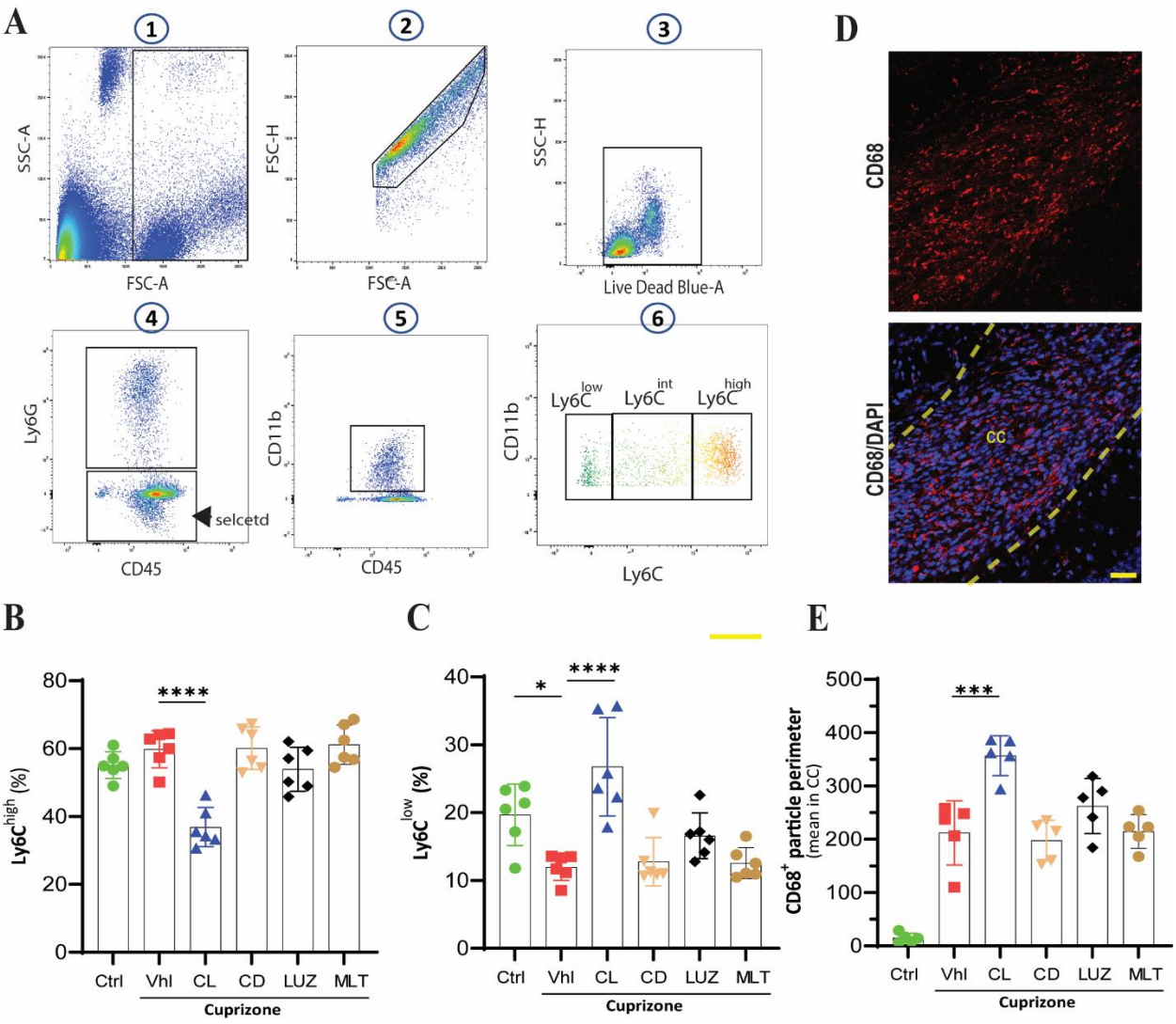

A
SOX2
Olig2
merged
C
LFB
No-Cuprioznе
Ctrl
Cuprioznе
Vhl
CL
CD
LUZ
MLT
B
% Olig2+
(among SOX2 cells)
40
30
20
10
0
**
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone
D
demyelination
(% of uncovered CC with myelin)
80
70
60
50
40
30
0
**
**
****
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone

Figure 15: Olig2 recruitment to the demyelinated site and restoration of myelin sheaths. (A) Fluorescent staining of Olig2, oligodendroglia marker, and SOX2, whose expression by Olig2 cells increases the recruitment of Olig2+ cells to the site of injury. **(B)** Quantification of the frequency of SOX2-expressing Olig2 cells (SOX2+/Olig2+). Blue arrow indicates SOX2+/Olig2+ and yellow indicates SOX2+/Olig2$^-$. **(C)** LFB staining of the corpus callosum to measure the extent of demyelination. **(D)** Quantification of the percentage of uncovered area of corpus callosum, which indicates the demyelinated area. Olig2, SOX2 and LFB quantifications were performed in the same area of the corpus callosum. Blue arrow shows the Olig2-expressing SOX2 cells and yellow arrow shows SOX2 lacking Olig2 expression. (n=5 mice/group; 3 slides per mouse). Significance is indicated by $**p < 0.01$; and $***p < 0.0001$. Scale bars, 20 μm in **A**; 100 μm in the **C**.


A
1
SSC-A
FSC-A
2
FSC-H
FSC-A
3
SSC-H
Live Dead Blue-A
4
Ly6G
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CD45
5
CD11b
CD45
6
CD11b
Ly6C low
Ly6C int
Ly6C high
Ly6C
D
CD68
CD68/DAPI
CC
B
Ly6C high (%)
****
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone
C
Ly6C low (%)
*
****
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone
E
CD68+ particle perimeter (mean in CC)
***
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone

Figure 16: Analysis of monocytes categories by FACS and phagocytosis. **(A)** Gating Strategy for monocytes. After gating for live single cells, neutrophiles are excluded from populations (CD45+/Ly6G-) and then gated for CD11b+/CD45+ populations. This was further gated with CD11b+/Ly6C to analyze the percentage of $Ly6C^{low}$, $Ly6C^{int}$, and $Ly6C^{high}$. **(B)** Percentage of classical inflammatory monocytes ($Ly6C^{high}$) **(C)** Percentage of patrolling monocytes ($Ly6C^{high}$). **(D)** Fluorescent staining of phagocytosis marker, CD68, and **(E)** The corresponding quantifications. All quantifications were performed in the peripheral blood samples collected at same time (n=5 mice/group). Significance is indicated by $**p < 0.01$; and $***p < 0.0001$. Scale bars, 20 μm.

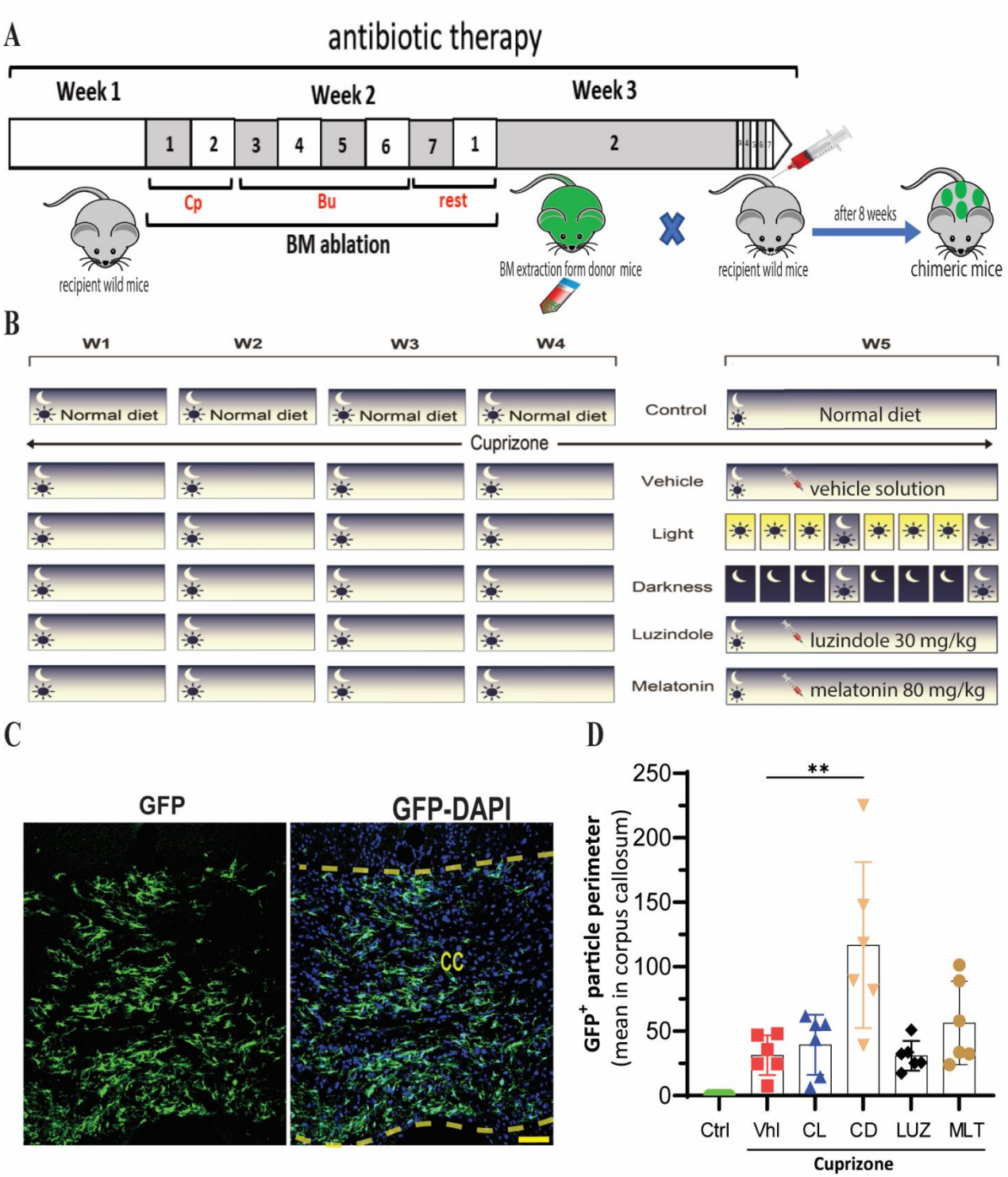
A
antibiotic therapy
Week 1
Week 2
Week 3
1
2
3
4
5
6
7
1
2
Cp
Bu
rest
BM ablation
recipient wild mice
BM extraction form donor mice
recipient wild mice
after 8 weeks
chimeric mice
B
W1
W2
W3
W4
W5
Normal diet
Normal diet
Normal diet
Normal diet
Control
Normal diet
Cuprizone
Vehicle
vehicle solution
Light
Darkness
Luzindole
luzindole 30 mg/kg
Melatonin
melatonin 80 mg/kg
C
GFP
GFP-DAPI
CC
D
**
GFP+ particle perimeter
(mean in corpus callosum)
250
200
150
100
50
0
Ctrl
Vhl
CL
CD
LUZ
MLT
Cuprizone

Figure 17: The infiltration rate in the CNS. (A) Schematic illustration of establishing chimeric mice and **(B)** protocol for all the treatments in chimeric mice. (antibiotic, SPtra; Busulfan, Bu; Cyclophosphamide, Cp) **(C)** A representative example of infiltrated GFP-tagged cells into the brain settled in the corpus callosum, and **(D)** the corresponding quantifications. (n=5 mice/group; 3 slides per mouse). Significance is indicated by $**p < 0.01$; $***p < 0.001$, and $****p < 0.0001$. Scale bars, 20 μm.

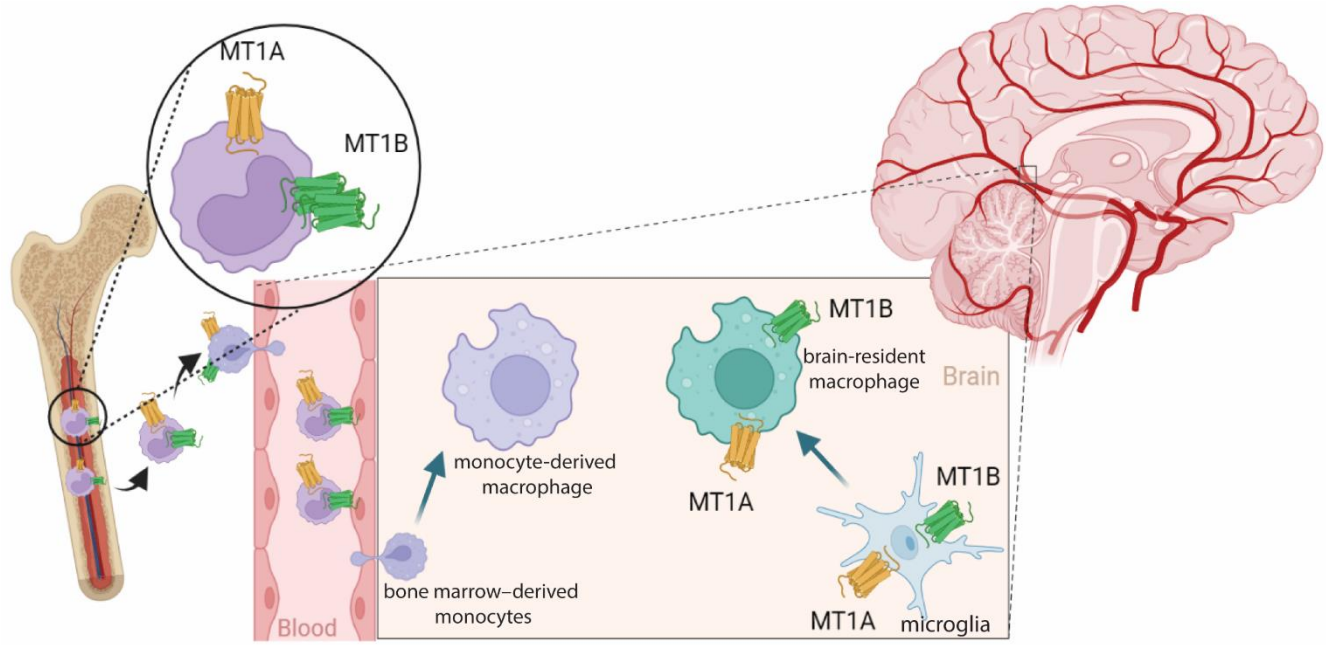

Figure 18: Schematic illustration of the melatonin receptors (MT1A and MT1B) by monocytes/macrophages.

Monocytes express melatonin receptors in the BM and the circulation. However, monocytes lose this expression upon infiltration into the CNS and differentiation into macrophages. On the other hand, microglia and brain-resident macrophages express melatonin receptors while BM-derived macrophages lack this expression in demyelinated mice.

6. Future experiments

As a pitfall of the first study, we did not use genetic manipulation to modify the expression of melatonin receptors; hence, we expect further experiments on melatonin and MS to use genetic mice, including MT1A/B knockout mice to induce EAE or use cuprizone. This allows further confirmation of rejection of the idea that melatonin mediates its effect on the cell by these two receptors or how overexpression or suppression of receptors affects MS induction and development.

Since bone-derived macrophages lose the expression of melatonin receptors, it is expected to use CRISPR/CAS 9 technology to specifically target the melatonin receptor expression in macrophages to induce its expression. This might allow melatonin to control the function of these macrophages and improve MS.

In the current study, we have checked the melatonin level only at a one-time point. But the following studies need to check it at different days of demyelination or at different times on the same day to see how melatonin alters throughout demyelination.

CHAPTER 2 : PDK4 inhibition ameliorates melatonin therapy by modulating cerebral metabolism and remyelination in an EAE demyelinating mouse model of multiple sclerosis

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Running title: PDK4 inhibition improves melatonin therapy

Résumé

Nous avons récemment montré que la mélatonine atténue la gravité de l'encéphalomyélite auto-immune expérimentale (EAE), un modèle animal de la SP. Cependant, l'efficacité du traitement à la mélatonine était associée à des effets secondaires, qui se manifestaient par un ralentissement de la remyélinisation, en raison de l'augmentation des effets inhibiteurs de la pyruvate déshydrogénase kinase-4 (PDK-4) cérébral sur le complexe pyruvate déshydrogénase (PDC), une enzyme déterminante pour la synthèse des acides gras (AG) pendant la remyélinisation. Dans cette étude, nous avons examiné le profil métabolique de la synthèse des AG en utilisant un traitement combinant de la mélatonine et de la dichloroacétate de diisopropylamine (DADA), un inhibiteur de PDK4, chez des souris EAE. La progression de la maladie a été suivie en enregistrant les scores d'invalidité. Les facteurs immunologiques, oligodendrogenèse et les facteurs métaboliques ont également été évalués. Les résultats ont montré que la thérapie combinant la mélatonine et de la DADA réduisait significativement les scores d'invalidité de l'EAE, par rapport à la mélatonine, alors que la DADA seule n'avait aucun effet. En outre, la co-thérapie a inhibé les cytokines pro-inflammatoires tout en augmentant les cytokines anti-inflammatoires, de façon nettement plus efficace que la mélatonine seule. De plus, la combinaison de ces médicaments a permis de rétablir l'expression des marqueurs oligodendrocytaires dans l'EAE, de manière plus efficace que la mélatonine. Par ailleurs, la co-thérapie a affecté le métabolisme énergétique cérébral en réduisant significativement les niveaux de lactate tout en augmentant les niveaux de N-acétylaspartate (NAA) et de 3-hydroxy-3-méthyl-glutaryl-coenzyme-A réductase (HMGCR). Enfin, alors que la mélatonine augmente les niveaux d'expression du lactate et de PDK4 et réduit fortement l'activité de la PDC, la co-thérapie rétablit significativement la fonction de la PDC tout en réduisant les niveaux de lactate. En résumé, l'administration de la mélatonine avec la DADA a augmenté l'efficacité du traitement à la mélatonine en éliminant les effets inhibiteurs de PDK4 sur la fonction de la PDC, une étape critique pour la synthèse correcte des AG pendant la remyélinisation.

Abstract

We recently showed that melatonin ameliorates the severity of experimental autoimmune encephalomyelitis (EAE), an animal model of MS. However, efficiency of melatonin therapy was associated with side effects, manifested by slowing down of remyelination, through increasing the inhibitory effects of brain pyruvate dehydrogenase kinase-4 (PDK-4) on pyruvate dehydrogenase complex (PDC), a key enzyme in fatty acid (FA) synthesis during remyelination. In this study, we investigated the metabolic profile of FA synthesis using combination therapy of melatonin and diisopropylamine dichloroacetate (DADA), a PDK4 inhibitor, in EAE mice. Disease progression was monitored by recording the disability scores. Immunological, oligodendrogenesis and metabolic factors were also evaluated. Results showed that combination therapy of melatonin and DADA significantly reduced EAE disability scores, compared to melatonin, whereas DADA alone did not have any effect. In addition, co-therapy inhibited pro-inflammatory while increasing anti-inflammatory cytokines, significantly better than melatonin alone. Moreover, administration of combination drugs recovered the declined expression of oligodendrocytic markers in EAE, more potently than melatonin. Furthermore, co-therapy affected cerebral energy metabolism by significantly reducing lactate levels while increasing N-acetylaspartate (NAA) and 3-hydroxy-3-methyl-glutaryl-coenzyme-A reductase (HMGCR) levels. Finally, while melatonin increased lactate and PDK4 expression levels and greatly reduced PDC activity, co-therapy significantly restored PDC function while reducing the lactate levels. In summary, administration of melatonin with DADA increased the efficiency of melatonin treatment by eliminating the inhibitory effects of PDK4 on PDC's function, a critical step for proper FA synthesis during remyelination.

Keywords :Melatonin; Diisopropylamine Dichloroacetate (DADA); Experimental Autoimmune Encephalomyelitis (EAE); fatty acids; multiple sclerosis; neuroinflammation; PDK4

1. Introduction

Multiple sclerosis (MS) is a neuroinflammatory disorder, characterized by the attack of immune cells to the central nervous system (CNS) resulting in myelin and axonal damage (Klocke *et al.* 2019). More than 2.2 million patients are suffering from MS worldwide (Wallin *et al.* 2019). MS is revealed by demyelinated axons (plaques) whose lipid structure is produced by oligodendrocyte cells in the CNS. Failure in oligodendrocyte production leads to a pathological situation with a wide range of disabilities and paralysis, depending on the amount and severity of axonal function (Trapp *et al.* 2009). Although the main causes of MS are still unknown, numerous factors have been proposed to affect disease progression. Among these, oxidative stress, mitochondrial dysfunction, and abnormality in fatty acid (FA) beta-oxidation have been proved to be involved in the disease state (Pastural *et al.* 2009; Senanayake *et al.* 2015; Jellinger 2010). FA synthesis within oligodendrocytes plays a key role in myelination and remyelination (Dimas *et al.* 2019). In fact, the current immunomodulatory treatments in MS managed only to slow down the progression of the disease and to reduce the number of relapses (Tillery *et al.* 2018). However, our group showed that FA synthesis in the remyelination process could be impaired following MS therapy, which seems to be one of the side effects of the current medications. Indeed, our previous animal study showed that while melatonin ameliorates the severity of the disease; however, its efficiency is reduced owing to its effect on FA synthesis, which is required for proper remyelination.

Melatonin is a hormone that is naturally synthesized by the pineal gland in the CNS in response to darkness as well as by the choroid plexus in a circadian-independent manner. Previous studies reported that melatonin increases oligodendrogenesis and modulates the function of the immune system (Ghareghani, Sadeghi, *et al.* 2017a; Ghareghani *et al.* 2019a). For instance, it reduces the amounts of pro-inflammatory cytokines (IL-1β and TNF) and increases those of anti-inflammatory cytokines (IL-4 and IL-10) (Ghareghani *et al.* 2019b).

The beneficial effects of melatonin in animal models of MS made it a potential candidate for clinical investigation on MS patients. Our previous study on the experimental autoimmune encephalomyelitis (EAE) animal model of MS showed that melatonin therapy ameliorates EAE symptoms. However, melatonin increases the levels of pyruvate

dehydrogenase kinase-4 (PDK-4) in the brain, which inhibits the function of the pyruvate dehydrogenase complex (PDC). The latter is known to be the main control point of energy metabolism which connects glycolysis to the tricarboxylic acid cycle (TCA) by producing NADH, FADH2, and subsequent oxidative phosphorylation. In addition, PDC is involved in acetyl-CoA production (Wu *et al.* 2018), which is a substrate for FA synthesis in the remyelination process. We showed that melatonin inhibits PDC and subsequently leads to a reduction in the substrate required for FA synthesis, which we suggested is a side effect of melatonin therapy. However, we observed that melatonin improved oligodendrogenesis and modulated the immune system function. Therefore, we proposed that during remyelination, oligodendrocytes use an alternative pathway to prepare enough substrate for FA synthesis. This alternative pathway appeared to be slower than the main FA synthesis pathway that is regulated by PDC (Ghareghani *et al.* 2019b).

The role of melatonin at the experimental level is still controversial. Melatonin is currently tested in a clinical trial for MS patients (ClinicalTrials.gov Identifier: NCT03498131). In the current study, we aimed to investigate whether an inhibitor of PDK4, diisopropylamine dichloroacetate (DADA), would improve the efficiency of melatonin therapy by minimizing the side effects of melatonin on FA synthesis.

2. Materials and Methods

i. Reagent or resource

EAE induction kit (Cat# EK-2110, Hooke Laboratories); melatonin (Cat#3550, Tocris Bioscience™); DADA (Cat#660-27-5, Tokyo Chemical Industry Co., Ltd. (TCI)); PDK4 (Cat# PA5-13776, Thermofisher); β-actin (Cat# sc-8432, Santa Cruz Biotechnology Inc); MOBP (Cat# WH0004336M1, SigmaAldrich); MBP (Cat# ab218011, Abcam); IL-1β (Cat#BMS6002, Thermofisher); TNF (Cat#BMS607-3, Thermofisher), IL-4 (Cat#BMS613, Thermofisher), IL-10 (Cat#BMS614INST, Thermofisher); Lactate (Ca#79-33-4, SigmaAldrich); N-acetylaspartate (Ca#997-55-7, SigmaAldrich).

ii. Animals

Adult female C57BL/6 mice (10-12 weeks old, 20-25 g) were purchased from Iran Pasteur Institute (Pasteur's Institute, Tehran, Iran). Mice were maintained at the animal breeding center under an artificial 12:12 light: dark cycle and pathogen-free conditions. The animal experimental procedures were carried out in accordance with the protocols of the Iranian Agriculture Ministry, which conforms to the provisions of the Declaration of Helsinki (as revised in Brazil in 2013), and of the European Communities Council Directive (86/609/EEC). All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Yasuj University of Medical Science (Protocol Permission number; IR.YUMS.REC.1395.2).

iii. Induction of EAE

EAE was induced using EAE's induction kit, according to the manufacturers' protocol. Briefly, the immunization solution containing the immunogenic epitope myelin oligodendrocyte glycoprotein-$^{35-55}$ (MOG$^{35-55}$) was emulsified with complete Freund's adjuvant (CFA, Sigma Aldrich) and Mycobacterium tuberculosis, and was injected subcutaneously over the flank. Booster pertussis toxin (PTX, 200 ng) was injected intraperitoneally on the day of immunization and 3 days later.

iv. Clinical EAE score

The weight of mice was evaluated daily, from day 7 post immunization until the end of study. In addition, mice were evaluated and scored for clinical signs of the disease from day 7 to day 23 post immunization, by at least 2 independent investigators, using 0-5 point scale (Nashold *et al.* 2013) as follows: 0: no clinical disease, 0.5: partial tail paralysis, 1.0: complete tail paralysis or limp tail, 1.5: complete tail paralysis and partial paralysis of one hind limb, 2.0: complete tail paralysis and partial paralysis of both hind limbs, 2.5: partial paralysis of one hind limb and complete paralysis of one hind limb, 3.0: paralysis of both hind limbs without forelimb weakness, 4.0: hind limbs and one forelimb paralysis, 5.0: moribund/dead signs.

v. Experimental groups

A total of 32 mice were randomly divided into 4 groups of 8 mice each, as follows: (A), Control mice treated with phosphate buffered saline (Ctrl-PBS); (B) EAE mice treated with vehicle PBS (EAE-PBS); (C) EAE mice treated with melatonin at a pharmacological dose of 10 mg/kg/day (EAE-Mel); (D) EAE mice treated with a combination of melatonin (10 mg/kg/day) and DADA (50 mg/kg/day, orally) (EAE-Mel-DADA). Treatment was initiated following induction of demyelination to the same extent in each mouse. This was attained when mice reached the score of ≥ 3, which corresponds to paralysis of both hind limbs without forelimb weakness. This allowed us to study the therapeutic effect of our drugs. The mice that showed early or late disease onset were excluded from the study. Clinical scores were recorded in a manner that was blinded to mouse groups. All treatments were performed between 8:00 and 9:00 AM, when the melatonin level is at its lowest. Mice were sacrificed at day 23 between 9:00 to 11:00 AM. Melatonin was freshly prepared by dissolving it in PBS and 5% dimethyl sulfoxide (DMSO). The pharmacological dose of melatonin and that of DADA were chosen based on previous studies (Hamdi 1998; Ghareghani, Sadeghi, *et al.* 2017b) (Yamane *et al.* 2014). Control, vehicle (EAE mice), and experimental groups all received the same percentage of 5% DMSO. The experimental procedures are summarized

in a schematic representation in **Figure 18A.** At the end of the study, mice were anesthetized, and brain tissues were quickly excised, immediately frozen on dry ice, and stored at −80◦ C until further use.

vi. Western Blotting

Briefly, western blotting steps were carried out as follows: brain frozen tissues were homogenized on ice and lysed in a lysis buffer. The contents of the lysing buffer included 50 mM Tris – HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P40, 0.1% SDS, 1 mM PMSF, and 100 mg/ml leupeptin. A Bio-Rad colorimetric protein assay kit (Bio-Rad, United States) was used to measure protein content. Then, proteins were separated on 8-15% SDS page. Next, gels were transferred to nitrocellulose membranes which were blocked in blocking buffer (5% skim milk solution) for 1 hour at room temperature. Then, primary antibodies were diluted in the blocking buffer and incubated at 4° C overnight on a shaker. Antibody target proteins included: Pyruvate Dehydrogenase Kinase isoform 4 (PDK4, 1:400), β-actin (1:1000), Myelin-associated oligodendrocytic basic protein (MOBP; 1:400) and myelin basic protein (MBP; 1:400). After washing steps, secondary antibodies coupled with horseradish peroxidase (HRP) were added and incubated at room temperature. An enhanced chemiluminescence detection system was used for detecting proteins bound to HRP conjugated antibodies. For measuring bands density, the MyImage software was used, and then relative images were quantified by image analysis software for gel documentation (LabWorks Software Version3.0, UVP Inc., United States).

vii. Enzyme-Linked Immunosorbent Assay (ELISA)

Brain samples were used to quantify the levels of pro-inflammatory cytokines IL-1β and TNF and anti-inflammatory cytokines IL-4 and IL-10 using ELISA kits according to the manufacturer's instructions (R&D Systems, and Abcam, United States).

viii. Real-Time PCR

Quantification of PDK4 and 3-hydroxy3-methylglutaryl-coenzyme-A reductase (HMGCR) genes was performed by quantitative real-time PCR (qRT-PCR), using a StepOne Real-Time PCR system (Applied Biosystems). Reagents and supplies included RealQ Plus 2x Master Mix Green (Ampliqon, Denmark), TaqMan primer/probe assays, TRI Reagent® solution (Sigma-Aldrich, the Netherlands), Sequence Detection System. To extract total RNA, Tri-Reagent was used following the manufacturer's instructions after homogenization of brain samples. On the other hand, the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, United States) was used in accordance with manufacturer's protocol to synthesize cDNA using random primers. Primer sequences were designed as follows: **PDK4**: F, CCGCTTAGTGAACACTCCTTC, and R, TCTACAAACTCT GACAGGGCTTT, **HMGCR**: F TGATTGGAGTTGGCACCAT, and R, TGGCCAACACTGACATGC. The specificity of PCR products was confirmed by melting curve analysis. The PCR was carried out as follows: initial activation at 95°C for 15 min, then 35 amplification cycles consisting of denaturation at 95°C for 15s, annealing at 57°C for 30s, and extension at 72°C for 30s. Data generated from the qPCR reactions were analyzed using the Comparative CT (ΔΔCT) method. All reactions were performed in triplicate using β-actin as an internal control for normalization.

ix. High-Performance Liquid Chromatography (HPLC)

Briefly, frozen samples of brain homogenates were centrifuged for 15 min at 40,000×g and supernatants were placed in an ice bath and neutralized to pH 4–5 with potassium hydroxide (KOH), and then pellets were weighed for analysis. Samples were centrifuged for another 15 min at 40,000 × g for 15 min to sediment the precipitant, potassium perchlorate (KClO4), which formed after neutralization with KOH. Supernatants were retained and filtered (0.2 µm) before lyophilization. The chromatographic measurements of brain lactate and N-acetylaspartate (NAA) were carried out with a KNAUER Smartline High Performance Liquid Chromatography (HPLC) system equipped with micro vacuum degasser, LPG system, UV-VIS Detector (2550 was set at 220 nm) and a MZ ODS-C18 (250 mm × 4.6 mm, 5 µm) column. The EZCHROM elite system was used for chromatographic calculations.

Determination of lactate and NAA were performed by HPLC, as previously described (Kehr, 1999; Shannon et al., 2016). The accuracy of extraction and determination of lactate and NAA in the brain were investigated using the standard addition method.

x. PDC Activity Determination

Pyruvate dehydrogenase complex (PDC) exists in two forms: active or dephosphorylated as well as inactive or phosphorylated forms. Inter-conversion between these forms can readily alter the flux through this complex. Both active and total PDC activities were measured based on the evolution or fixation of $^{14}CO_2$ [^{1-14}C] on ice freeze-thawed homogenates of brains of all groups on day 23, as described previously (Johnson et al., 2001; Pliss et al., 2004, 2013). Enzymatic activity of PDC in tissue was determined. For 'active' PDC activity, dichloroacetate as an inhibitor of PDH kinases and sodium fluoride as an inhibitor of PDH phosphatases were used in the homogenizing buffer to preserve the phosphorylation status for active PDC activity assessment; however, purified PDH phosphatase 1 was used in the homogenates to reach the complete dephosphorylation of PDH for total activity assessment of PDC. PDC activity is expressed as munits/mg protein.

xi. Data analysis

All groups were blinded to the experimenters for all the quantifications. Results are expressed as mean ± SD (standard deviation). The data distribution was analyzed by the Shapiro–Wilk normality test in addition to Brown–Forsythe which was used to check the homogeneity of variance for ANOVA test analyses. All data presented in the manuscript passed both tests and were analyzed as normally distributed and with equal variances. Descriptive and inferential statistics was applied to the data using GraphPad Prism version 6.01 (San Diego, CA, United States). P values less than 0.05 ($p<0.05$) were considered to be statistically significant. Significance is indicated by $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, and$****p < 0.0001$.

3. Results

i. Combination therapy of melatonin and DADA ameliorated the disability scores of EAE mice

The disease course in the EAE mouse model exhibits a chronic progressive-relapsing phenotype. To assess the combination therapy of melatonin and DADA on EAE severity, mice were treated by daily *i.p.* injections of melatonin at pharmacological doses (10 mg/kg/day) or DADA (50 mg/kg/day) or their combination, from day 13 post first immunization until the end of the study at day 23. Results of neurological disability scores showed lower scores in the whole experimental period for melatonin treatment (EAE+Mel) compared to the vehicle group (EAE+PBS). However, DADA did not have any effect when administered alone (EAE+DADA). On the other hand, combination therapy of melatonin and DADA (EAE+Mel+DADA) considerably ameliorated and reduced EAE disability scores all the days, compared to the melatonin group alone **(Figure 19. B)**.

ii. Combination therapy modulated the neuroinflammation

To investigate the role of neuroinflammation in combination therapy-induced amelioration of the disease, key cytokines of both pro-inflammatory (IL-1β and TNF) and anti-inflammatory (IL-4 and IL-10) pathways were evaluated by ELISA **(Figure 20)**. Analysis of variance showed that the effect of combination therapy was significant for IL-1β, ($F(4, 35) = 15.27$, $p<0.000001$); TNF ($F(4, 35) = 19.88$, $p <0.000001$), IL-4 ($F(4, 35) = 23.814$, $p<0.000001$), and IL-10 ($F(4, 35) = 28.18$, $p<0.000001$).

Indeed, brain levels of pro-inflammatory cytokines IL-1β (275.5±46.75) and TNF (434.1±61.36) were significantly (***p=0.000266 and ****p=0.000002, respectively) higher in EAE mice, compared with controls (156.1±31.35 and 263.9±54.31, respectively) **(Figure 20A and 20B**). EAE mice treated with DADA alone did not cause any effect on pro-inflammatory markers, compared to PBS treated-EAE mice, at day 23 **(Figure 20A and 20B**). However, melatonin therapy alone significantly reduced the expression of inflammatory cytokines of IL-1β (201.6± 51.06; *p= 0.039767) and TNF (339.5±53.27; **p= 0.008897), in comparison to EAE mice. Importantly, combination therapy of melatonin and

DADA significantly reduced even further the expression levels of IL-1β (125.6± 40.44; ****p=0.000007) and TNF (254.0±44.55; ****p=0.000002), in comparison to the control EAE group, but also compared to melatonin alone (*p=0.032395 and *p=0.021640, respectively; **Figure 20A and 20B).**

On the other hand, the levels of IL-4 (50.625±19.95; ****p=0.000001) and IL-10 (73.38±19.99; ****p=0.000001) in untreated EAE mice were significantly decreased compared with controls (208.75±69.09 and 281.9±84.55, respectively) **(Figure 20C and 20D**). Treatment with DADA alone did not affect the expression levels of these anti-inflammatory cytokines. However, treatment with melatonin alone significantly increased the expression of IL-4 (124.88±30.42; *p= 0.013284) and IL-10 (180.5±54.99; **p= 0.001381), in comparison to EAE mice (**Figure 20C and 20D**). Importantly, the combination treatment of melatonin and DADA significantly increased even further the expression levels of IL-4 (201.88±61.33; ****p=0.000001) and IL-10 (256.1±23.39; ****p=0.000001), in comparison to the control EAE group, but also compared to melatonin alone (**p= 0.009537 and *p= 0.037946, respectively).

In summary, these results indicate that the beneficial effect of a combination treatment of melatonin and DADA in EAE mice is better than melatonin alone and is linked to an inhibition of inflammatory cytokines and an enhanced production of anti-inflammatory cytokines. Since we did not observe any effect of DADA therapy alone in EAE disability scores, or inflammatory and anti-inflammatory cytokines levels, therefore, the DADA group alone was not maintained in subsequent experiments.

iii. Combination therapy potentiated the remyelination process

To test whether the combination therapy affects the protein expression levels of mature oligodendrocytic markers, brain lysates were used to perform western blot analysis on myelin basic protein (MBP) and myelin-associated oligodendrocytic basic protein (MOBP) (**Figure 21A)**. One-way ANOVA analysis of variance showed the significant impact of combination therapy on MBP ($F(3, 28) = 38.31$, $p<0.0001$) and MOBP ($F(3, 28) = 32.13$, $p<0.0001$). Results showed a significant decrease in protein expression levels of MBP (0.2463±0.12;

****p=<0.0001) and MOBP (0.3438±0.15; ****p=<0.0001) in untreated EAE mice, compared to controls (1.349±0.27 and 1.684±0.33, respectively), demonstrating the loss of oligodendrocytes following EAE induction **(Figure 21B and 21C**). However, melatonin treatment resulted in a significant increase in protein expression levels of MBP (0.6750±0.18; **p=0.0033) and MOBP (0.8900±0.17; **p=0.0056), compared to the untreated EAE group. Importantly, combination therapy of melatonin and DADA significantly increased protein levels of MBP (1.114±0.27; ****p<0.0001) and MOBP (1.456±0.43; ****p<0.0001) in comparison to EAE mice, but also in comparison to melatonin alone (**p= 0.002640 and **p= 0.004009, respectively) (**Figure 21B and 21C**). Therefore, administration of the combination drugs melatonin and DADA appears to induce the expression of oligodendrocytic proteins in EAE mice and to recover oligodendrocytes reduction better than melatonin treatment alone.

iv. Combination therapy modulates the cerebral energy metabolism

It has been demonstrated that lactate can be utilized by PDC activity (Parikh *et al.* 2009) and that NAA can be converted to acetyl-CoA in oligodendrocytes, which is required for FA synthesis in myelin (Chakraborty *et al.* 2001). Therefore, we measured brain lactate and NAA, using HPLC, in order to assess the effect of combination therapy on brain metabolism and mitochondrial function which indicated the significant effect of combination therapy on lactate ($F(3, 28) = 9.716$, $P=0.000147$) and NAA ($F(3, 28) = 9.400$, $P=0.0002$). Assessment of brain lactate levels showed a rising tendency in EAE mice, compared with controls (0.9425±0.37 vs 0.7188±0.44), however statistical significance (p=0.653479) was not reached (**Figure 22A**). Administration of melatonin resulted in a significant increase in brain lactate concentrations, in comparison to untreated EAE mice (1.706±0.39; **p<0.01) (**Figure 22A**). However, combination therapy of melatonin and DADA caused a significant reduction in lactate levels (1.075±0.32; **p=0.013737), compared to melatonin treatment alone (**Figure 22A**).

On the other hand, NAA brain concentrations significantly decreased in untreated EAE mice, compared to the control group (1.013±0.69 vs 2.659±0.4548; ****p<0.0001, **Figure 22B**). However, melatonin treatment alone (1.900±0.44; *p=0.0384) or in combination with

DADA (1.975±0.81; *p=0.0220) significantly increased NAA levels, compared to untreated EAE mice (*p<0.05, **Figure 22B**). Combination therapy was not better than melatonin alone in increasing NAA levels (p=0.9950). Together, these results demonstrate that combination therapy of melatonin and DADA has an effect on cerebral energy metabolism.

v. Combination therapy restores HMGCR expression in the brain

The effects of combination treatment on cholesterol biosynthesis were assessed by monitoring HMGCR enzyme activity. One-way ANOVA analysis of variance showed a significant alternation of the HMGCR ($F(3, 28) = 17.13$, $P<0.0001$). Results showed that HMGCR mRNA levels were significantly reduced in EAE mice, compared to the control group (0.4475±0.17 vs 1.223±0.28; ****p<0.0001) (**Figure 23**). In contrast, melatonin therapy alone (0.9338±0.25) or in combination with DADA (1.150±0.23) significantly increased HMGCR levels, compared with untreated EAE mice (**p=0.0019 and ****p<0.0001, respectively) (**Figure 23)**. However, the difference between melatonin (EAE+Mel) and the combination treatment (EAE+Mel+DADA) was not significant (p=0.2895), although combination therapy considerably increased the potential of melatonin in restoring HMGCR expression.

vi. Melatonin therapy increased PDK4 expression, but not combination therapy

The effect of combination therapy on PDK4 mRNA and protein expression levels was then investigated by quantitative real time-PCR and Western blot, respectively, and analyzed with one-way ANOVA (mRNA level; $F(3, 28) = 19.45$, $P<0.0001$ and protein level; $F(3, 28) = 19.45$, $P<0.0001$). Induction of EAE mice did not affect PDK4 mRNA and protein expression levels compared with controls, at day 23 (p= 0.754325 and p= 0.897514) (**Figure 24A and 24B**). However, melatonin treatment resulted in a significant ~2-fold increase in PDK4 mRNA (1.400±0.25) and protein expression levels (1.258±0.27), compared with untreated EAE mice (***p=0.000156 and **p=0.002260; respectively) (**Figure 24A and Figure 24B**). In contrast, combination therapy of melatonin and DADA did not have any

added effect, compared to melatonin treatment alone (p=0.991453 and p=0.990333; respectively**) (Figure 24A and Figure 24B**).

vii. Combination therapy eliminated the inhibitory effect of PDK4 on PDC activity

Given that PDK4 controls the activity of PDC, PDC activities were assessed in both "Active" and "Total" states, in brain homogenates at day 23 using one-way ANOVA for data analysis ($F(3, 28) = 26.54$, $P<0.0001$). Results showed a significant increase (***$p < 0.001$) in active (10.33±2.16) and total (12.74±2.14) PDC activities in untreated EAE mice, compared with the control group (5.363±1.87 and 7.213±2.62, respectively) **(Figure 25A and 25B)**. On the other hand, this increase in PDC activity was inhibited by melatonin. Indeed, melatonin caused a significant decrease, by ~3.6 and ~2.6 –fold respectively, in active (2.83±0.86) and total (4.95±2.01) PDC activities, in comparison to untreated EAE mice. These inhibitory effects of melatonin were significantly abrogated by combination therapy of melatonin and DADA. Indeed, the combination treatment significantly increased active (11.70±3.42; ****$p<0.0001$) and total (14.13±3.49; ****$p<0.0001$) PDC activities, compared with melatonin-treated EAE mice **(Figure 25A and 25B).**

4. Discussion

In this study, we investigated the effect of a combination therapy of melatonin and DADA, a PDK4 inhibitor, on the metabolic profile of FA synthesis in EAE mice. Our results showed that combination therapy significantly reduced EAE disability scores, compared to melatonin, whereas DADA alone did not have any effect. In addition, co-therapy decreased pro-inflammatory while increasing anti-inflammatory cytokines, significantly better than melatonin alone. Moreover, combination drugs recovered the expression of oligodendrocytic markers, more potently than melatonin. Furthermore, co-therapy affected cerebral energy metabolism by significantly reducing lactate levels while increasing NAA and HMGCR levels. Finally, co-therapy significantly restored PDC function while reducing the lactate levels in comparison to melatonin which increased lactate and PDK4 levels while reducing PDC activity. In summary, the administration of melatonin with DADA increased the

efficiency of melatonin treatment by eliminating the inhibitory effects of PDK4 on PDC function, a critical step for proper FA synthesis during remyelination.

The immune system is influenced by melatonin, a master circadian rhythm hormone in the body. However, the effects of melatonin are sometimes contradictory since they depend on several factors including age, sex, species of animals, time and method of melatonin administration, and various stressor factors. For instance, while most studies on animal models of MS reported that melatonin modulated the immune system and improved oligodendrogenesis (Kang *et al.* 2001; Álvarez-Sánchez *et al.* 2015; Chen *et al.* 2016; Wen *et al.* 2016; Dokoohaki *et al.* 2017; Ghareghani, Scavo, *et al.* 2018a; Ghareghani, Zibara, *et al.* 2018; Long *et al.* 2018; Ramos González *et al.* 2018; Ghareghani *et al.* 2019a; Mahmoodi *et al.* 2020), our previous study on Lewis rats showed an age-dependent effect of melatonin in EAE treatment since treating young EAE rats with melatonin (10 mg/kg/d) exacerbated the severity of EAE. However, as a drawback of the study, melatonin was administered for a short period of 8 subsequent days (Ghareghani, Dokoohaki, *et al.* 2017a). In fact, longer treatment periods and different ages are suggested for a better understanding of the age-dependent effect of melatonin. Consistent with this observation, another study showed that inhibition of the direct effects of melatonin on cells, using Luzindole as an antagonist of melatonin receptors, suppressed EAE development after immunization. Therefore, it has been proposed that the immune-enhancing effects of melatonin in demyelination may be suppressed by the inhibition of melatonin receptors (Constantinescu *et al.* 1997). In the current study, we showed the beneficial role of melatonin in immune cell modulation and in increasing the remyelination process. Indeed, treatment of EAE mice by melatonin resulted in a significant reduction in pro-inflammatory cytokines IL-1β and TNF and a significant increase in anti-inflammatory cytokines IL-4 and IL-10, which was associated with an elevation in protein levels of oligodendrocyte markers MBP and MOBP.

On the other hand, the current study showed that melatonin treatment affects the key enzymes involved in FA synthesis and is required for proper myelin synthesis. Although no significant difference in PDK4 expression level was observed following EAE induction, the activity of PDC increased significantly, which reflects the natural potential of mice brains in pathological conditions of myelin loss for self-regeneration. This increase in PDC was impaired by melatonin. Indeed, melatonin administration increased the PDK4 levels both at

the transcriptional and translational levels in EAE mice while reducing the PDC activity. This observation was in line with our previous study (Ghareghani *et al.* 2019b), and that of Sharman et al. who also reported an increase in PDK4 mRNA levels in the CNS of mice following melatonin treatment (Sharman *et al.* 2007). We suggest that this alteration in PDK4 level and PDC activity is in fact a side effect of melatonin therapy.

On the other hand, we previously measured the level of lactate in the brain of EAE Lewis rats and we proposed lactate as a potential biomarker in the diagnosis of MS progression (Ghareghani, Dokoohaki, *et al.* 2017b). However, lactate levels in EAE mice in the current study were higher than in control mice, although not statistically significant. It is noteworthy that a clinical study on MS patients already showed that serum lactate levels is 2.8 times higher in MS than in controls (Amorini *et al.* 2014). While melatonin exacerbated the severity of monophasic EAE in young Lewis rats, highlighting the age-dependent action of melatonin, this exacerbation in EAE symptoms was associated with an increase in lactate levels (Ghareghani, Dokoohaki, *et al.* 2017b). Since boosting PDC activity leads to a reduction in lactate accumulation (Parikh *et al.* 2009), this study demonstrated that a reduction in PDC activity, caused by an increase in PDK levels following melatonin therapy, was accompanied by accumulation of lactate in the brain. Considering the fact that pyruvate in oligodendrocytes, obtained from the blood or lactate and glucose conversion, needs first to be converted to acetyl-CoA (Fransen *et al.* 2017), we hypothesized that inhibition of PDC, because of PDK elevation, limits the pyruvate conversion to acetyl-CoA, which leads to the accumulation of pyruvate (Ghareghani *et al.* 2019b). This acetyl-CoA is required to produce metabolites for FA synthesis. While it is already suggested that both aerobic and anaerobic glycolysis convert pyruvate to lactate (Schurr *et al.* 2007), this could explain why PDC inhibition by melatonin increases lactate levels.

In addition to the role of PDK and PDC in FA synthesis, cholesterol is another factor that is located in myelin sheaths and cell membranes of neurons and astrocytes (Snipes *et al.* 1997). The synthesis of cholesterol is regulated by the key enzyme HMGCR. It is already suggested that cholesterol synthesis is limited during demyelination; however, its synthesis is upregulated during remyelination as it is required for myelin synthesis (Lavrnja *et al.* 2017). Investigations on EAE and lysolecithin demyelination models showed that HMGCR expression is downregulated at the peak of the disease (Mueller *et al.* 2008; Raddatz *et al.*

2016; Lavrnja *et al.* 2017) and that this is associated with a reduction in the expression of myelin proteins (Mueller *et al.* 2008). Consistent with these observations, we demonstrated that while HMGCR expression is reduced in EAE mice, compared to controls, it was correlated with demyelination, as reported by quantifying the expression of oligodendrocyte markers MBP and MOBP. In addition, we observed that melatonin restored HMGCR expression as well as MBP and MOBP. This suggests that increased HMGCR expression is an indicator of ongoing remyelination which needs more cholesterol to be synthesized by HMGCR.

Although melatonin reduced the activity of PDC, which is required for proper and efficient FA synthesis during remyelination; however, it still improved EAE severity. We considered this suppressive effect of melatonin on PDC as a side effect of melatonin, caused by using a pharmacological dose of melatonin; hence, we tried to minimize this side effect in the EAE model by combination therapy with DADA. DADA, also called "Vitamin B15" is the active component of many formulations of pangamic acid (Gelernt *et al.* 1982). DADA acts as a safe inhibitor of PDK4 and its affinity to PDK4 is 12.5-fold more than other isoforms of PDK such as PDK2 (Yamane *et al.* 2014). There are limited studies regarding DADA's effects on the body. An experimental study in a severe influenza model in mice showed the high potential of DADA in reducing the cytokine storm including IL-2, IL-6, IFN-α, TNF, and IFN-γ, in addition to its role in restoring the down-regulated PDH activity caused by influenza (Yamane *et al.* 2014).

In the current study, we observed no difference between EAE mice and DADA-treated EAE mice regarding clinical scores and cytokines expression. The most possible explanation for this observation could be the unchanged level of PDK4 in EAE mice, which does not involve PDK4 modulation as a potential factor for treatment. However, melatonin increased the PDK4 level, which highlights the possible effect of PDK4 modulation in EAE progression following melatonin therapy. Indeed, DADA potentiated the beneficial effects of melatonin on reducing the pro-inflammatory cytokines (IL-1β and TNF) and increasing the anti-inflammatory cytokines (IL-4 and IL-10). In fact, DADA therapy alone would not be a potential therapeutic candidate for ameliorating EAE symptoms. DADA improved the potential of melatonin and increased oligodendrogenesis which was assayed by protein expressions of MBP and MOBP. Lactate levels, but not NAA and HMGCR, were increased

by melatonin treatment, however, they were reduced after combination therapy with melatonin and DADA.

On the other hand, NAA, a nervous system-specific metabolite (Moffett *et al.* 2007) whose role in remyelination has not been fully elucidated yet, is known to get transferred into oligodendrocytes, converted to acetate and then acetyl-CoA, a substrate for FA and cholesterol synthesis. This seems to be the alternative pathway that is potentiated following the decrease in PDC activity. This alternative pathway appeared to be slower than the main FA synthesis pathway regulated by PDC. Given that the measurement of axonal injury can be carried out by magnetic resonance spectroscopy of NAA for quantification of the resonance intensity of a neuronal marker (Narayanan *et al.* 2001), we showed here that NAA significantly decreased flowing EAE induction. Similarly, previous studies reported a reduction of brain NAA in MS and which precedes neuronal atrophy (Moffett *et al.* 2007; Cader *et al.* 2007). Although melatonin slowed down the main pathway of FA synthesis by inhibition of PDC activity, leading to lower levels of acetyl-CoA synthesis required for FA synthesis; however, melatonin caused an increase in NAA, which is another source of acetyl-CoA production mainly exported from neuron to oligodendrocytes. This can partly explain why melatonin still increases the remyelination process despite its inhibitory role in PDC activity. However, adding DADA to melatonin therapy did not boost NAA levels. Therapeutically, it is reported that treatments of MS patients with interferon beta-1b, fluoxetine, and glatiramer acetate were associated with partial recovery of NAA levels (Narayanan *et al.* 2001; Mostert *et al.* 2006; Khan *et al.* 2005).

Although there is a limited number of clinical studies regarding PDK/PDC axis in MS patients, Nijland and colleagues' demonstrated increased expression of axonal PDC in demyelinated lesions of MS patients and suggested that glucose metabolism is increased in axonal lesions (Nijland *et al.* 2015). Consistent with these data, we showed an increased PDC activity following EAE induction.

In the current study, we examined the alternation of many factors, especially PDK4 and PDC, in whole-brain homogenates. However, to uncover the exact source of PDK4 and PDC changes, it would be crucial to investigate these factors in specific cell populations including oligodendrocytes, microglia, and astrocytes. Interestingly, while the main pathway of acetyl-

CoA production in oligodendrocytes was thought to be PDC, whose function is under the control of PDK-4, a study using impairment of PDC activity in oligodendrocytes showed that inhibition of acetyl-CoA production through the PDC pathway is not necessary for myelin maintenance (Della-Flora Nunes *et al.* 2017). One limitation of the current study is the need to assess PDC when cells require more acetyl-CoA for the synthesis of the new myelin sheets, which necessitates investigating PDC in pathological conditions of demyelination. Furthermore, the source of acetyl-CoA used by oligodendrocytes for the maintenance of myelin was not explored. In fact, high amounts of fatty acids could be provided by oligodendrocytes or dietary sources, in cases of deficiency of fluxed fatty acids from astrocytes (Camargo *et al.* 2017).

5. Conclusion

Although melatonin therapy ameliorates the severity of EAE by modulating the immune system function and by increasing oligodendrogenesis, it reduces the activity of PDC through PDK4 elevation, which seems to slow down the FA synthesis required for re-myelination in EAE. This inhibition of PDC activity by melatonin was eliminated by combination therapy with DADA, which inhibits the PDK4 activity and hence does not allow PDK4 to inhibit PDC. This potentiated the beneficial role of melatonin in EAE therapy. The main findings of this study have been summarized as schematic models in **Figure 26.** To date, no clinical studies in MS patients have investigated the PDK/PDC axis in lesions of different MS types. Further experimental and clinical studies are required to elucidate the function of this axis in MS pathogenesis in the aim of finding a new therapeutic strategy.

6. Declarations

Funding: This work was supported by a grant from Lebanese University (KZ).

Conflicts of interest/Competing interests: The authors declare no conflict of interest.

Availability of data and material: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability: Not applicable

Authors' contributions: All authors contributed to the acquisition and analysis of data. MG and KZ, SR contributed to the conception, design of the study, writing-original draft, and writing-review & editing. All authors read and approved the final version of the manuscript.

Ethics approval: The animal experimental procedures were carried out in accordance with the protocols of the Iranian Agriculture Ministry, which conforms to the provisions of the Declaration of Helsinki, and of the European Communities Council Directive (86/609/EEC). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Yasuj University of Medical Science.

Consent to participate: Not applicable

Consent for publication: Not applicable

Acknowledgments: This work was supported by a grant from Lebanese University (KZ).

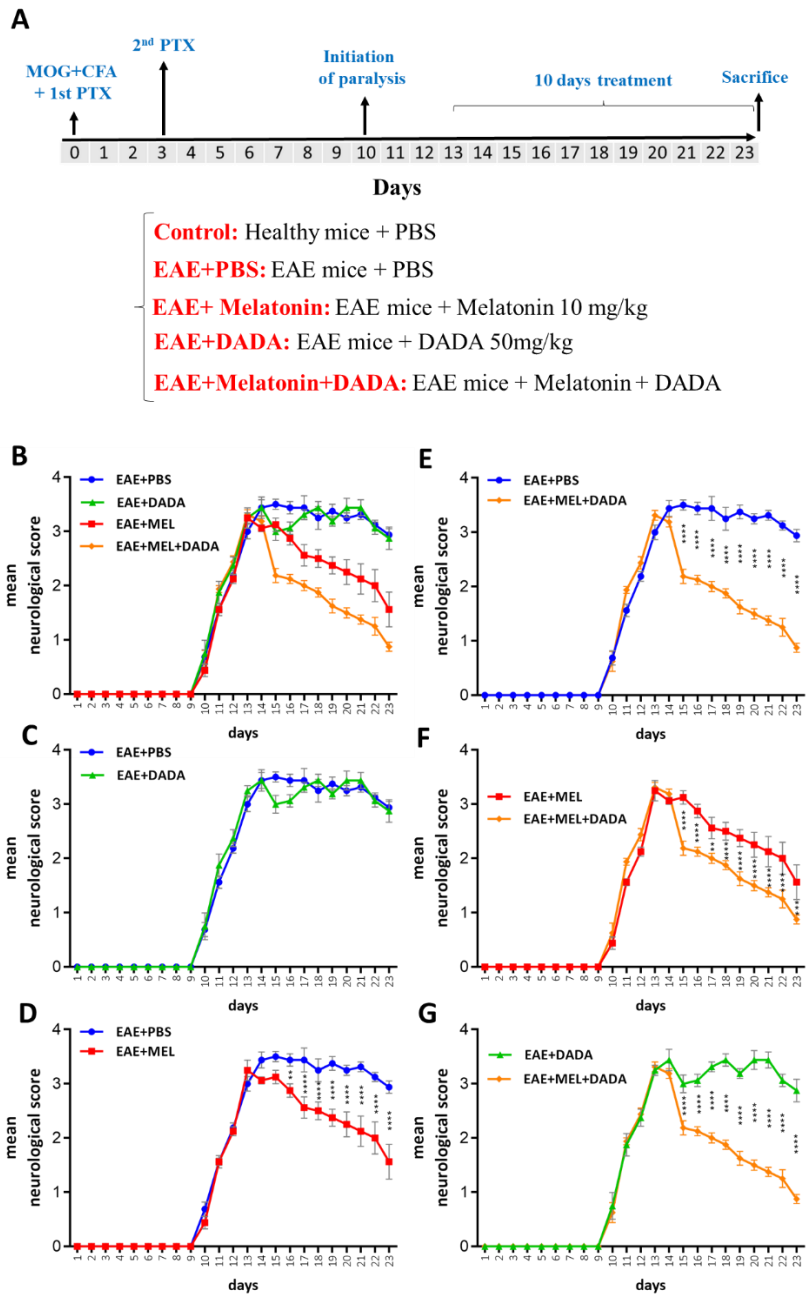


Figure 19: (A) Schematic representation of experiment procedures and (B-G) daily assessment of EAE severity. Neurological disability analysis displayed an amelioration in EAE disease severity in melatonin (EAE+MEL) and combined therapy of melatonin and DADA (EAE-MEL+DADA), but not DADA alone. Treatment was initiated from day 13 post-immunization for 10 consecutive days. (n=8 mice/group)

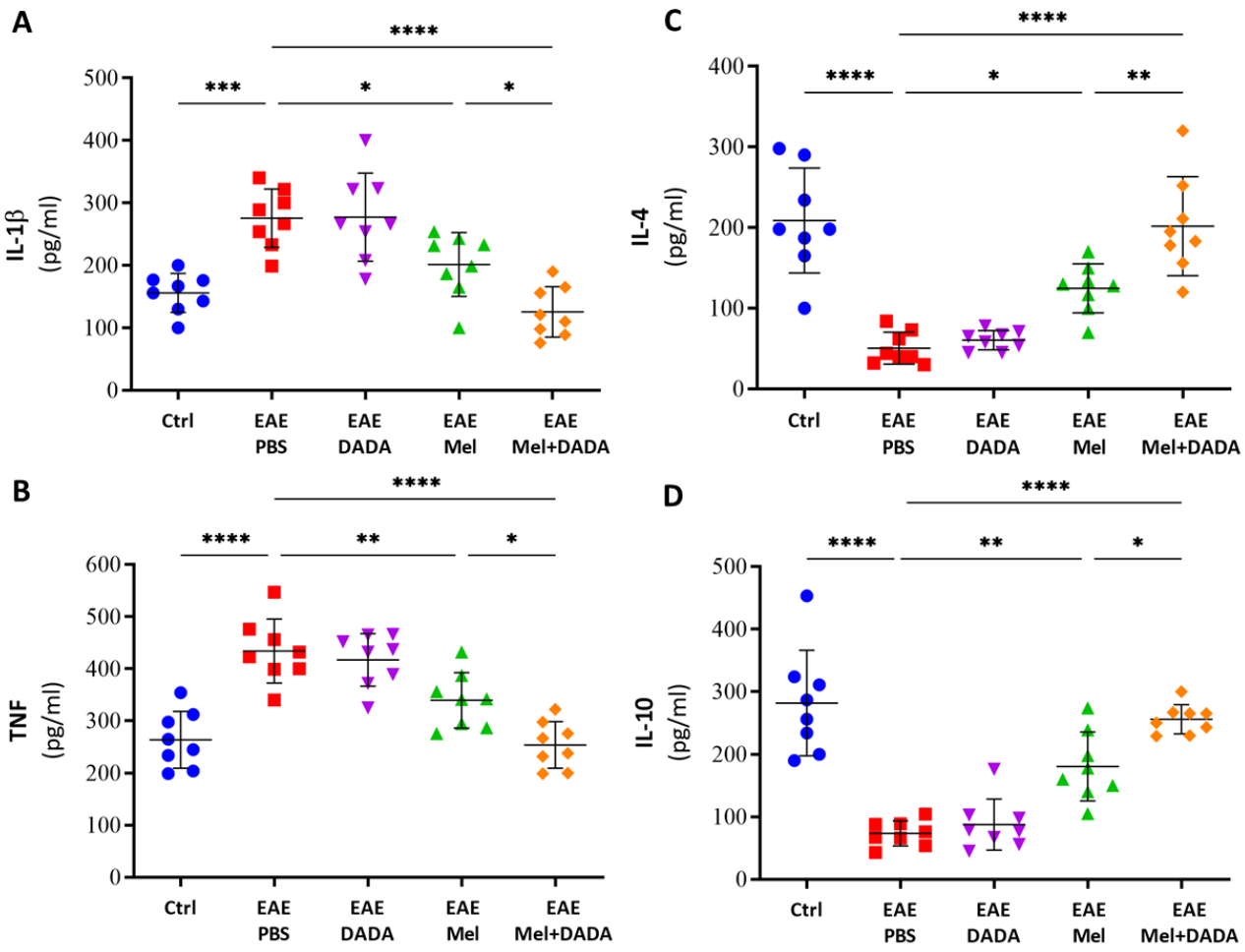
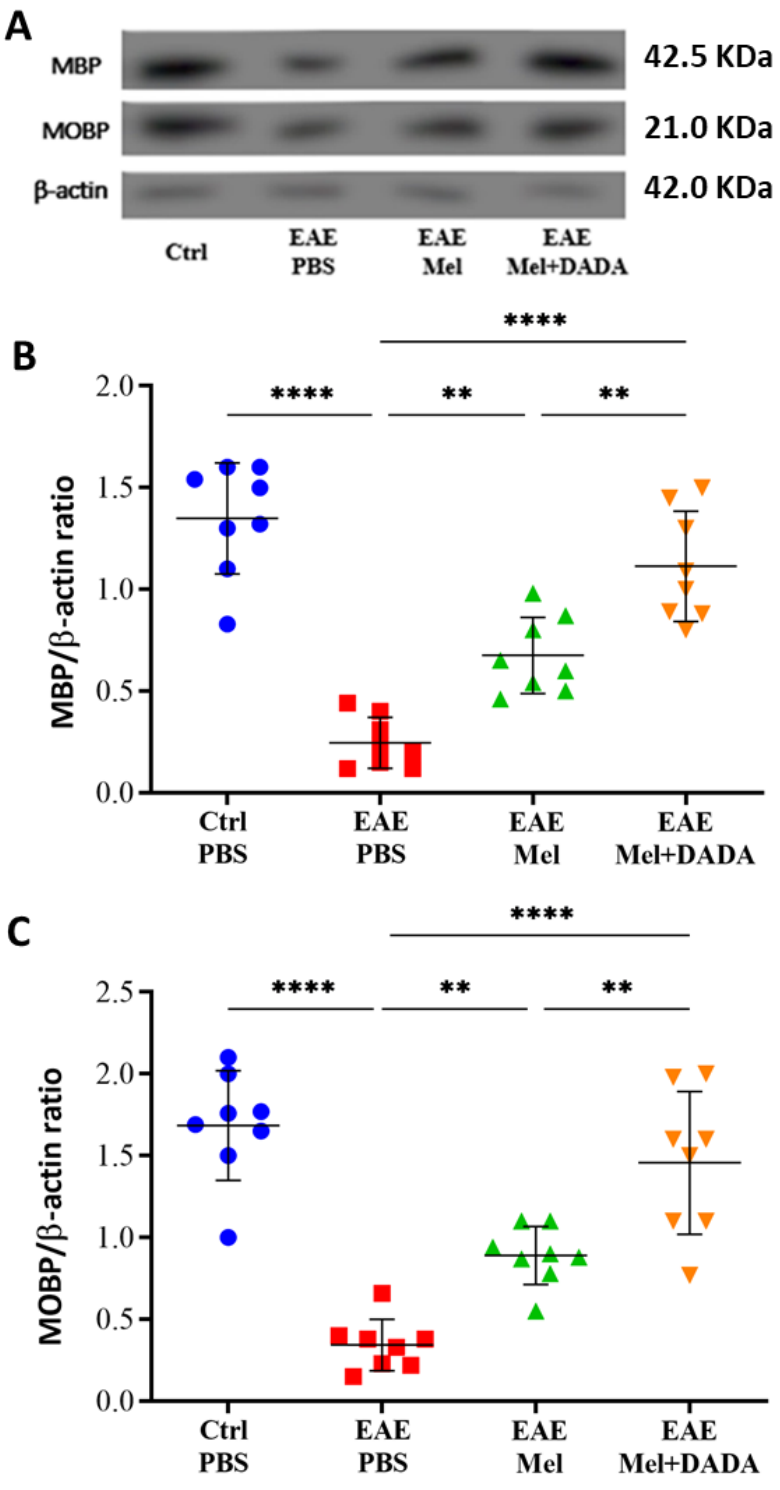


Figure 20: The effects of melatonin, DADA, and combination therapy on cytokine levels in the brain. Brain homogenates were used to quantify the levels of pro-inflammatory cytokines **(A)** IL-1β and **(B)** TNF and anti-inflammatory cytokines **(C)** IL-4 and **(D)** IL-10. Significance is indicated by $*p < 0.05$; $**p < 0.01$; and $****p < 0.0001$.


A
MBP
MOBP
β-actin
42.5 KDa
21.0 KDa
42.0 KDa
Ctrl
EAE PBS
EAE Mel
EAE Mel+DADA
B
MBP/β-actin ratio
2.0
1.5
1.0
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Ctrl PBS
EAE PBS
EAE Mel
EAE Mel+DADA
C
MOBP/β-actin ratio
2.5
2.0
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1.0
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0.0
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Ctrl PBS
EAE PBS
EAE Mel
EAE Mel+DADA

Figure 21: Oligodendrocyte's expression levels. (A) Western blot analysis of myelin basic protein (MBP) and myelin-associated oligodendrocytic basic protein (MOBP) in the brain. The two markers (MBP and MOBP) were run alongside the same β-actin as housekeeping control. **(B and C)** Quantitative analysis for MBP and MOBP proteins, respectively. Values are expressed as the Mean±SEM. Each group included 8 mice (n = 8). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test. Significance is indicated by $*p < 0.05$; $**p < 0.01$; and $****p < 0.0001$.

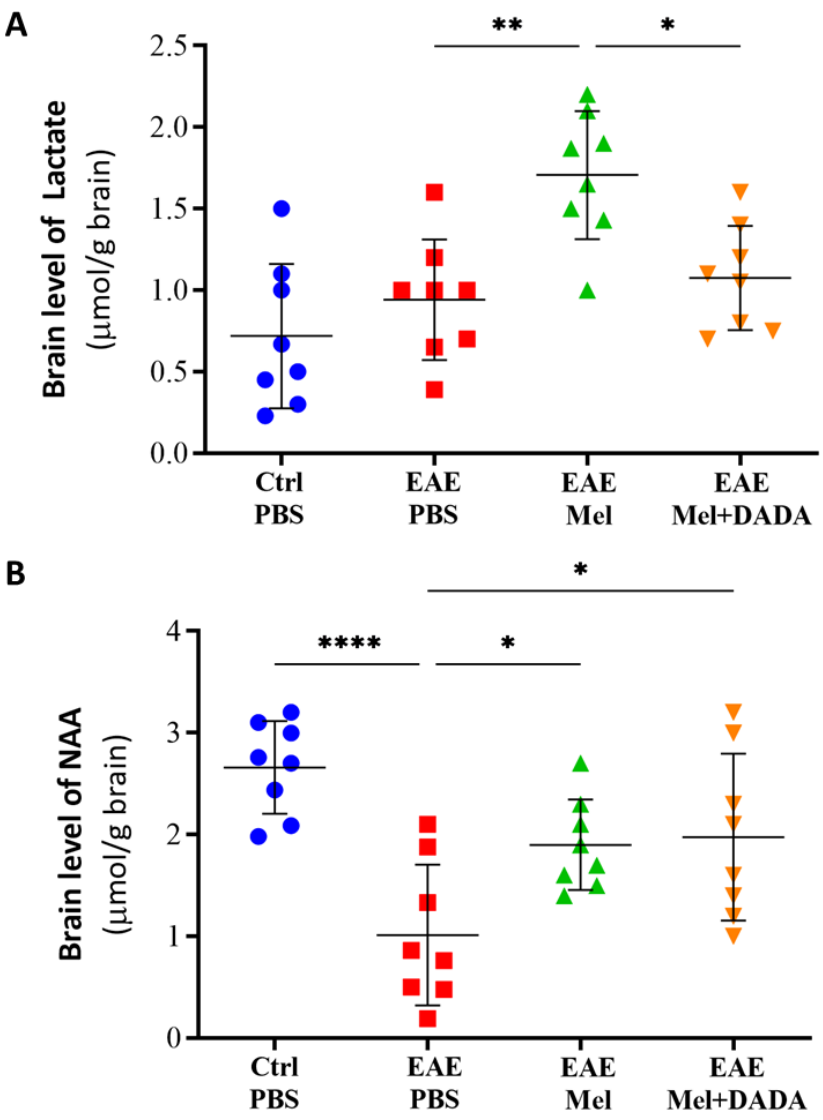

Figure 22: Brain levels of (A) lactate and (B) N-acetylaspartate (NAA), using HPLC. Values are expressed as the Mean±SEM. Each group included 8 mice (n = 8). Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Tukey's test. Significance is indicated by ∗p < 0.05; ∗∗p < 0.01; and ∗∗∗∗p < 0.0001.

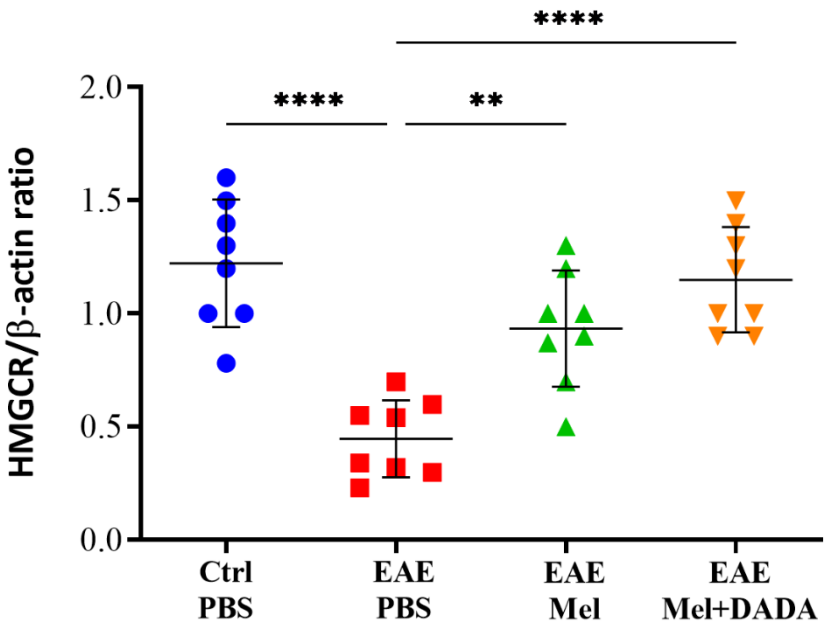

Figure 23: mRNA expression levels of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR) in brain homogenates. β-actin was used as an internal control. Quantification of HMGCR expression levels was normalized to controls. Values are expressed as the Mean±SEM. Each group included 8 mice (n = 8). Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Tukey's test. Significance is indicated by $*p < 0.05$ and $***p < 0.001$.

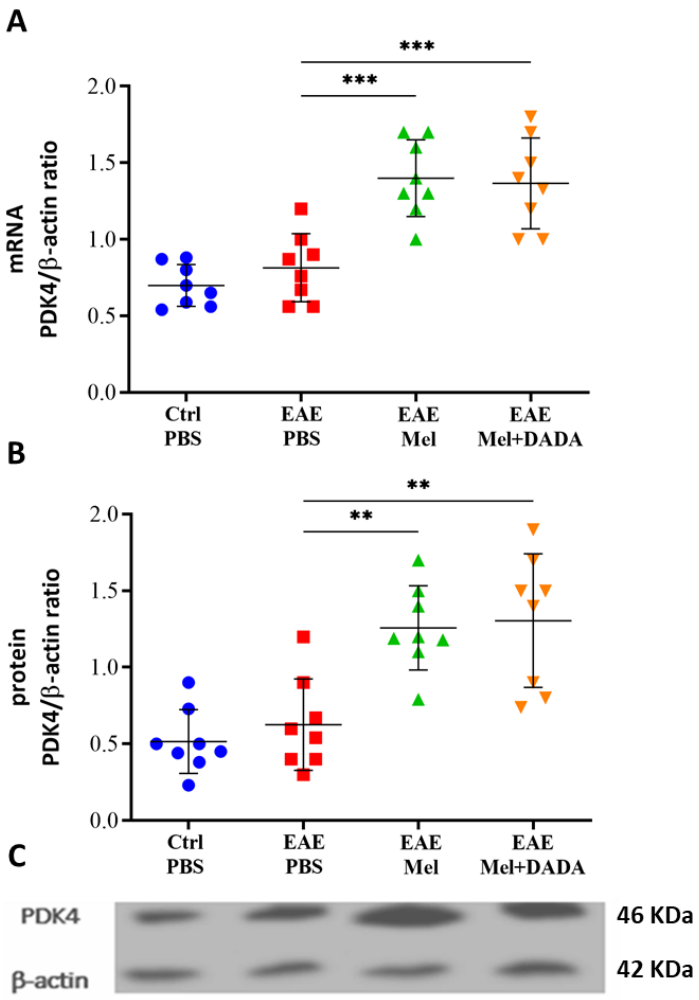

Figure 24: Expression of PDK4. PDK4 levels after administration of melatonin with DADA in brain homogenates **(A)** mRNA expression levels of PDK4 assessed by real time-PCR. **(B)** Protein expression levels of PDK4 assessed by western blot. β-actin was used as an internal control for normalization for both real time-PCR and western blot. PDK4 expression levels was normalized to controls.

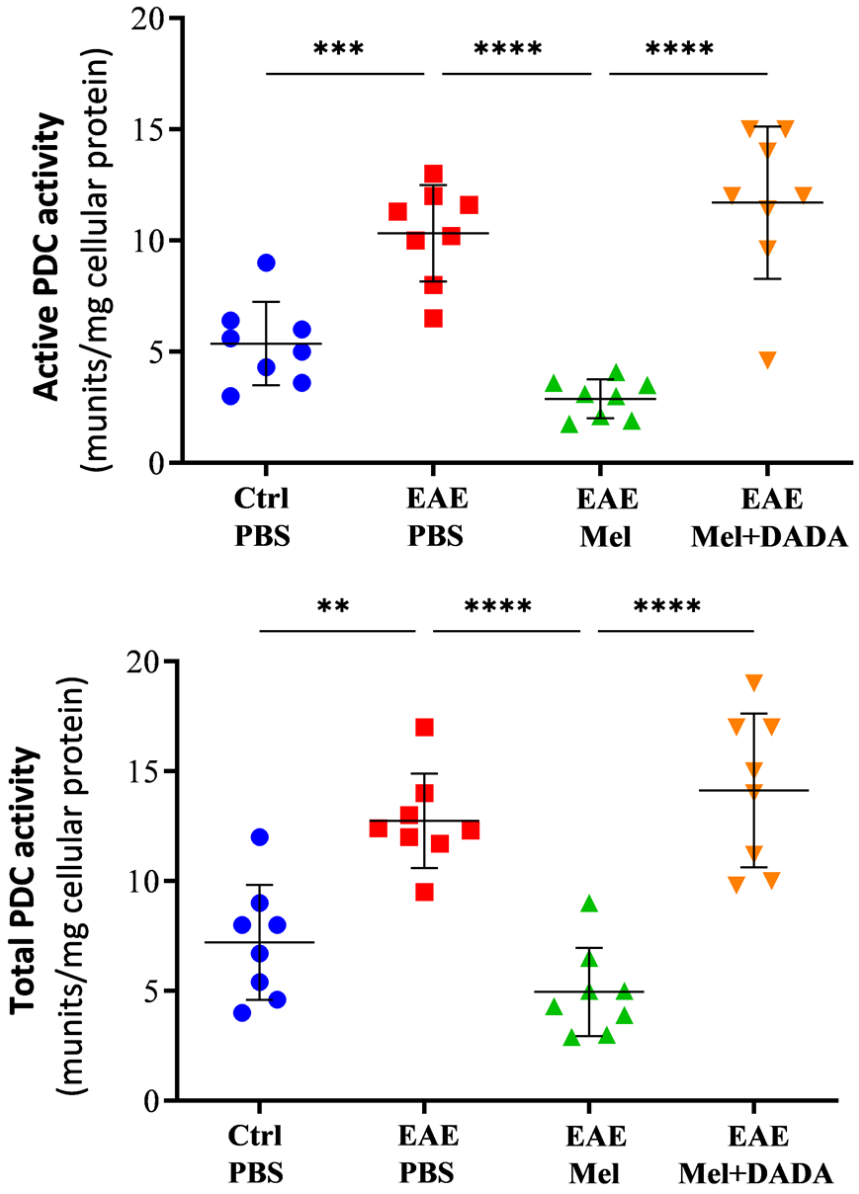

Figure 25: Activity of PDC. The change in the activity of the PDC enzyme was measured either at the **(A)** "active" or **(B)** "total" forms of PDC. Values are expressed as the Mean±SEM. Each group included 8 mice (n = 8). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test. Significance is indicated by $*p < 0.05$ and $**p < 0.001$.

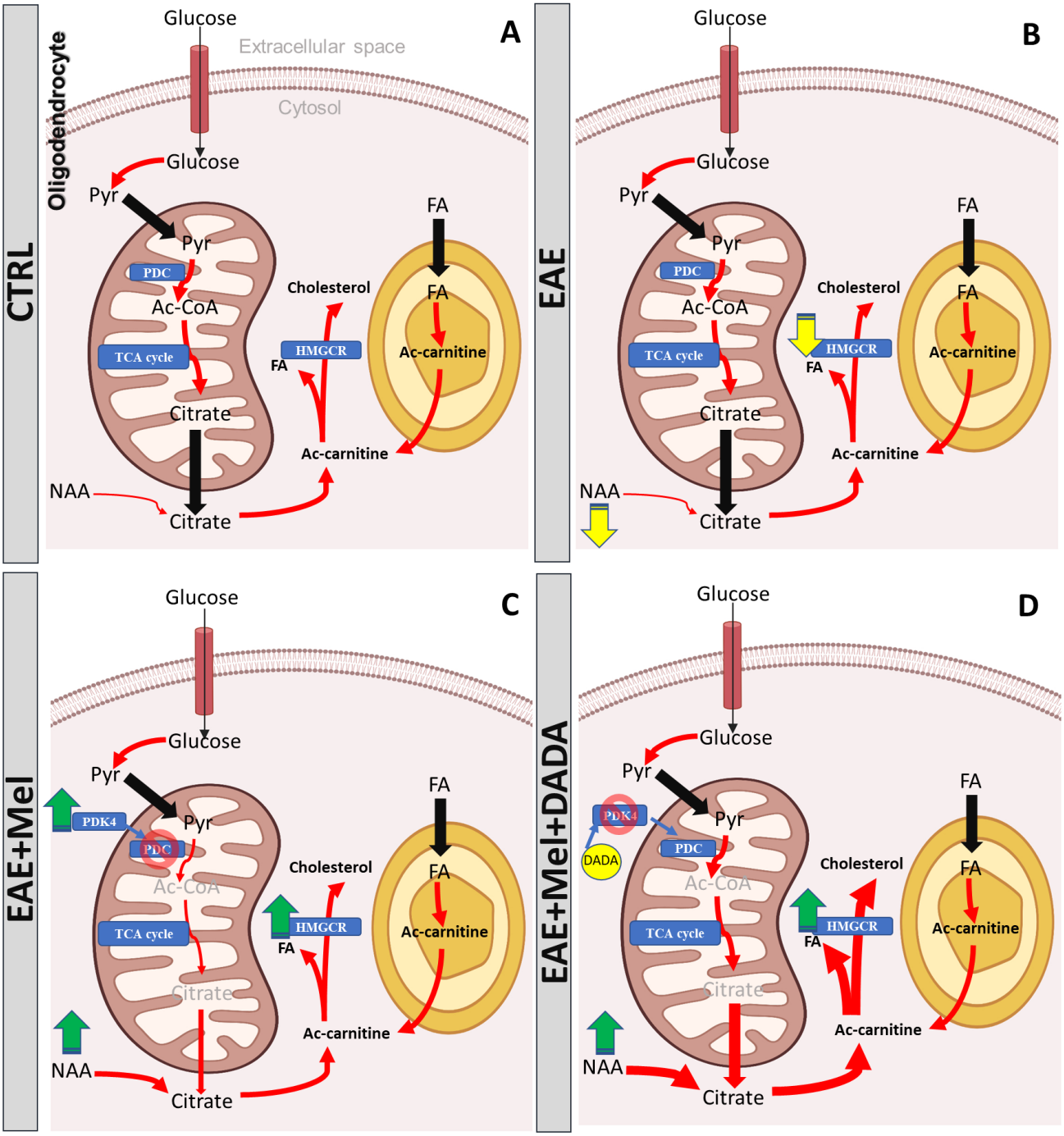
CTRL
Oligodendrocyte
Glucose
Extracellular space
Cytosol
A
Glucose
Pyr
Pyr
PDC
Ac-CoA
TCA cycle
Citrate
NAA
Citrate
FA
FA
Ac-carnitine
Cholesterol
HMGCR
FA
Ac-carnitine
EAE
B
Glucose
Pyr
Pyr
PDC
Ac-CoA
TCA cycle
Citrate
NAA
Citrate
FA
FA
Ac-carnitine
Cholesterol
HMGCR
FA
Ac-carnitine
EAE+Mel
C
Glucose
Pyr
PDK4
Pyr
PDC
Ac-CoA
TCA cycle
Citrate
NAA
Citrate
FA
FA
Ac-carnitine
Cholesterol
HMGCR
FA
Ac-carnitine
EAE+Mel+DADA
D
Glucose
Pyr
PDK4
DADA
Pyr
PDC
Ac-CoA
TCA cycle
Citrate
NAA
Citrate
FA
FA
Ac-carnitine
Cholesterol
HMGCR
FA
Ac-carnitine

Figure 26: The models of Melatonin and DADA effects on FA synthesis during remyelination. (A) In physiological conditions, citrate is produced from imported glucose or pyruvate and from imported NAA into the oligodendrocyte to be utilized as a substrate for FA synthesis required from myelination. Proper function of PDC warrants the synthesis of this citrate. **(B)** During EAE, the PDC function remains intact, which is involved in pyruvate conversion into citrate. The other precursor of citrate, NAA, shows significant reduction, which is suggested to reduce the citrate level, which might slow down FA synthesis and subsequently the remyelination in EAE mice. **(C)** Melatonin therapy recovers the reduced level of NAA in EAE mice in order to increase the citrate levels required for FA synthesis. On the other hand, melatonin increases PDK4 expression which acts as an inhibitor of PDC, leading to the suppression of citrate synthesis. However, the oligodendrocyte still imports the NAA from axons or any other unknown sources to have enough citrate for FA synthesis. **(D)** Since PDC inhibition by melatonin could be considered as a side effect of melatonin therapy, DADA administered in combination with melatonin would suppress the function of PDK4 and rescue the PDC from inhibiting PDK4 function. This leads to the recovery of citrate synthesis by the PDC-dependent pathway, while NAA, another source of citrate, is already high due to melatonin, resulting together into the fast formation of sufficient sources for FA synthesis in remyelination. The yellow arrow shows reduction, the green arrow shows an increase, red arrow shows conversions; blue rectangle shows the enzymes, Pyr; pyruvate, FA; Fatty acid, PDK-4; pyruvate dehydrogenase kinase-4, PDC; pyruvate dehydrogenase complex, Ac-CoA; acetyl-CoA, HMGCR; 3-hydroxy3-methylglutaryl-coenzyme-A reductase, TCA; tricarboxylic acid cycle, NAA; N-acetylaspartate.

7. Future experiments

As a drawback of this study, we used whole-brain homogenate to check the concentrations of metabolites, but it is highly important to use cell sorting for isolation of specific cell types to check the levels of the metabolites in each cell like oligodendrocytes, astrocytes, etc.

This can be better evaluated by verifying the metabolites alter at different stages of the disease to discriminate the differences in demyelination and remyelination stages.

General Discussion

While the etiology of MS is still unknown, a combination of risk factors has been reported to be involved in MS pathogenesis including stress, age, sex, genetical predisposition, etc. Among the wide range of these factors, melatonin, a hormone produced by the pineal gland in response to darkness in circadian dependent manner, is involved in MS pathogenesis; however, its role in MS is still controversial. Previous studies on MS patients showed conflict results possibly due to latitude effects, different lifestyle, and diet that is variable from person to person in the same latitude. For instance, two studies in two different countries at the same latitude (31N) -Isfahan, from Iran, and Haifa from Israel- showed that the level of 6-sulphatoxy-melatonin (6-SMT) in the urine of MS patients is lower than controls; 6-SMT reflects the serum melatonin level and can be used for evaluation of melatonin status (Melamud *et al.* 2012). In contrast, a study in Isfahan showed no significant difference between MS and controls in terms of saliva melatonin (Ghorbani *et al.* 2013). A subsequent study in the same latitude in Iran showed that melatonin is almost 3-fold higher in control than in MS patients (Farhadi *et al.* 2014).

Since some studies reported that melatonin is reduced in MS and melatonin therapy may be helpful for disease amelioration, some studies examined the effects of melatonin on an animal model of EAE, which is a well-accepted model for MS. Our previous study showed that melatonin level is dropped by EAE induction and recovering the melatonin level by exogenous melatonin improve the severity of EAE model (Ghareghani, Dokoohaki, *et al.* 2017a). In this study we found that though melatonin ameliorates the EAE model in adult rats, it exacerbates the severity in younger mice; a finding that proposed the age-dependent role of melatonin in MS. Furthermore, we observed a direct correlation between EAE exacerbation and brain level of lactate. In search of the possible mechanistic pathway for the increase of lactate by melatonin therapy, our following study further confirmed the beneficial role of melatonin therapy in EAE mice, but we noticed that melatonin suppresses the function of PDC which caused an increase in lactate accumulation (Ghareghani *et al.* 2019a).

On the other hand, we studied how corticosteroids affect the melatonin and lactate levels in MS while it improves MS. We uncovered that corticosteroids therapy in EAE,

caused a significant decline in melatonin levels (Dokoohaki *et al.* 2017) and this reduction in melatonin also was associated with a decline in lactate level (Ghareghani *et al.* 2016). This finding shed light on the idea that reducing melatonin level may cause a less inhibitory effect on the PDC pathway which explains why lactate accumulates less and its level decreases. This study directed us to carry out the current thesis study on the EAE model to improve the efficiency of melatonin therapy. Despite the increase in expression of PDK4 by melatonin therapy in EAE which suppresses PDC and inhibit the main fatty acid synthesis pathway, still melatonin removed the EAE as measured by an increase in remyelination, reduction in expression of inflammatory cytokines and markers of oxidative stress (Ghareghani *et al.* 2019a). We found that oligodendrocyte cells use an alternative substrate, possibly imported NAA from axons, to produce the required fatty acid for remyelination. Therefore, in the current study of the thesis, we used a combination of melatonin and DADA to treat the EAE mice. It demonstrated that while melatonin increases the PDK4 level, it couldn't inhibit the PDC activity and affect the main pathway of fatty acid synthesis; hence co-therapy improved the efficiency of melatonin therapy.

Since some studies reported that melatonin in lower in MS patients and melatonin therapy can reduce the MS symptoms and severity, we think that melatonin could play an immunoregulatory role if exogenous melatonin therapy is used to recover the dropped melatonin level in MS patients or EAE model. Relevant studies on the EAE model uncovered some of the mechanistic pathways by which melatonin improves EAE. Kang et al showed that melatonin therapy reduces the $ED1^+$ macrophages and $CD4^+$ T cells entry into the CNS of the EAE model. Given that intercellular adhesion molecule (ICAM-1) plays a key role in recruiting the inflammatory cells to the CNS, Kang et al observed that melatonin decreases the ICAM-1 immunoreactivity in the blood vessels of the EAE model (Kang *et al.* 2001). In continue, Álvarez-Sánchez et al showed that melatonin decreases immune cell infiltration and Th1/Th17 responses in the CNS of EAE mice. Furthermore, they showed that the expression of CD44 significantly decreased by melatonin, a marker that is involved in Th cell survival, polarization, and differentiation whose reduction or genetic suppression improves the EAE severity (Álvarez-Sánchez et al. 2015). It is also found that melatonin caused a reduction in the infiltration of inflammatory Th17 cells into the CNS and an increment in splenic interleukin (IL)-10 (Chen *et al.* 2016).

On the other hand, studies on autoimmune diseases showed that melatonin may be involved in the exacerbation of diseases, as discussed in the introduction. Here in the current thesis project, we have reported that melatonin can be deleterious for MS if it is located in a concentration higher than normal level. Contrary to the EAE model, whose melatonin is drooped, melatonin is increased by the cuprizone model; hence led melatonin to show immunoenhancing function in MS instead of immunoregulatory which is expected in EAE mice.

On the other hand, considering northern countries such as the USA (above 37°N), Canada (42°N to 83°N), Denmark (58°N), and Sweden (60°N), whose individuals have higher melatonin levels s are still among the high MS prevalence countries and are ranked with the highest scores. Furthermore, it is reported that USA and Canada have uncontrolled over-the-counter usage of melatonin. Surprisingly, while melatonin is not approved by FDA as a medication, a 7-fold increase has been reported in the over-the-counter purchase of melatonin among young children within the last 5 years. The same trend has been described in developed countries that also have high MS prevalence. Intriguingly, how would melatonin be a universal remedy and be beneficial for MS treatment while northern countries, with long-term natural higher endogenous melatonin because of the climate and high usage of melatonin, are among the high-risk MS countries?

To answer this question, in this thesis, our cuprizone model simulated higher endogenous melatonin in murine models of MS. Data showed that this model has the potential to mimic the higher levels of melatonin seen in the habitants of higher latitudes. While the EAE model of MS showed a reduction in melatonin levels, the cuprizone model of demyelination showed a tendency to increase them. Furthermore, the cuprizone model mimics several characteristics of progressive MS, for which there is no FDA-approved medication. In addition, the current treatment for RRMS does not show efficient outcomes for these patients. Moreover, we observed that keeping mice in constant darkness, to boost endogenous melatonin, or treating them with exogenous melatonin caused amplification of all disease pathological outcomes including higher monocytes infiltration into the brain, suppression of neural stem cells proliferation and their differentiation to oligodendrocyte precursor cells, and inhibition of maturation and recruitment of oligodendrocytes to the sites of demyelination. Surprisingly, keeping mice in constant light, to suppress the pineal

melatonin synthesis, and treating them with an antagonist of melatonin receptors, to suppress the function of melatonin, caused a rescue of all the above-mentioned events and improved the severity of the disease. Besides, this was associated with suppression of nighttime melatonin, near physiological concentration, and boosting cortisol levels (Ghareghani M *et al.* 2022). Therefore, we suggest the suppression of melatonin for the improvement of MS, however, we claim that keeping melatonin at physiological concentrations is vital for improving the disease.

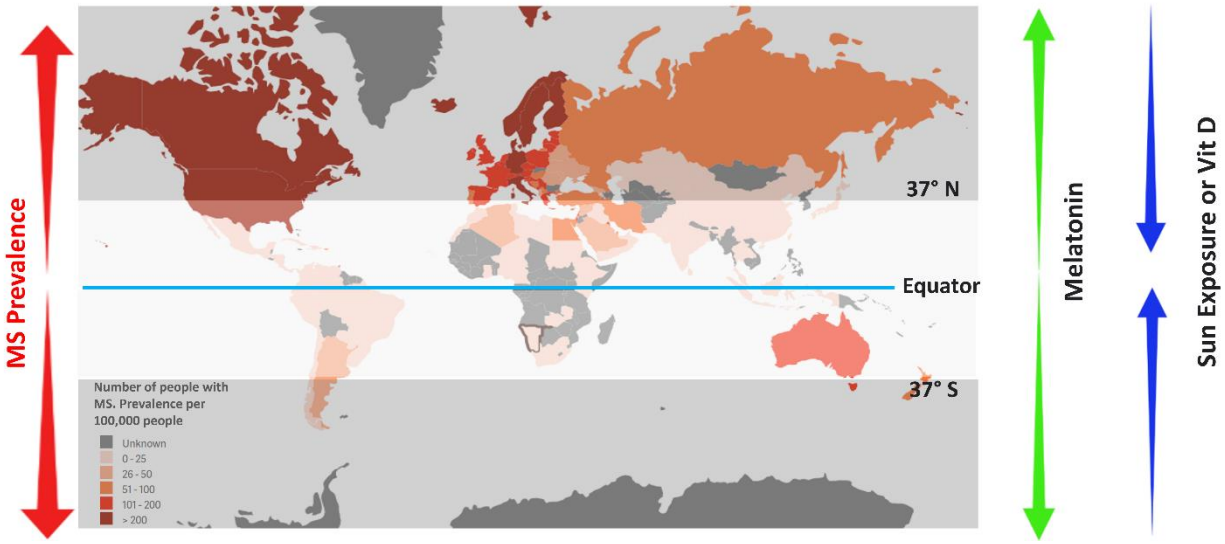

Figure 27. Schematic diagram demonstrating the epidemiological prevalence of MS. MS prevalence is increased by moving from the equator to the poles, which is associated with a decline in sunlight exposure and vitamin D levels with an opposite rise in melatonin levels.

Since we have also reviewed the link between vitamin D and melatonin in MS pathogenesis and treatment, here we propose how we can achieve the best therapeutic efficiency of melatonin manipulation in MS which is tightly connected to the level of vitamin D. Although we have not studied the role of vitamin D in the current thesis, we proposed a mechanistic pathway by which melatonin and vitamin D affect MS in the manuscript which is under review in PNAS. We consider melatonin and vitamin D as two sides of the same coin that affect life through diet, seasons, and day/night cycles. During the summer, vitamin

D is produced in higher amounts due to more exposure to sunlight, while melatonin is further suppressed either by longer day length or boosted vitamin D, which seem to apply inhibitory effects on melatonin release. This trend is reversed in non-summer seasons, especially during winter when the night is longer, and skin exposure to sunlight is extremely reduced. This reduction in sun exposure is due to cold temperature, which necessitates body coverage, as well as to light angle, which does not come straight, resulting in boosting melatonin and reducing vitamin D synthesis. Indeed, evidence shows that a cold climate causes an increase in melatonin synthesis enzymes **(Fig. 27).**

Therefore, when the coin lands on the vitamin D side during winter, it is more likely that vitamin D declines and melatonin rise **(Fig. 28F).** This mimics the situation in northern countries where the night is longer, and skin exposure to sunlight is extremely reduced due to cold temperature, causing a surge in melatonin levels. A high melatonin diet can synergistically boost melatonin levels. This uncontrolled increase in melatonin may be associated with an immunoenhancing role in MS and an increase in inflammatory cytokines at night by the pineal gland. It is more likely that recovering the declined vitamin D for these patients improves the disease in a short term; however, it would be more efficient to induce a balance in vitamin D and melatonin levels, as seen in healthy cases. Therefore, boosting vitamin D levels, preferably by exposure to sunlight or vitamin D supplements, and avoiding the melatonin-enhancing lifestyle and diet, may work for balancing these two when the coin lands on its edge **(Fig. 28A).** Based on our most recent experimental study, keeping the murine model of MS at light during night inhibited the surge of night melatonin and caused a remarkable improvement in disease severity. To translate this study to the clinical level, we propose that habitants in higher latitude, whose melatonin levels are high for the long term, to sleep in a lighted room without sleep disturbance, which is important for the proper function of the brain **(Fig. 29; upper panel).** It is already revealed that our pineal gland can perceive light even with close eyelids when we are at either REM or no-REM phase, which accounts for roughly 50% in nighttime melatonin.(Figueiro *et al.* 2012) On the other hand, Vitamin D provided by lifestyle and diet do not need adjustment if it is already at the normal range **(Fig. 28F).** However, patients should remain on their lifestyle in case they have normal melatonin levels; instead, they may only increase vitamin D levels **(Fig. 28D).**

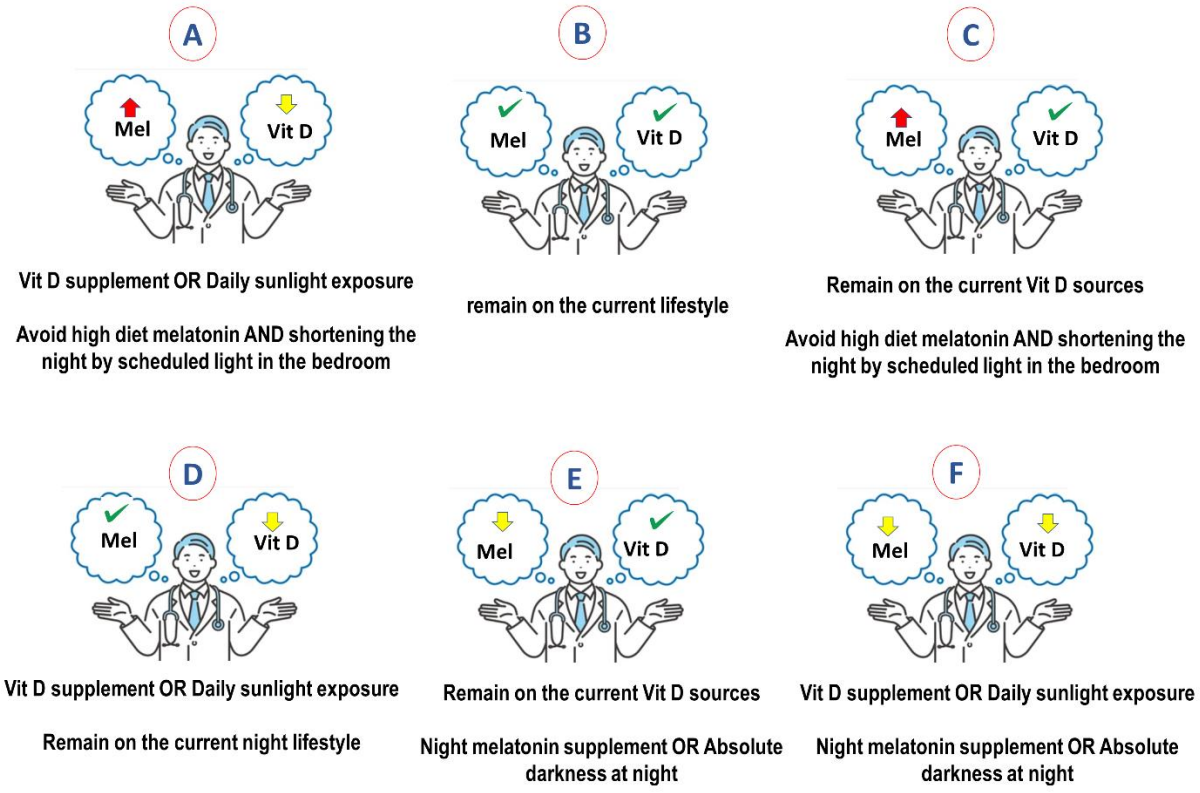

Figure 28. Proposed strategy for adjusting vitamin D and melatonin levels to reach a balance for the proper function of the immune system in MS.

When the coin lands on melatonin's side during summer, it is more likely that vitamin D be normal and melatonin drops. This mimics the situation in countries located near the equator where longer, warmer days, as well as high and easy exposure to sunlight all, are beneficial in boosting vitamin D and increasing the natural immune suppression of cortisol in patients. A low melatonin diet and shorter nights may decrease melatonin levels. It may be suggested to patients to change their night diet and lifestyle to boost melatonin **(Fig. 28B);** for instance, by sleeping 7-8 hours in absolute darkness **(Fig. 29; upper panel).** However, if patients are both below the normal range of melatonin and vitamin D, they will need a daytime 25-OH-D booster in addition to melatonin by taking a vitamin D supplement or preferably exposure to sunlight **(Fig. 28C and Fig. 29; lower panel).**

Furthermore, instead of prescribing vitamin D or melatonin supplements, we highly suggest asking the patient to adjust vitamin D and melatonin levels through sunlight or night, which seems to be more efficient and with less side effects. It should not be forgotten that sunlight may recover the dropped cortisol level in MS patients, which is expected to significantly suppress disease progression

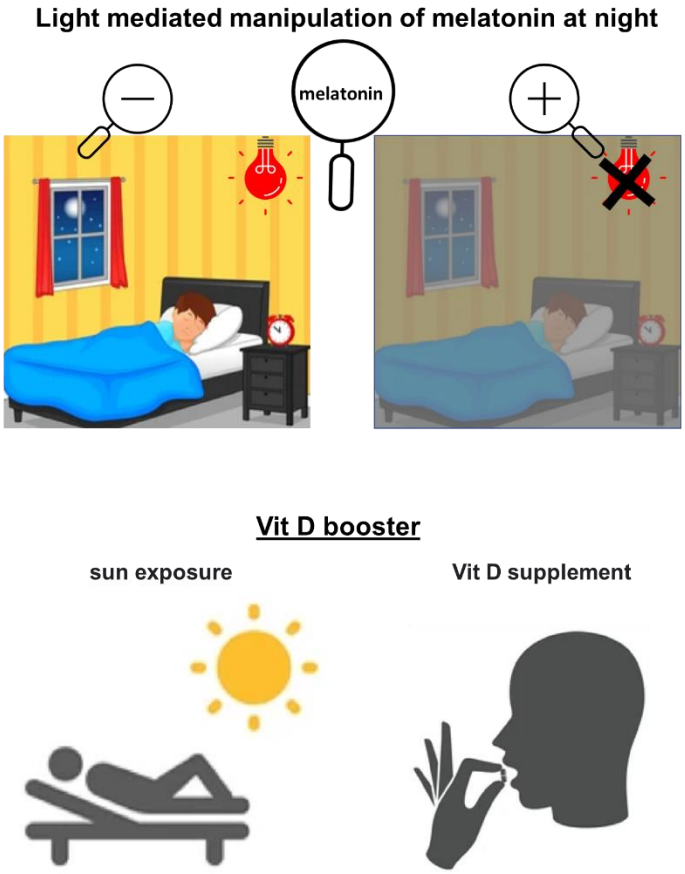

Figure 29: Simple strategies for manipulation of endogenous melatonin or vitamin D. Upper panel; sleeping at lighted room markedly inhibits the increase in night melatonin while absolute darkness boosts it. Lower panel: both sun exposure and vitamin D supplement increase the vitamin D level; however, sun exposure is preferred.

General conclusion

The current knowledge regarding the role of melatonin in autoimmune diseases such as MS is not enough due to the lack of long-term studies on both animal models and humans. On the other hand, an increase, or a decrease in the average levels of melatonin in a specific population and at a specific time does not mean that all individuals have the same trend. Instead, every person has his own diet and lifestyle also varies overtime. Therefore, instead of using melatonin supplements, it should be better to check melatonin's oscillation and to consider the most efficient strategy for adjusting its levels. Melatonin therapy can improve MS symptoms and severity if we recover the decline melatonin level, and it exacerbates it if we increase the melatonin to a value higher than what is expected in normal subjects. It's worth mentioning that in case the animal model or patient shows a decline in melatonin level, it might be more efficient if melatonin is prescribed with DADA.

General perspective

Assessment of the melatonin level and comparing it to the age and sex-matched control before using melatonin therapy in autoimmune disease and model to adjust the level to study the effect when melatonin is lower and higher than normal level.

Given that the intensity of light has a huge impact on the synthesis of melatonin, and melatonin plays a significant role in autoimmune diseases, it would be helpful to design new studies and check the light intensity inside the cages to make sure all mice receive the same intensity.

Designing the studies to use light manipulation, in terms of duration and intensity of light, instead of melatonin supplement, to increase or decrease melatonin levels which also affect cortisol levels.

APPENDIX A: Reduced Melatonin Levels May Facilitate Glioblastoma Initiation in the Subventricular Zone

Review paper

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Running head: Circadian Melatonin in Glioblastoma

i. Résumé

Il existe de plus en plus de preuves que le glioblastome, une tumeur cérébrale très agressive, provient de cellules souches neurales (CSN) situées dans la zone sous-ventriculaire (SVZ) du ventricule cérébral latéral. En utilisant les techniques avancées d'imagerie *in vivo*, Gengatharan et ses collègues ont récemment identifié une différence entre le jour et la nuit dans la division des CSN dans la SVZ chez les adultes. Ils ont rapporté que le rythme circadien de la mélatonine et son récepteur contrôlent la différence jour/nuit de la division des NSC, avec une forte activité mitotique pendant le jour et une faible activité la nuit. L'expression de la mélatonine et de son récepteur diminue au cours du vieillissement, ce qui élimine l'effet régulateur de la mélatonine sur la mitose des CSN. De plus, le rythme circadien de la mélatonine est atténué par la lumière la nuit, ce qui pourrait modifier le cycle mitotique circadien des CSN dans la SVZ. En outre, les hommes, dont le taux de la mélatonine est inférieur à celui des femmes, présentent un taux d'incidence de glioblastome 60 % plus élevé. Étant donné que le vieillissement contribue de manière significative à l'initiation et à la progression des glioblastomes, nous suggérons que le déclin de la synthèse et de la libération circadienne de mélatonine ainsi que celui de ses récepteurs dans la SVZ, qui diminuent également avec le vieillissement, agissent de pair avec d'autres facteurs pour faciliter l'initiation et la croissance des glioblastomes.

ii. Abstract

There is increasing evidence that glioblastoma, a highly aggressive brain tumor, originates from neural stem cell (NSC) located in the subventricular zone (SVZ) of the lateral cerebral ventricle. Using the most advanced *in vivo* imaging techniques, Gengatharan and colleagues recently identified a day/night difference in the adult SVZ-NSC division. They reported that the circadian melatonin rhythm and its receptor control the day/night difference in NSC division with high mitotic activity during the day and low activity at night. The expression of melatonin and its receptor diminishes during aging, which eliminates the regulatory effect of melatonin on NSC mitosis. Moreover, the circadian melatonin rhythm is dampened by light-at-night with the potential of altering the circadian mitotic cycle of NSC in the SVZ. Also, men, with a lower melatonin amplitude than women, exhibit a 60% higher rate of glioblastoma incidence. Given that aging contributes significantly to glioblastoma initiation and progression, we suggest that the decline in circadian melatonin synthesis and release as well as its receptors in the SVZ which also diminish with aging act in concert with other factors to facilitate glioblastoma initiation and growth.

APPENDIX B: Melatonin And Vitamin D, Two Sides of The Same Coin, better to Land on Its Edge to Improve Multiple Sclerosis

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Review Paper: Under review in PNAS

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Classification: Biological Sciences/Immunology and Inflammation

Keywords: Multiple sclerosis, MS, melatonin, latitude, vitamin D

i. Résumé

Les études actuelles ont révélé un gradient latitudinal de la prévalence de la sclérose en plaques (SP), qui augmente en allant de l'équateur vers les pôles. La durée et la qualité de l'exposition d'un individu à la lumière du soleil varient en fonction de la latitude. L'exposition de la peau à la lumière du soleil active la synthèse de la vitamine D, tandis que l'absence de lumière, telle que perçue par les yeux, active la synthèse de la mélatonine dans la glande pinéale. Une carence/insuffisance ou un surdosage en vitamine D ou en mélatonine peut survenir à n'importe quelle latitude en raison de modes de vie et de régimes alimentaires spécifiques. Le fait de s'éloigner de l'équateur, surtout au-delà de 37°, augmente la carence/insuffisance en vitamine D tout en augmentant la mélatonine. De plus, la synthèse de mélatonine augmente dans les habitats froids comme les pays nordiques. Étant donné que certaines études ont montré le rôle bénéfique de la mélatonine dans la SP, on s'attend à ce que les pays nordiques, y compris le nord des États-Unis, le Canada, le Danemark et la Suède, tous situés au-dessus de 37°, dont les individus ont une mélatonine endogène plus élevée, présentent une prévalence de la SP plus faible ; cependant, ces pays sont classés avec les scores les plus élevés. En plus d'une mélatonine élevée, des pays comme les États-Unis et le Canada ont un usage non contrôlé des médicaments en vente libre. Sous les hautes latitudes, la carence en vitamine D et une prévalence plus élevée de la SP persistent, même si l'insuffisance/la carence en vitamine D est généralement compensée par une prise de suppléments et non par la lumière du soleil. Récemment, nous avons découvert que l'obscurité prolongée augmentait les niveaux de mélatonine du modèle de SP, imitant l'augmentation à long terme dans les pays nordiques. Cela réduisait le cortisol et augmentait l'infiltration, l'inflammation et la démyélinisation. La thérapie par la lumière constante a sauvé tout cela. Dans cette revue, nous expliquons les rôles possibles de la mélatonine et de la vitamine D dans la prévalence de la SP. Nous expliquons ensuite les causes possibles de la prévalence élevée de la SP dans les pays du Nord. Enfin, nous suggérons des stratégies pour traiter la SP en manipulant la vitamine D et la mélatonine, de préférence avec la lumière du soleil ou l'obscurité, et non avec des suppléments.

ii. Abstract

Current studies revealed a latitudinal gradient of multiple sclerosis (MS) prevalence, increasing by moving from the equator to the poles. The duration and quality of an individual's exposure to sunlight vary with latitude. Skin exposure to sunlight activates vitamin D synthesis, while light absence, as perceived by the eyes, activates melatonin synthesis in the pineal gland. Vitamin D or melatonin deficiency/insufficiency or overdose can occur at any latitude due to specific lifestyles and diets. Moving away from the equator, especially beyond 37°, increases vitamin D deficiency/insufficiency while raising melatonin. Furthermore, melatonin synthesis increases in cold habitats like northern countries. Given that some studies showed melatonin's beneficial role in MS, it's expected that northern countries including north of USA, Canada, Denmark, and Sweden, all above 37°, whose individuals have higher endogenous melatonin, should show lower MS prevalence; however, these are ranked with the highest scores. In addition to high melatonin, countries like USA and Canada have uncontrolled over-the-counter usage. In high latitudes, vitamin D deficiency and a higher MS prevalence persist even though vitamin D insufficiency/deficiency is typically compensated for by supplementation and not sunlight. Recently, we found that prolonged darkness increased the MS model's melatonin levels, mimicking the long-term increase in northern countries. This reduced cortisol and increased infiltration, inflammation, and demyelination. Constant-light therapy rescued all these. In this review, we explain melatonin and vitamin D's possible roles in MS prevalence. We then explain the possible causes for the high MS prevalence in northern countries. Finally, we suggest strategies to treat MS by manipulating vitamin D and melatonin, preferably with sunlight or darkness, not supplements.

Bibliography

Abu-Samak, M. S., M. E. AbuRuz, R. Masa'Deh, R. Khuzai, and S. Jarrah. 2019. 'Correlation of selected stress associated factors with vitamin D deficiency in Jordanian men and women', *Int J Gen Med*, 12: 225-33.

Ackerman, K. D., R. Heyman, B. S. Rabin, B. P. Anderson, P. R. Houck, E. Frank, and A. Baum. 2002. 'Stressful life events precede exacerbations of multiple sclerosis', *Psychosom Med*, 64: 916-20.

Adamczyk-Sowa, M., K. Pierzchala, P. Sowa, R. Polaniak, M. Kukla, and M. Hartel. 2014. 'Influence of melatonin supplementation on serum antioxidative properties and impact of the quality of life in multiple sclerosis patients', *J Physiol Pharmacol*, 65: 543-50.

Adamsson, M., T. Laike, and T. Morita. 2016. 'Annual variation in daily light exposure and circadian change of melatonin and cortisol concentrations at a northern latitude with large seasonal differences in photoperiod length', *J Physiol Anthropol*, 36: 6.

Al-Dujaili, E. A., N. Munir, and R. R. Iniesta. 2016. 'Effect of vitamin D supplementation on cardiovascular disease risk factors and exercise performance in healthy participants: a randomized placebo-controlled preliminary study', *Ther Adv Endocrinol Metab*, 7: 153-65.

Alcina, A., M. Abad-Grau Mdel, M. Fedetz, G. Izquierdo, M. Lucas, O. Fernández, D. Ndagire, A. Catalá-Rabasa, A. Ruiz, J. Gayán, C. Delgado, C. Arnal, and F. Matesanz. 2012. 'Multiple sclerosis risk variant HLA-DRB1*1501 associates with high expression of DRB1 gene in different human populations', *PLoS One*, 7: e29819.

Alluri, H., R. L. Wilson, C. Anasooya Shaji, K. Wiggins-Dohlvik, S. Patel, Y. Liu, X. Peng, M. R. Beeram, M. L. Davis, J. H. Huang, and B. Tharakan. 2016. 'Melatonin Preserves Blood-Brain Barrier Integrity and Permeability via Matrix Metalloproteinase-9 Inhibition', *PLoS One*, 11: e0154427.

Aloia, J. F., R. Dhaliwal, A. Shieh, M. Mikhail, M. Fazzari, L. Ragolia, and S. A. Abrams. 2014. 'Vitamin D supplementation increases calcium absorption without a threshold effect', *Am J Clin Nutr*, 99: 624-31.

Álvarez-Sánchez, N., I. Cruz-Chamorro, A. López-González, J. C. Utrilla, J. M. Fernández-Santos, A. Martínez-López, P. J. Lardone, J. M. Guerrero, and A. Carrillo-Vico. 2015. 'Melatonin controls experimental autoimmune encephalomyelitis by altering the T effector/regulatory balance', *Brain Behav Immun*, 50: 101-14.

Amorini, Angela M., Viviana Nociti, Axel Petzold, Claudio Gasperini, Esmeralda Quartuccio, Giacomo Lazzarino, Valentina Di Pietro, Antonio Belli, Stefano Signoretti, Roberto Vagnozzi, Giuseppe Lazzarino, and Barbara Tavazzi. 2014. 'Serum lactate as a novel potential biomarker in multiple sclerosis', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1842: 1137-43.

Arendt, J. 1986. 'Role of the pineal gland and melatonin in seasonal reproductive function in mammals', *Oxf Rev Reprod Biol*, 8: 266-320.

———. 1988. 'Melatonin', *Clin Endocrinol (Oxf)*, 29: 205-29.

Arneth, Borros M. 2019. 'Impact of B cells to the pathophysiology of multiple sclerosis', *Journal of Neuroinflammation*, 16: 128.

Ayoglu, B., N. Mitsios, I. Kockum, M. Khademi, A. Zandian, R. Sjöberg, B. Forsström, J. Bredenberg, I. Lima Bomfim, E. Holmgren, H. Grönlund, A. O. Guerreiro-Cacais, N. Abdelmagid, M. Uhlén, T. Waterboer, L. Alfredsson, J. Mulder, J. M. Schwenk, T. Olsson, and P. Nilsson. 2016. 'Anoctamin 2 identified as an autoimmune target in multiple sclerosis', *Proc Natl Acad Sci U S A*, 113: 2188-93.

Bäärnhielm, M., A. K. Hedström, I. Kockum, E. Sundqvist, S. A. Gustafsson, J. Hillert, T. Olsson, and L. Alfredsson. 2012. 'Sunlight is associated with decreased multiple sclerosis risk: no interaction with human leukocyte antigen-DRB1*15', *Eur J Neurol*, 19: 955-62.

Baba, K., A. Benleulmi-Chaachoua, A. S. Journé, M. Kamal, J. L. Guillaume, S. Dussaud, F. Gbahou, K. Yettou, C. Liu, S. Contreras-Alcantara, R. Jockers, and G. Tosini. 2013. 'Heteromeric MT1/MT2 melatonin receptors modulate photoreceptor function', *Sci Signal*, 6: ra89.

Balusu, S., E. Van Wonterghem, R. De Rycke, K. Raemdonck, S. Stremersch, K. Gevaert, M. Brkic, D. Demeestere, V. Vanhooren, A. Hendrix, C. Libert, and R. E. Vandenbroucke. 2016. 'Identification of a novel mechanism of blood-brain communication during peripheral inflammation via choroid plexus-derived extracellular vesicles', *EMBO Mol Med*, 8: 1162-83.

Barjavel, M. J., Z. Mamdouh, N. Raghbate, and O. Bakouche. 1998. 'Differential expression of the melatonin receptor in human monocytes', *J Immunol*, 160: 1191-7.

Barnes, P. J. 2006. 'How corticosteroids control inflammation: Quintiles Prize Lecture 2005', *Br J Pharmacol*, 148: 245-54.

Bartsch, H., A. Buchberger, H. Franz, C. Bartsch, I. Maidonis, D. Mecke, and E. Bayer. 2000. 'Effect of melatonin and pineal extracts on human ovarian and mammary tumor cells in a chemosensitivity assay', *Life Sci*, 67: 2953-60.

Beretich, B. D., and T. M. Beretich. 2009. 'Explaining multiple sclerosis prevalence by ultraviolet exposure: a geospatial analysis', *Mult Scler*, 15: 891-8.

Berghoff, S. A., T. Düking, L. Spieth, J. Winchenbach, S. K. Stumpf, N. Gerndt, K. Kusch, T. Ruhwedel, W. Möbius, and G. Saher. 2017. 'Blood-brain barrier hyperpermeability precedes demyelination in the cuprizone model', *Acta Neuropathol Commun*, 5: 94.

Bjelobaba, Ivana, Vesna Begovic-Kupresanin, Sanja Pekovic, and Irena Lavrnja. 2018. 'Animal models of multiple sclerosis: Focus on experimental autoimmune encephalomyelitis', *Journal of Neuroscience Research*, 96: 1021-42.

Bjornevik, Kjetil, Marianna Cortese, Brian C. Healy, Jens Kuhle, Michael J. Mina, Yumei Leng, Stephen J. Elledge, David W. Niebuhr, Ann I. Scher, Kassandra L. Munger, and Alberto Ascherio. 2022. 'Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis', *Science*, 375: 296-301.

Bjornsson, Christopher S., Maria Apostolopoulou, Yangzi Tian, and Sally Temple. 2015. 'It takes a village: constructing the neurogenic niche', *Developmental cell*, 32: 435-46.

Black, L. I., T. C. Clarke, P. M. Barnes, B. J. Stussman, and R. L. Nahin. 2015. 'Use of complementary health approaches among children aged 4-17 years in the United States: National Health Interview Survey, 2007-2012', *Natl Health Stat Report*: 1-19.

Blask, D. E., R. T. Dauchy, L. A. Sauer, J. A. Krause, and G. C. Brainard. 2002. 'Light during darkness, melatonin suppression and cancer progression', *Neuro Endocrinol Lett*, 23 Suppl 2: 52-6.

Bliddal, M., H. Kildegaard, L. Rasmussen, M. Ernst, P. J. Jennum, S. H. Mogensen, A. Pottegård, and R. Wesselhoeft. 2022. 'Melatonin use among children, adolescents, and young adults: a Danish nationwide drug utilization study', *Eur Child Adolesc Psychiatry*.

Braniste, V., M. Al-Asmakh, C. Kowal, F. Anuar, A. Abbaspour, M. Tóth, A. Korecka, N. Bakocevic, L. G. Ng, P. Kundu, B. Gulyás, C. Halldin, K. Hultenby, H. Nilsson, H. Hebert, B. T. Volpe, B. Diamond, and S. Pettersson. 2014. 'The gut microbiota influences blood-brain barrier permeability in mice', *Sci Transl Med*, 6: 263ra158.

Brousse, B., O. Mercier, K. Magalon, F. Daian, P. Durbec, and M. Cayre. 2021. 'Endogenous neural stem cells modulate microglia and protect against demyelination', *Stem Cell Reports*, 16: 1792-804.

Butt, A. M., A. D. Rivera, D. Fulton, and K. Azim. 2022. 'Targeting the Subventricular Zone to Promote Myelin Repair in the Aging Brain', *Cells*, 11.

Butti, E., M. Bacigaluppi, L. Chaabane, F. Ruffini, E. Brambilla, G. Berera, C. Montonati, A. Quattrini, and G. Martino. 2019. 'Neural Stem Cells of the Subventricular Zone Contribute to Neuroprotection of the Corpus Callosum after Cuprizone-Induced Demyelination', *J Neurosci*, 39: 5481-92.

Cader, S, Heidi Johansen-Berg, M Wylezinska, Jackie Palace, TE Behrens, S Smith, and Paul M Matthews. 2007. 'Discordant white matter N-acetylasparate and diffusion MRI measures suggest that chronic metabolic dysfunction contributes to axonal pathology in multiple sclerosis', *Neuroimage*, 36: 19-27.

Cagnacci, A., R. Soldani, and S. S. Yen. 1995. 'Hypothermic effect of melatonin and nocturnal core body temperature decline are reduced in aged women', *Journal of Applied Physiology*, 78: 314-17.

Cain, S. W., C. F. Dennison, J. M. Zeitzer, A. M. Guzik, S. B. Khalsa, N. Santhi, M. W. Schoen, C. A. Czeisler, and J. F. Duffy. 2010. 'Sex differences in phase angle of entrainment and melatonin amplitude in humans', *J Biol Rhythms*, 25: 288-96.

Camara-Lemarroy, C. R., L. M. Metz, and V. W. Yong. 2018. 'Focus on the gut-brain axis: Multiple sclerosis, the intestinal barrier and the microbiome', *World J Gastroenterol*, 24: 4217-23.

Camargo, Nutabi, Andrea Goudriaan, Anne-Lieke F. van Deijk, Willem M. Otte, Jos F. Brouwers, Hans Lodder, David H. Gutmann, Klaus-Armin Nave, Rick M. Dijkhuizen, Huibert D. Mansvelder, Roman Chrast, August B. Smit, and Mark H. G. Verheijen. 2017. 'Oligodendroglial myelination requires astrocyte-derived lipids', *PLoS biology*, 15: e1002605-e05.

Campino, C., F. Valenzuela, E. Arteaga, C. Torres-Farfán, C. Trucco, A. Velasco, S. Guzmán, and M. Serón-Ferré. 2008. '[Melatonin reduces cortisol response to ACTH in humans]', *Rev Med Chil*, 136: 1390-7.

Carrano, A., J. J. Juarez, D. Incontri, A. Ibarra, and H. Guerrero Cazares. 2021. 'Sex-Specific Differences in Glioblastoma', *Cells*, 10.

Chakraborty, Goutam, Praveen Mekala, Daniel Yahya, Gusheng Wu, and Robert W Ledeen. 2001. 'Intraneuronal N-acetylaspartate supplies acetyl groups for myelin lipid synthesis: evidence for myelin-associated aspartoacylase', *Journal of neurochemistry*, 78: 736-45.

Chen, S. J., S. H. Huang, J. W. Chen, K. C. Wang, Y. R. Yang, P. F. Liu, G. J. Lin, and H. K. Sytwu. 2016. 'Melatonin enhances interleukin-10 expression and suppresses chemotaxis to inhibit inflammation in situ and reduce the severity of experimental autoimmune encephalomyelitis', *Int Immunopharmacol*, 31: 169-77.

Christakos, S., P. Dhawan, A. Porta, L. J. Mady, and T. Seth. 2011. 'Vitamin D and intestinal calcium absorption', *Mol Cell Endocrinol*, 347: 25-9.

Codega, P., V. Silva-Vargas, A. Paul, A. R. Maldonado-Soto, A. M. Deleo, E. Pastrana, and F. Doetsch. 2014. 'Prospective identification and purification of quiescent adult neural stem cells from their in vivo niche', *Neuron*, 82: 545-59.

Codenotti, S., M. Battistelli, S. Burattini, S. Salucci, E. Falcieri, R. Rezzani, F. Faggi, M. Colombi, E. Monti, and A. Fanzani. 2015. 'Melatonin decreases cell proliferation, impairs myogenic differentiation and triggers apoptotic cell death in rhabdomyosarcoma cell lines', *Oncol Rep*, 34: 279-87.

Cole, R. J., D. F. Kripke, J. Wisbey, W. J. Mason, W. Gruen, P. J. Hauri, and S. Juarez. 1995. 'Seasonal variation in human illumination exposure at two different latitudes', *J Biol Rhythms*, 10: 324-34.

Constantinescu, C. S., B. Hilliard, E. Ventura, and A. Rostami. 1997. 'Luzindole, a melatonin receptor antagonist, suppresses experimental autoimmune encephalomyelitis', *Pathobiology*, 65: 190-4.

Cutolo, M., G. J. Maestroni, K. Otsa, O. Aakre, B. Villaggio, S. Capellino, P. Montagna, L. Fazzuoli, T. Veldi, T. Peets, E. Hertens, and A. Sulli. 2005. 'Circadian melatonin and cortisol levels in rheumatoid arthritis patients in winter time: a north and south Europe comparison', *Ann Rheum Dis*, 64: 212-6.

Dalla Costa, G., A. Giordano, M. Romeo, F. Sangalli, G. Comi, and V. Martinelli. 2018. 'Digital epidemiology confirms a latitude gradient of MS in France', *Mult Scler Relat Disord*, 20: 129-31.

David-Bercholz, J., C. T. Kuo, and B. Deneen. 2021. 'Astrocyte and Oligodendrocyte Responses From the Subventricular Zone After Injury', *Front Cell Neurosci*, 15: 797553.

Deacon, Stephen, and Josephine Arendt. 1995. 'Melatonin-induced temperature suppression and its acute phase-shifting effects correlate in a dose-dependent manner in humans', *Brain research*, 688: 77-85.

Della-Flora Nunes, Gustavo, Lauren Mueller, Nicholas Silvestri, Mulchand S. Patel, Lawrence Wrabetz, M. Laura Feltri, and Yannick Poitelon. 2017. 'Acetyl-CoA production from pyruvate is not necessary for preservation of myelin', *Glia*, 65: 1626-39.

Dimas, P., L. Montani, J. A. Pereira, D. Moreno, M. Trötzmüller, J. Gerber, C. F. Semenkovich, H. C. Köfeler, and U. Suter. 2019a. 'CNS myelination and remyelination depend on fatty acid synthesis by oligodendrocytes', *Elife*, 8.

Dimas, Penelope, Laura Montani, Jorge A. Pereira, Daniel Moreno, Martin Trötzmüller, Joanne Gerber, Clay F. Semenkovich, Harald C. Köfeler, and Ueli Suter. 2019b. 'CNS myelination and remyelination depend on fatty acid synthesis by oligodendrocytes', *eLife*, 8: e44702.

Disanto, G., J. Pakpoor, J. M. Morahan, C. Hall, U. C. Meier, G. Giovannoni, and S. V. Ramagopalan. 2013. 'Epstein-Barr virus, latitude and multiple sclerosis', *Mult Scler*, 19: 362-5.

Doetsch, Fiona, Jose Manuel García-Verdugo, and Arturo Alvarez-Buylla. 1999. 'Regeneration of a germinal layer in the adult mammalian brain', *Proceedings of the National Academy of Sciences*, 96: 11619.

Doetsch, Fiona, Jose Manuel García-Verdugo, and Arturo Alvarez-Buylla. 1997. 'Cellular Composition and Three-Dimensional Organization of the Subventricular Germinal Zone in the Adult Mammalian Brain', *The Journal of Neuroscience*, 17: 5046.

Dokoohaki, S., M. Ghareghani, A. Ghanbari, N. Farhadi, K. Zibara, and H. Sadeghi. 2017. 'Corticosteroid therapy exacerbates the reduction of melatonin in multiple sclerosis', *Steroids*, 128: 32-36.

Ecker, Josef, Gerhard Liebisch, Marion Englmaier, Margot Grandl, Horst Robenek, and Gerd Schmitz. 2010. 'Induction of fatty acid synthesis is a key requirement for phagocytic differentiation of human monocytes', *Proceedings of the National Academy of Sciences of the United States of America*, 107: 7817-22.

Ejaz, H., J. K. Figaro, A. M. F. Woolner, B. M. V. Thottakam, and H. F. Galley. 2020. 'Maternal Serum Melatonin Increases During Pregnancy and Falls Immediately After Delivery Implicating the Placenta as a Major Source of Melatonin', *Front Endocrinol (Lausanne)*, 11: 623038.

el-Maghraby, T. A., N. M. Shalaby, M. H. Al-Tawdy, and S. S. Salem. 2005. 'Gastric motility dysfunction in patients with multiple sclerosis assessed by gastric emptying scintigraphy', *Can J Gastroenterol*, 19: 141-5.

Erland, L. A., and P. K. Saxena. 2017. 'Melatonin Natural Health Products and Supplements: Presence of Serotonin and Significant Variability of Melatonin Content', *J Clin Sleep Med*, 13: 275-81.

Faraco, G., L. Park, J. Anrather, and C. Iadecola. 2017. 'Brain perivascular macrophages: characterization and functional roles in health and disease', *J Mol Med (Berl)*, 95: 1143-52.

Farez, M. F., I. D. Mascanfroni, S. P. Méndez-Huergo, A. Yeste, G. Murugaiyan, L. P. Garo, M. E. Balbuena Aguirre, B. Patel, M. C. Ysrraelit, C. Zhu, V. K. Kuchroo, G. A. Rabinovich, F. J. Quintana, and J. Correale. 2015. 'Melatonin Contributes to the Seasonality of Multiple Sclerosis Relapses', *Cell*, 162: 1338-52.

Farhadi, N., M. Gharghani, and Z. Farhadi. 2016. 'Effects of long-term light, darkness and oral administration of melatonin on serum levels of melatonin', *Biomed J*, 39: 81-4.

Farhadi, N., S. Oryan, and M. Nabiuni. 2014. 'Serum levels of melatonin and cytokines in multiple sclerosis', *Biomed J*, 37: 90-2.

Fernandez-Castaneda, A., and A. Gaultier. 2016. 'Adult oligodendrocyte progenitor cells - Multifaceted regulators of the CNS in health and disease', *Brain Behav Immun*, 57: 1-7.

Fernandez-Gil, Beatriz Irene, Carla Vazquez-Ramos, Alexandra Bechtle, Paola Suarez-Meade, Neda Qosja, Paula Schiapparelli, Rachel Sarabia-Estrada, Germaine Escames, and Alfredo Quinones-Hinojosa. 2021. 'DDRE-33. MELATONIN AS A MASTER METABOLIC SWITCH FOR GLIOBLASTOMA', *Neuro-oncology advances*, 3: i13-i14.

Figueiro, M. G., and M. S. Rea. 2012. 'Preliminary evidence that light through the eyelids can suppress melatonin and phase shift dim light melatonin onset', *BMC Res Notes*, 5: 221.

Frank, Matthew G., Laura K. Fonken, Linda R. Watkins, and Steven F. Maier. 2019. 'Microglia: Neuroimmune-sensors of stress', *Seminars in Cell & Developmental Biology*, 94: 176-85.

Franklin, R. J., and C. Ffrench-Constant. 2008. 'Remyelination in the CNS: from biology to therapy', *Nat Rev Neurosci*, 9: 839-55.

Fransen, Marc, Celien Lismont, and Paul Walton. 2017. 'The Peroxisome-Mitochondria Connection: How and Why?', *International journal of molecular sciences*, 18: 1126.

Fu, J., Y. Wu, Y. Miao, Y. Xu, E. Zhao, J. Wang, H. Sun, Q. Liu, Y. Xue, Y. Xu, and T. Hu. 2017. 'Improved cold tolerance in Elymus nutans by exogenous application of melatonin may involve ABA-dependent and ABA-independent pathways', *Sci Rep*, 7: 39865.

Fujinami, R. S., and M. B. Oldstone. 1985. 'Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity', *Science*, 230: 1043-5.

Gallagher, L. G., S. Ilango, A. Wundes, G. A. Stobbe, K. W. Turk, G. M. Franklin, M. S. Linet, D. M. Freedman, B. H. Alexander, and H. Checkoway. 2019. 'Lifetime exposure to ultraviolet radiation and the risk of multiple sclerosis in the US radiologic technologists cohort study', *Mult Scler*, 25: 1162-69.

Garcia-Mauriño, S., M. G. Gonzalez-Haba, J. R. Calvo, M. Rafii-El-Idrissi, V. Sanchez-Margalet, R. Goberna, and J. M. Guerrero. 1997. 'Melatonin enhances IL-2, IL-6, and IFN-gamma production by human circulating CD4+ cells: a possible nuclear receptor-mediated mechanism involving T helper type 1 lymphocytes and monocytes', *J Immunol*, 159: 574-81.

García-Mauriño, S., D. Pozo, J. R. Calvo, and J. M. Guerrero. 2000. 'Correlation between nuclear melatonin receptor expression and enhanced cytokine production in human lymphocytic and monocytic cell lines', *J Pineal Res*, 29: 129-37.

Garcia-Saenz, A., A. Sánchez de Miguel, A. Espinosa, A. Valentin, N. Aragonés, J. Llorca, P. Amiano, V. Martín Sánchez, M. Guevara, R. Capelo, A. Tardón, R. Peiró-Perez, J. J. Jiménez-Moleón, A. Roca-Barceló, B. Pérez-Gómez, T. Dierssen-Sotos, T. Fernández-Villa, C. Moreno-Iribas, V. Moreno, J. García-Pérez, G. Castaño-Vinyals, M. Pollán, M. Aubé, and M. Kogevinas. 2018. 'Evaluating the Association between Artificial Light-at-Night Exposure and Breast and Prostate Cancer Risk in Spain (MCC-Spain Study)', *Environ Health Perspect*, 126: 047011.

Gelernt, M. D., and V. Herbert. 1982. 'Mutagenicity of diisopropylamine dichloroacetate, the "active constituent" of vitamin B15 (pangamic acid)', *Nutr Cancer*, 3: 129-33.

Generali, E., A. Ceribelli, M. A. Stazi, and C. Selmi. 2017. 'Lessons learned from twins in autoimmune and chronic inflammatory diseases', *J Autoimmun*, 83: 51-61.

Gengatharan, A., S. Malvaut, A. Marymonchyk, M. Ghareghani, M. Snapyan, J. Fischer-Sternjak, J. Ninkovic, M. Götz, and A. Saghatelyan. 2021. 'Adult neural stem cell activation in mice is regulated by the day/night cycle and intracellular calcium dynamics', *Cell*, 184: 709-22.e13.

Gerrard, B., V. Singh, O. Babenko, I. Gauthier, V. Wee Yong, I. Kovalchuk, A. Luczak, and G. A. S. Metz. 2017. 'Chronic mild stress exacerbates severity of experimental autoimmune encephalomyelitis in association with altered non-coding RNA and metabolic biomarkers', *Neuroscience*, 359: 299-307.

Ghareghani M, Pons V, Laflamme N, Zibara K, and Rivest S. 2022. "Inhibiting Nighttime Melatonin and Boosting Cortisol Increase Patrolling Monocytes, Phagocytosis, and Myelination in a Murine Model of Multiple Sclerosis." In. Exp Mol Med.

Ghareghani, M., S. Dokoohaki, A. Ghanbari, N. Farhadi, K. Zibara, S. Khodadoust, M. Parishani, M. Ghavamizadeh, and H. Sadeghi. 2017a. 'Melatonin exacerbates acute experimental autoimmune encephalomyelitis by enhancing the serum levels of lactate: A potential biomarker of multiple sclerosis progression', *Clin Exp Pharmacol Physiol*, 44: 52-61.

Ghareghani, M., A. Ghanbari, S. Dokoohaki, N. Farhadi, S. M. Hosseini, R. Mohammadi, and H. Sadeghi. 2016. 'Methylprednisolone improves lactate metabolism through reduction of elevated serum lactate in rat model of multiple sclerosis', *Biomed Pharmacother*, 84: 1504-09.

Ghareghani, M., R. J. Reiter, K. Zibara, and N. Farhadi. 2018a. 'Latitude, Vitamin D, Melatonin, and Gut Microbiota Act in Concert to Initiate Multiple Sclerosis: A New Mechanistic Pathway', *Front Immunol*, 9: 2484.

Ghareghani, M., H. Sadeghi, K. Zibara, N. Danaei, H. Azari, and A. Ghanbari. 2017a. 'Melatonin Increases Oligodendrocyte Differentiation in Cultured Neural Stem Cells', *Cell Mol Neurobiol*, 37: 1319-24.

Ghareghani, M., L. Scavo, D. Arnoult, K. Zibara, and N. Farhadi. 2018a. 'Melatonin therapy reduces the risk of osteoporosis and normalizes bone formation in multiple sclerosis', *Fundam Clin Pharmacol*, 32: 181-87.

Ghareghani, M., L. Scavo, Y. Jand, N. Farhadi, H. Sadeghi, A. Ghanbari, S. Mondello, D. Arnoult, S. Gharaghani, and K. Zibara. 2019a. 'Melatonin Therapy Modulates Cerebral Metabolism and Enhances Remyelination by Increasing PDK4 in a Mouse Model of Multiple Sclerosis', *Front Pharmacol*, 10: 147.

Ghareghani, M., K. Zibara, R. J. Reiter, and S. Rivest. 2022. 'Reduced melatonin levels may facilitate glioblastoma initiation in the subventricular zone', *Expert Rev Mol Med*, 24: e24.

Ghareghani, M., K. Zibara, H. Sadeghi, and N. Farhadi. 2018. 'Spasticity Treatment Ameliorates the Efficacy of Melatonin Therapy in Experimental Autoimmune Encephalomyelitis (EAE) Mouse Model of Multiple Sclerosis', *Cell Mol Neurobiol*, 38: 1145-51.

Ghareghani, Majid, Shima Dokoohaki, Amir Ghanbari, Naser Farhadi, Kazem Zibara, Saeid Khodadoust, Mohammad Parishani, Mehdi Ghavamizadeh, and Heibatollah Sadeghi. 2017b. 'Melatonin exacerbates acute experimental autoimmune encephalomyelitis by enhancing the serum levels of lactate: A potential biomarker of multiple sclerosis progression', *Clinical and Experimental Pharmacology and Physiology*, 44: 52-61.

Ghareghani, Majid, Zahra Farhadi, Serge Rivest, and Kazem Zibara. 2022. 'PDK4 Inhibition Ameliorates Melatonin Therapy by Modulating Cerebral Metabolism and Remyelination in an EAE Demyelinating Mouse Model of Multiple Sclerosis', *Frontiers in Immunology*, 13.

Ghareghani, Majid, Russel J. Reiter, Kazem Zibara, and Naser Farhadi. 2018b. 'Latitude, Vitamin D, Melatonin, and Gut Microbiota Act in Concert to Initiate Multiple Sclerosis: A New Mechanistic Pathway', *Frontiers in immunology*, 9: 2484-84.

Ghareghani, Majid, Heibatollah Sadeghi, Kazem Zibara, Nazanin Danaei, Hassan Azari, and Amir Ghanbari. 2017b. 'Melatonin Increases Oligodendrocyte Differentiation in Cultured Neural Stem Cells', *Cellular and Molecular Neurobiology*, 37: 1319-24.

Ghareghani, Majid, Linda Scavo, Damien Arnoult, Kazem Zibara, and Naser Farhadi. 2018b. 'Melatonin therapy reduces the risk of osteoporosis and normalizes bone formation in multiple sclerosis', *Fundamental & Clinical Pharmacology*, 32: 181-87.

Ghareghani, Majid, Linda Scavo, Yahya Jand, Naser Farhadi, Hossein Sadeghi, Amir Ghanbari, Stefania Mondello, Damien Arnoult, Sajjad Gharaghani, and Kazem Zibara. 2019b. 'Melatonin Therapy Modulates Cerebral Metabolism and Enhances Remyelination by Increasing PDK4 in a Mouse Model of Multiple Sclerosis', *Frontiers in pharmacology*, 10: 147-47.

Ghorbani, A., M. Salari, V. Shaygannejad, and R. Norouzi. 2013. 'The role of melatonin in the pathogenesis of multiple sclerosis: a case-control study', *Int J Prev Med*, 4: S180-4.

Golan, D., E. Staun-Ram, L. Glass-Marmor, I. Lavi, O. Rozenberg, S. Dishon, M. Barak, S. Ish-Shalom, and A. Miller. 2013. 'The influence of vitamin D supplementation on melatonin status in patients with multiple sclerosis', *Brain Behav Immun*, 32: 180-5.

Goldmann, T., P. Wieghofer, M. J. Jordão, F. Prutek, N. Hagemeyer, K. Frenzel, L. Amann, O. Staszewski, K. Kierdorf, M. Krueger, G. Locatelli, H. Hochgerner, R. Zeiser, S. Epelman, F. Geissmann, J. Priller, F. M. Rossi, I. Bechmann, M. Kerschensteiner, S. Linnarsson, S. Jung, and M. Prinz. 2016. 'Origin, fate and dynamics of macrophages at central nervous system interfaces', *Nat Immunol*, 17: 797-805.

Gong, S., Y. L. Miao, G. Z. Jiao, M. J. Sun, H. Li, J. Lin, M. J. Luo, and J. H. Tan. 2015. 'Dynamics and correlation of serum cortisol and corticosterone under different physiological or stressful conditions in mice', *PLoS One*, 10: e0117503.

Gonzalez-Perez, O. 2014. 'The ventricular-subventricular zone: a source of oligodendrocytes in the adult brain', *Front Cell Neurosci*, 8: 137.

Goodin, Douglas S. 2009. 'The Causal Cascade to Multiple Sclerosis: A Model for MS Pathogenesis', *PLoS One*, 4: e4565.

Gooley, J. J., K. Chamberlain, K. A. Smith, S. B. Khalsa, S. M. Rajaratnam, E. Van Reen, J. M. Zeitzer, C. A. Czeisler, and S. W. Lockley. 2011a. 'Exposure to room light before bedtime suppresses melatonin onset and shortens melatonin duration in humans', *J Clin Endocrinol Metab*, 96: E463-72.

Gooley, Joshua J., Kyle Chamberlain, Kurt A. Smith, Sat Bir S. Khalsa, Shantha M. W. Rajaratnam, Eliza Van Reen, Jamie M. Zeitzer, Charles A. Czeisler, and Steven W. Lockley. 2011b. 'Exposure to Room Light before Bedtime Suppresses Melatonin Onset and Shortens Melatonin Duration in Humans', *The Journal of Clinical Endocrinology & Metabolism*, 96: E463-E72.

Gorlé, N., C. Blaecher, E. Bauwens, C. Vandendriessche, S. Balusu, J. Vandewalle, C. Van Cauwenberghe, E. Van Wonterghem, G. Van Imschoot, C. Liu, R. Ducatelle, C. Libert, F. Haesebrouck, A. Smet, and R. E. Vandenbroucke. 2018. 'The choroid plexus epithelium as a

novel player in the stomach-brain axis during Helicobacter infection', *Brain, Behavior, and Immunity*, 69: 35-47.

Grivas, T. B., and O. D. Savvidou. 2007a. 'Melatonin the "light of night" in human biology and adolescent idiopathic scoliosis', *Scoliosis*, 2: 6.

Grivas, Theodoros B., and Olga D. Savvidou. 2007b. 'Melatonin the "light of night" in human biology and adolescent idiopathic scoliosis', *Scoliosis*, 2: 6-6.

Groh, Laszlo, Samuel T. Keating, Leo A. B. Joosten, Mihai G. Netea, and Niels P. Riksen. 2018. 'Monocyte and macrophage immunometabolism in atherosclerosis', *Seminars in Immunopathology*, 40: 203-14.

Gudewill, S., T. Pollmächer, H. Vedder, W. Schreiber, K. Fassbender, and F. Holsboer. 1992. 'Nocturnal plasma levels of cytokines in healthy men', *Eur Arch Psychiatry Clin Neurosci*, 242: 53-6.

Guerrero-Vargas, Natalí N., Raful Navarro-Espíndola, Mara A. Guzmán-Ruíz, María Del Carmen Basualdo, Estefania Espitia-Bautista, Ana López-Bago, Ricardo Lascurain, Cinthya Córdoba-Manilla, Ruud M. Buijs, and Carolina Escobar. 2017. 'Circadian disruption promotes tumor growth by anabolic host metabolism; experimental evidence in a rat model', *BMC cancer*, 17: 625-25.

Hamdi, Anwar. 1998. 'Melatonin administration increases the affinity of D2 dopamine receptors in the rat striatum', *Life Sciences*, 63: 2115-20.

Handel, A. E., A. J. Williamson, G. Disanto, L. Handunnetthi, G. Giovannoni, and S. V. Ramagopalan. 2010. 'An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis', *PLoS One*, 5.

Hansson, I., R. Holmdahl, and R. Mattsson. 1990. 'Constant darkness enhances autoimmunity to type II collagen and exaggerates development of collagen-induced arthritis in DBA/1 mice', *J Neuroimmunol*, 27: 79-84.

———. 1992. 'The pineal hormone melatonin exaggerates development of collagen-induced arthritis in mice', *J Neuroimmunol*, 39: 23-30.

———. 1993. 'Pinealectomy ameliorates collagen II-induced arthritis in mice', *Clin Exp Immunol*, 92: 432-6.

Häusler, D., S. Torke, E. Peelen, T. Bertsch, M. Djukic, R. Nau, C. Larochelle, S. S. Zamvil, W. Brück, and M. S. Weber. 2019. 'High dose vitamin D exacerbates central nervous system autoimmunity by raising T-cell excitatory calcium', *Brain*, 142: 2737-55.

Hedström, A. K., T. Olsson, I. Kockum, J. Hillert, and L. Alfredsson. 2020. 'Low sun exposure increases multiple sclerosis risk both directly and indirectly', *J Neurol*, 267: 1045-52.

Hofman, Florence M., and Thomas C. Chen. 2016. 'Chapter 3 - Choroid Plexus: Structure and Function.' in Josh Neman and Thomas C. Chen (eds.), *The Choroid Plexus and Cerebrospinal Fluid* (Academic Press: San Diego).

Holick, M. F. 2003. 'Vitamin D: A millenium perspective', *J Cell Biochem*, 88: 296-307.

———. 2004. 'Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease', *Am J Clin Nutr*, 80: 1678s-88s.

Hong, Y., Y. Jin, K. Park, J. Choi, H. Kang, S. R. Lee, and Y. Hong. 2019. 'Elevated Serum Melatonin under Constant Darkness Enhances Neural Repair in Spinal Cord Injury through Regulation of Circadian Clock Proteins Expression', *J Clin Med*, 8.

Hossain, M. F., M. S. Uddin, G. M. S. Uddin, D. M. Sumsuzzman, M. S. Islam, G. E. Barreto, B. Mathew, and G. M. Ashraf. 2019. 'Melatonin in Alzheimer's Disease: A Latent Endogenous Regulator of Neurogenesis to Mitigate Alzheimer's Neuropathology', *Mol Neurobiol*, 56: 8255-76.

Howatson, Glyn, Phillip G. Bell, Jamie Tallent, Benita Middleton, Malachy P. McHugh, and Jason Ellis. 2012. 'Effect of tart cherry juice (Prunus cerasus) on melatonin levels and enhanced sleep quality', *European Journal of Nutrition*, 51: 909-16.

Hsu, C. N., and Y. L. Tain. 2020. 'Light and Circadian Signaling Pathway in Pregnancy: Programming of Adult Health and Disease', *Int J Mol Sci*, 21.

Huang, Yu-Ming, Chih-Wen Chi, Pao-Shu Wu, Hung-Chi Tai, Ming-Nan Chien, and Yu-Jen Chen. 2022. 'Adrenal Gland Irradiation Causes Fatigue Accompanied by Reactive Changes in Cortisol Levels', *Journal of Clinical Medicine*, 11.

Ichihara, Y., T. Doi, Y. Ryu, M. Nagao, Y. Sawada, and T. Ogata. 2017. 'Oligodendrocyte Progenitor Cells Directly Utilize Lactate for Promoting Cell Cycling and Differentiation', *J Cell Physiol*, 232: 986-95.

Irving, A. A., S. J. Marling, J. Seeman, L. A. Plum, and H. F. DeLuca. 2019. 'UV light suppression of EAE (a mouse model of multiple sclerosis) is independent of vitamin D and its receptor', *Proc Natl Acad Sci U S A*, 116: 22552-55.

Ishida, A., T. Mutoh, T. Ueyama, H. Bando, S. Masubuchi, D. Nakahara, G. Tsujimoto, and H. Okamura. 2005. 'Light activates the adrenal gland: timing of gene expression and glucocorticoid release', *Cell Metab*, 2: 297-307.

Ismailova, K., P. Poudel, A. Parlesak, P. Frederiksen, and B. L. Heitmann. 2019. 'Vitamin D in early life and later risk of multiple sclerosis-A systematic review, meta-analysis', *PLoS One*, 14: e0221645.

Jand, Y., M. H. Ghahremani, A. Ghanbari, S. Ejtemaei-Mehr, G. J. Guillemin, and M. Ghazi-Khansari. 2022. 'Melatonin ameliorates disease severity in a mouse model of multiple sclerosis by modulating the kynurenine pathway', *Sci Rep*, 12: 15963.

Jang, T., N. S. Litofsky, T. W. Smith, A. H. Ross, and L. D. Recht. 2004. 'Aberrant nestin expression during ethylnitrosourea-(ENU)-induced neurocarcinogenesis', *Neurobiol Dis*, 15: 544-52.

Janković, B. D., K. Isaković, and S. Petrović. 1970. 'Effect of pinealectomy on immune reactions in the rat', *Immunology*, 18: 1-6.

Jelinek, G. A., C. H. Marck, T. J. Weiland, N. Pereira, D. M. van der Meer, and E. J. Hadgkiss. 2015. 'Latitude, sun exposure and vitamin D supplementation: associations with quality of life and disease outcomes in a large international cohort of people with multiple sclerosis', *BMC Neurol*, 15: 132.

Jellinger, Kurt A. 2010. 'Basic mechanisms of neurodegeneration: a critical update', *Journal of cellular and molecular medicine*, 14: 457-87.

Jung, C. M., S. B. Khalsa, F. A. Scheer, C. Cajochen, S. W. Lockley, C. A. Czeisler, and K. P. Wright, Jr. 2010. 'Acute effects of bright light exposure on cortisol levels', *J Biol Rhythms*, 25: 208-16.

Kadena, M., Y. Kumagai, A. Vandenbon, H. Matsushima, H. Fukamachi, N. Maruta, H. Kataoka, T. Arimoto, H. Morisaki, T. Funatsu, and H. Kuwata. 2017. 'Microarray and gene co-expression analysis reveals that melatonin attenuates immune responses and modulates actin rearrangement in macrophages', *Biochem Biophys Res Commun*, 485: 414-20.

Kadry, H., B. Noorani, and L. Cucullo. 2020. 'A blood-brain barrier overview on structure, function, impairment, and biomarkers of integrity', *Fluids Barriers CNS*, 17: 69.

Kang, J. C., M. Ahn, Y. S. Kim, C. Moon, Y. Lee, M. B. Wie, Y. J. Lee, and T. Shin. 2001. 'Melatonin ameliorates autoimmune encephalomyelitis through suppression of intercellular adhesion molecule-1', *J Vet Sci*, 2: 85-9.

Kanikowska, Dominika, Magdalena Roszak, Rafał Rutkowski, Maki Sato, Dorota Sikorska, Zofia Orzechowska, Andrzej Bręborowicz, and Janusz Witowski. 2019. 'Seasonal differences in rhythmicity of salivary cortisol in healthy adults', *Journal of Applied Physiology*, 126: 764-70.

Karaki, H., and G. B. Weiss. 1984. 'Calcium channels in smooth muscle', *Gastroenterology*, 87: 960-70.

Karasek, M. 2004. 'Melatonin, human aging, and age-related diseases', *Experimental Gerontology*, 39: 1723-29.

Kashani, I. R., Z. Rajabi, M. Akbari, G. Hassanzadeh, A. Mohseni, M. K. Eramsadati, K. Rafiee, C. Beyer, M. Kipp, and A. Zendedel. 2014. 'Protective effects of melatonin against mitochondrial injury in a mouse model of multiple sclerosis', *Exp Brain Res*, 232: 2835-46.

Kauppila, A., A. Kivelä, A. Pakarinen, and O. Vakkuri. 1987. 'Inverse seasonal relationship between melatonin and ovarian activity in humans in a region with a strong seasonal contrast in luminosity', *J Clin Endocrinol Metab*, 65: 823-8.

Khan, Omar, Yimin Shen, Christina Caon, F en Bao, Wendy Ching, Melissa Reznar, Alyssa Buccheister, Jiani Hu, Zahid Latif, and Alexandros Tselis. 2005. 'Axonal metabolic recovery and potential neuroprotective effect of glatiramer acetate in relapsing-remitting multiple sclerosis', *Multiple Sclerosis Journal*, 11: 646-51.

Khanna, L., B. Zeydan, O. H. Kantarci, and M. Camilleri. 2022. 'Gastrointestinal motility disorders in patients with multiple sclerosis: A single-center study', *Neurogastroenterol Motil*, 34: e14326.

Kim, M., E. Ladomersky, A. Mozny, M. Kocherginsky, K. O'Shea, Z. Z. Reinstein, L. Zhai, A. Bell, K. L. Lauing, L. Bollu, E. Rabin, K. Dixit, P. Kumthekar, L. C. Platanias, L. Hou, Y. Zheng, J. Wu, B. Zhang, M. Hrachova, S. A. Merrill, M. M. Mrugala, V. C. Prabhu, C. Horbinski, C. D. James, B. Yamini, Q. T. Ostrom, M. O. Johnson, D. A. Reardon, R. V. Lukas, and D. A. Wainwright. 2021. 'Glioblastoma as an age-related neurological disorder in adults', *Neuro-oncology advances*, 3: vdab125.

Kinker, G. S., L. H. Ostrowski, P. A. C. Ribeiro, R. Chanoch, S. M. Muxel, I. Tirosh, G. Spadoni, S. Rivara, V. R. Martins, T. G. Santos, R. P. Markus, and Pacm Fernandes. 2021. 'MT1 and MT2 melatonin receptors play opposite roles in brain cancer progression', *J Mol Med (Berl)*, 99: 289-301.

Kivelä, A. 1991. 'Serum melatonin during human pregnancy', *Acta Endocrinol (Copenh)*, 124: 233-7.

Klocke, Shilpa, and Nicole Hahn. 2019. 'Multiple sclerosis', *The mental health clinician*, 9: 349-58.

Kubicka-Baczyk, K., B. Labuz-Roszak, K. Pierzchala, M. Adamczyk-Sowa, and A. Machowska-Majchrzak. 2015. 'Calcium-phosphate metabolism in patients with multiple sclerosis', *J Endocrinol Invest*, 38: 635-42.

Kuklina, E. M. 2016. '[Melatonin as an inducing factor for multiple sclerosis]', *Zh Nevrol Psikhiatr Im S S Korsakova*, 116: 102-05.

Labunets, I. F., and A. E. Rodnichenko. 2019. '[Melatonin effects in young and aged mice with toxic cuprizone-induced model of demyelination.]', *Adv Gerontol*, 32: 338-46.

Ladomersky, E., D. M. Scholtens, M. Kocherginsky, E. A. Hibler, E. T. Bartom, S. Otto-Meyer, L. Zhai, K. L. Lauing, J. Choi, J. A. Sosman, J. D. Wu, B. Zhang, R. V. Lukas, and D. A. Wainwright. 2019. 'The Coincidence Between Increasing Age, Immunosuppression, and the Incidence of Patients With Glioblastoma', *Front Pharmacol*, 10: 200.

Lampron, A., A. Larochelle, N. Laflamme, P. Préfontaine, M. M. Plante, M. G. Sánchez, V. W. Yong, P. K. Stys, MÈ Tremblay, and S. Rivest. 2015. 'Inefficient clearance of myelin debris by microglia impairs remyelinating processes', *J Exp Med*, 212: 481-95.

Laursen, J. H., H. B. Søndergaard, P. S. Sørensen, F. Sellebjerg, and A. B. Oturai. 2016. 'Association between age at onset of multiple sclerosis and vitamin D level-related factors', *Neurology*, 86: 88-93.

Lavrnja, Irena, Kosara Smiljanic, Danijela Savic, Aleksandra Mladenovic-Djordjevic, Katarina Tesovic, Selma Kanazir, and Sanja Pekovic. 2017. 'Expression profiles of cholesterol metabolism-related genes are altered during development of experimental autoimmune encephalomyelitis in the rat spinal cord', *Scientific Reports*, 7: 2702.

Lee, Joo Ho, Jeong Eun Lee, Jee Ye Kahng, Se Hoon Kim, Jun Sung Park, Seon Jin Yoon, Ji-Yong Um, Woo Kyeong Kim, June-Koo Lee, Junseong Park, Eui Hyun Kim, Ji-Hyun Lee, Joon-Hyuk Lee, Won-Suk Chung, Young Seok Ju, Sung-Hong Park, Jong Hee Chang, Seok-Gu Kang, and Jeong Ho Lee. 2018. 'Human glioblastoma arises from subventricular zone cells with low-level driver mutations', *Nature*, 560: 243-47.

Leppäluoto, J., K. Sikkilä, V. B. Meyer-Rochow, and J. Hassi. 2003. 'Low melatonin secretion associates with albedo in circumpolar environments', *J Pineal Res*, 35: 158-62.

Leproult, R., E. F. Colecchia, M. L'Hermite-Balériaux, and E. Van Cauter. 2001. 'Transition from dim to bright light in the morning induces an immediate elevation of cortisol levels', *J Clin Endocrinol Metab*, 86: 151-7.

Levenstein, S., C. Prantera, V. Varvo, M. L. Scribano, A. Andreoli, C. Luzi, M. Arcà, E. Berto, G. Milite, and A. Marcheggiano. 2000. 'Stress and exacerbation in ulcerative colitis: a prospective study of patients enrolled in remission', *Am J Gastroenterol*, 95: 1213-20.

Li, Jingen, Virend K. Somers, Hao Xu, Francisco Lopez-Jimenez, and Naima Covassin. 2022. 'Trends in Use of Melatonin Supplements Among US Adults, 1999-2018', *JAMA*, 327: 483-85.

Liguori, M., M. G. Marrosu, M. Pugliatti, F. Giuliani, F. De Robertis, E. Cocco, G. B. Zimatore, P. Livrea, and M. Trojano. 2000. 'Age at onset in multiple sclerosis', *Neurol Sci*, 21: S825-9.

Lin, Po-Han, Yen-Ting Tung, Hsin-Yuan Chen, Yi-Fen Chiang, Hui-Chih Hong, Ko-Chieh Huang, Sung-Po Hsu, Tsui-Chin Huang, and Shih-Min Hsia. 2020. 'Melatonin activates cell death programs for the suppression of uterine leiomyoma cell proliferation', *Journal of Pineal Research*, 68: e12620.

Long, T., Y. Yang, L. Peng, and Z. Li. 2018. 'Neuroprotective Effects of Melatonin on Experimental Allergic Encephalomyelitis Mice Via Anti-Oxidative Stress Activity', *J Mol Neurosci*, 64: 233-41.

Lublin, F. D., S. C. Reingold, J. A. Cohen, G. R. Cutter, P. S. Sørensen, A. J. Thompson, J. S. Wolinsky, L. J. Balcer, B. Banwell, F. Barkhof, B. Bebo, Jr., P. A. Calabresi, M. Clanet, G. Comi, R. J. Fox, M. S. Freedman, A. D. Goodman, M. Inglese, L. Kappos, B. C. Kieseier, J. A. Lincoln, C. Lubetzki, A. E. Miller, X. Montalban, P. W. O'Connor, J. Petkau, C. Pozzilli, R. A. Rudick, M. P. Sormani, O. Stüve, E. Waubant, and C. H. Polman. 2014. 'Defining the clinical course of multiple sclerosis: the 2013 revisions', *Neurology*, 83: 278-86.

Maeser, Danielle, and Nicole Maeser. 2018. 'Sunlight Exposure, Vitamin D Synthesis, and Multiple Sclerosis in the Northern and Southern Regions of the United States', *Minnesota Undergraduate Research & Academic Journal*, 1.

Mahad, D. J., and R. M. Ransohoff. 2003. 'The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE)', *Semin Immunol*, 15: 23-32.

Mahmoodi, M., F. Sedghy, and A. Jafarzadeh. 2020. 'Effect of Treatment with Thyme Extract on Urinary Levels of Melatonin in an Experimental Autoimmune Encephalomyelitis Mouse Model', *Rep Biochem Mol Biol*, 8: 407-12.

Mansouri, A., I. Gaou, C. De Kerguenec, S. Amsellem, D. Haouzi, A. Berson, A. Moreau, G. Feldmann, P. Lettéron, D. Pessayre, and B. Fromenty. 1999. 'An alcoholic binge causes massive degradation of hepatic mitochondrial DNA in mice', *Gastroenterology*, 117: 181-90.

Mansouri, B., S. Asadollahi, K. Heidari, M. Fakhri, F. Assarzadegan, M. Nazari, and A. Divani. 2014. 'Risk factors for increased multiple sclerosis susceptibility in the Iranian population', *J Clin Neurosci*, 21: 2207-11.

Mao, Lulu, and Steven M. Hill. 2005. 'Inhibition of cell proliferation and blockade of cell invasion by melatonin in human breast cancer cells mediated through multiple signaling pathways', *Cancer Research*, 65: 58.

Marcus, J. F., S. M. Shalev, C. A. Harris, D. S. Goodin, and S. A. Josephson. 2012. 'Severe hypercalcemia following vitamin d supplementation in a patient with multiple sclerosis: a note of caution', *Arch Neurol*, 69: 129-32.

Marquez, E., S. Sánchez-Fidalgo, J. R. Calvo, C. A. la de Lastra, and V. Motilva. 2006. 'Acutely administered melatonin is beneficial while chronic melatonin treatment aggravates the evolution of TNBS-induced colitis', *J Pineal Res*, 40: 48-55.

Martín, M., M. Macías, G. Escames, R. J. Reiter, M. T. Agapito, G. G. Ortiz, and D. Acuña-Castroviejo. 2000. 'Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo', *J Pineal Res*, 28: 242-8.

Martin, R., H. F. McFarland, and D. E. McFarlin. 1992. 'Immunological aspects of demyelinating diseases', *Annu Rev Immunol*, 10: 153-87.

Masuda, T., R. Sankowski, O. Staszewski, C. Böttcher, L. Amann, Sagar, C. Scheiwe, S. Nessler, P. Kunz, G. van Loo, V. A. Coenen, P. C. Reinacher, A. Michel, U. Sure, R. Gold, D. Grün, J. Priller, C. Stadelmann, and M. Prinz. 2019. 'Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution', *Nature*, 566: 388-92.

Melamud, L., D. Golan, R. Luboshitzky, I. Lavi, and A. Miller. 2012. 'Melatonin dysregulation, sleep disturbances and fatigue in multiple sclerosis', *J Neurol Sci*, 314: 37-40.

Melief, J., J. W. Koper, E. Endert, H. J. Møller, J. Hamann, B. M. Uitdehaag, and I. Huitinga. 2016. 'Glucocorticoid receptor haplotypes conferring increased sensitivity (BclI and N363S) are associated with faster progression of multiple sclerosis', *J Neuroimmunol*, 299: 84-89.

Menendez-Menendez, J., and C. Martinez-Campa. 2018. 'Melatonin: An Anti-Tumor Agent in Hormone-Dependent Cancers', *Int J Endocrinol*, 2018: 3271948.

Meng, X., Y. Li, S. Li, Y. Zhou, R. Y. Gan, D. P. Xu, and H. B. Li. 2017. 'Dietary Sources and Bioactivities of Melatonin', *Nutrients*, 9.

Menn, B., J. M. Garcia-Verdugo, C. Yaschine, O. Gonzalez-Perez, D. Rowitch, and A. Alvarez-Buylla. 2006. 'Origin of oligodendrocytes in the subventricular zone of the adult brain', *J Neurosci*, 26: 7907-18.

Merrill, J. E., D. H. Kono, J. Clayton, D. G. Ando, D. R. Hinton, and F. M. Hofman. 1992. 'Inflammatory leukocytes and cytokines in the peptide-induced disease of experimental allergic encephalomyelitis in SJL and B10.PL mice', *Proc Natl Acad Sci U S A*, 89: 574-8.

Miller, E., A. Walczak, I. Majsterek, and J. Kędziora. 2013. 'Melatonin reduces oxidative stress in the erythrocytes of multiple sclerosis patients with secondary progressive clinical course', *J Neuroimmunol*, 257: 97-101.

Min, Byong-Keol, Sungmi Park, Hyeon-Ji Kang, Dong Wook Kim, Hye Jin Ham, Chae-Myeong Ha, Byung-Jun Choi, Jung Yi Lee, Chang Joo Oh, Eun Kyung Yoo, Hui Eon Kim, Byung-Gyu Kim, Jae-Han Jeon, Do Young Hyeon, Daehee Hwang, Yong-Hoon Kim, Chul-Ho Lee, Taeho Lee, Jung-whan Kim, Yeon-Kyung Choi, Keun-Gyu Park, Ajay Chawla, Jongsoon Lee, Robert A. Harris, and In-Kyu Lee. 2019. 'Pyruvate Dehydrogenase Kinase Is a Metabolic Checkpoint for Polarization of Macrophages to the M1 Phenotype', *Frontiers in Immunology*, 10.

Mirzadeh, Z., F. T. Merkle, M. Soriano-Navarro, J. M. Garcia-Verdugo, and A. Alvarez-Buylla. 2008. 'Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain', *Cell Stem Cell*, 3: 265-78.

Miyauchi, Eiji, Seok-Won Kim, Wataru Suda, Masami Kawasumi, Satoshi Onawa, Naoko Taguchi-Atarashi, Hidetoshi Morita, Todd D. Taylor, Masahira Hattori, and Hiroshi Ohno. 2020. 'Gut microorganisms act together to exacerbate inflammation in spinal cords', *Nature*, 585: 102-06.

Moffett, John R, Brian Ross, Peethambaran Arun, Chikkathur N Madhavarao, and Aryan MA Namboodiri. 2007. 'N-Acetylaspartate in the CNS: from neurodiagnostics to neurobiology', *Progress in neurobiology*, 81: 89-131.

Mohajeri, M. Hasan, Giorgio La Fata, Robert E. Steinert, and Peter Weber. 2018. 'Relationship between the gut microbiome and brain function', *Nutrition Reviews*, 76: 481-96.

Morales, Aixa V., and Helena Mira. 2019. 'Adult Neural Stem Cells: Born to Last', *Frontiers in Cell and Developmental Biology*, 7.

Moriya, T., N. Horie, M. Mitome, and K. Shinohara. 2007. 'Melatonin influences the proliferative and differentiative activity of neural stem cells', *J Pineal Res*, 42: 411-8.

Mostert, Jop P, Paul E Sijens, Matthijs Oudkerk, and Jacques De Keyser. 2006. 'Fluoxetine increases cerebral white matter NAA/Cr ratio in patients with multiple sclerosis', *Neuroscience letters*, 402: 22-24.

Mu, Q., J. Kirby, C. M. Reilly, and X. M. Luo. 2017. 'Leaky Gut As a Danger Signal for Autoimmune Diseases', *Front Immunol*, 8: 598.

Mueller, A. M., X. Pedré, T. Stempfl, I. Kleiter, S. Couillard-Despres, L. Aigner, G. Giegerich, and A. Steinbrecher. 2008. 'Novel role for SLPI in MOG-induced EAE revealed by spinal cord expression analysis', *J Neuroinflammation*, 5: 20.

Munk Nielsen, N., G. Corn, M. Frisch, E. Stenager, N. Koch-Henriksen, J. Wohlfahrt, M. Magyari, and M. Melbye. 2019. 'Multiple sclerosis among first- and second-generation immigrants in Denmark: a population-based cohort study', *Brain*, 142: 1587-97.

Na, Yi Rang, Daun Jung, Juha Song, Jong-Wan Park, Jung Joo Hong, and Seung Hyeok Seok. 2020. 'Pyruvate dehydrogenase kinase is a negative regulator of interleukin-10 production in macrophages', *Journal of Molecular Cell Biology*, 12: 543-55.

Nagelkerken, L. 1998. 'Role of Th1 and Th2 cells in autoimmune demyelinating disease', *Braz J Med Biol Res*, 31: 55-60.

Nait-Oumesmar, B., N. Picard-Riera, C. Kerninon, L. Decker, D. Seilhean, G. U. Höglinger, E. C. Hirsch, R. Reynolds, and A. Baron-Van Evercooren. 2007. 'Activation of the subventricular zone in multiple sclerosis: evidence for early glial progenitors', *Proc Natl Acad Sci U S A*, 104: 4694-9.

Nakahara, Daiichiro, Masato Nakamura, Masayuki Iigo, and Hitoshi Okamura. 2003. 'Bimodal circadian secretion of melatonin from the pineal gland in a living CBA mouse', *Proceedings of the National Academy of Sciences*, 100: 9584.

Nakatomi, H., T. Kuriu, S. Okabe, S. Yamamoto, O. Hatano, N. Kawahara, A. Tamura, T. Kirino, and M. Nakafuku. 2002. 'Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors', *Cell*, 110: 429-41.

Narayanan, Sridar, Nicola De Stefano, Gordon S Francis, Rozie Arnaoutelis, Zografos Caramanos, D Louis Collins, Daniel Pelletier, Barry GW Arnason, Jack P Antel, and Douglas L Arnold. 2001. 'Axonal metabolic recovery in multiple sclerosis patients treated with interferon β–1b', *Journal of neurology*, 248: 979-86.

Nashold, Faye E., Corwin D. Nelson, Lauren M. Brown, and Colleen E. Hayes. 2013. 'One calcitriol dose transiently increases Helios⁺FoxP3⁺ T cells and ameliorates autoimmune demyelinating disease', *Journal of Neuroimmunology*, 263: 64-74.

Newhook, L. A., S. Sloka, M. Grant, E. Randell, C. S. Kovacs, and L. K. Twells. 2009. 'Vitamin D insufficiency common in newborns, children and pregnant women living in Newfoundland and Labrador, Canada', *Matern Child Nutr*, 5: 186-91.

Nielsen, N. M., K. L. Munger, N. Koch-Henriksen, D. M. Hougaard, M. Magyari, K. T. Jørgensen, M. Lundqvist, J. Simonsen, T. Jess, A. Cohen, E. Stenager, and A. Ascherio. 2017. 'Neonatal vitamin D status and risk of multiple sclerosis: A population-based case-control study', *Neurology*, 88: 44-51.

Nijland, Philip G., Remco J. Molenaar, Susanne M. A. van der Pol, Paul van der Valk, Cornelis J. F. van Noorden, Helga E. de Vries, and Jack van Horssen. 2015. 'Differential expression of glucose-metabolizing enzymes in multiple sclerosis lesions', *Acta Neuropathologica Communications*, 3: 79.

Obrenovich, Mark E. M. 2018. 'Leaky Gut, Leaky Brain?', *Microorganisms*, 6: 107.

Olivier, Paul, Romain H. Fontaine, Gauthier Loron, Juliette Van Steenwinckel, Valérie Biran, Véronique Massonneau, Angela Kaindl, Jeremie Dalous, Christiane Charriaut-Marlangue, Marie-Stéphane Aigrot, Julien Pansiot, Catherine Verney, Pierre Gressens, and Olivier Baud. 2009. 'Melatonin Promotes Oligodendroglial Maturation of Injured White Matter in Neonatal Rats', *PLoS One*, 4: e7128.

Ostkamp, P., A. Salmen, B. Pignolet, D. Görlich, T. F. M. Andlauer, A. Schulte-Mecklenbeck, G. Gonzalez-Escamilla, F. Bucciarelli, I. Gennero, J. Breuer, G. Antony, T. Schneider-Hohendorf, N. Mykicki, A. Bayas, F. Then Bergh, S. Bittner, H. P. Hartung, M. A. Friese, R. A. Linker, F. Luessi, K. Lehmann-Horn, M. Mühlau, F. Paul, M. Stangel, B. Tackenberg, H. Tumani, C. Warnke, F. Weber, B. Wildemann, U. K. Zettl, U. Ziemann, B. Müller-Myhsok, T. Kümpfel, L. Klotz, S. G. Meuth, F. Zipp, B. Hemmer, R. Hohlfeld, D. Brassat, R. Gold, C. C. Gross, C. Lukas, S. Groppa, K. Loser, H. Wiendl, and N. Schwab. 2021. 'Sunlight exposure exerts immunomodulatory effects to reduce multiple sclerosis severity', *Proc Natl Acad Sci U S A*, 118.

Parikh, Sumit, Russell Saneto, Marni J Falk, Irina Anselm, Bruce H Cohen, and Richard Haas. 2009. 'A modern approach to the treatment of mitochondrial disease', *Current treatment options in neurology*, 11: 414.

Park, H., and N. H. Jeoung. 2016. 'Inflammation increases pyruvate dehydrogenase kinase 4 (PDK4) expression via the Jun N-Terminal Kinase (JNK) pathway in C2C12 cells', *Biochem Biophys Res Commun*, 469: 1049-54.

Parmar, P., and S. Daya. 2001. 'The effect of copper on (3H)-tryptophan metabolism in organ cultures of rat pineal glands', *Metab Brain Dis*, 16: 199-205.

Pastural, Élodie, Shawn Ritchie, Yingshen Lu, Wei Jin, Amir Kavianpour, Khine Khine Su-Myat, Doug Heath, Paul L. Wood, Maura Fisk, and Dayan B. Goodenowe. 2009. 'Novel plasma phospholipid biomarkers of autism: Mitochondrial dysfunction as a putative causative mechanism', *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 81: 253-64.

Pender, M. P. 2011. 'The essential role of Epstein-Barr virus in the pathogenesis of multiple sclerosis', *Neuroscientist*, 17: 351-67.

Pérez-Nievas, B. G., B. García-Bueno, J. L. Madrigal, and J. C. Leza. 2010. 'Chronic immobilisation stress ameliorates clinical score and neuroinflammation in a MOG-induced EAE in Dark Agouti rats: mechanisms implicated', *J Neuroinflammation*, 7: 60.

Petrovsky, N., and L. C. Harrison. 1998. 'The chronobiology of human cytokine production', *Int Rev Immunol*, 16: 635-49.

Petrovsky, N., P. McNair, and L. C. Harrison. 1998. 'Diurnal rhythms of pro-inflammatory cytokines: regulation by plasma cortisol and therapeutic implications', *Cytokine*, 10: 307-12.

Petrovsky, Nikolai, Luis Socha, Diego Silva, Ashley B. Grossman, Christine Metz, and Richard Bucala. 2003. 'Macrophage migration inhibitory factor exhibits a pronounced circadian rhythm relevant to its role as a glucocorticoid counter-regulator', *Immunology & Cell Biology*, 81: 137-43.

Phillips, R., C. Horsfield, S. Kuijper, S. F. Sarfo, J. Obeng-Baah, S. Etuaful, B. Nyamekye, P. Awuah, K. M. Nyarko, F. Osei-Sarpong, S. Lucas, A. H. Kolk, and M. Wansbrough-Jones. 2006. 'Cytokine response to antigen stimulation of whole blood from patients with Mycobacterium ulcerans disease compared to that from patients with tuberculosis', *Clin Vaccine Immunol*, 13: 253-7.

Pierrot-Deseilligny, Charles, and Jean-Claude Souberbielle. 2010. 'Is hypovitaminosis D one of the environmental risk factors for multiple sclerosis?', *Brain*, 133: 1869-88.

Polderman, Kees H. 2012. 'Is therapeutic hypothermia immunosuppressive?', *Critical Care*, 16: A8-A8.

Polito, A., and R. Reynolds. 2005. 'NG2-expressing cells as oligodendrocyte progenitors in the normal and demyelinated adult central nervous system', *J Anat*, 207: 707-16.

Pons, V., and S. Rivest. 2022. 'Targeting Systemic Innate Immune Cells as a Therapeutic Avenue for Alzheimer Disease', *Pharmacol Rev*, 74: 1-17.

Qu, Jiagui, Joshua D Rizak, Xiaomiao Li, Jiejing Li, and Yuanye Ma. 2013. 'Melatonin treatment increases the transcription of cell proliferation-related genes prior to inducing cell death in C6 glioma cells in vitro', *Oncology letters*, 6: 347-52.

Quiñones-Hinojosa, Alfredo, and Kaisorn Chaichana. 2007. 'The human subventricular zone: A source of new cells and a potential source of brain tumors', *Experimental Neurology*, 205: 313-24.

Raddatz, Barbara B., Wenhui Sun, Graham Brogden, Yanyong Sun, Patricia Kammeyer, Arno Kalkuhl, Florian Colbatzky, Ulrich Deschl, Hassan Y. Naim, Wolfgang Baumgärtner, and Reiner Ulrich. 2016. 'Central Nervous System Demyelination and Remyelination is Independent from Systemic Cholesterol Level in Theiler's Murine Encephalomyelitis', *Brain Pathology*, 26: 102-19.

Ralph, C. L. 1975. 'The pineal gland and geographical distribution of animals', *Int J Biometeorol*, 19: 289-303.

Ramos González, E. J., L. J. Ramirez Jirano, D. Z. García Martínez, G. G. Ortiz, L. F. Jave Suárez, C. A. Leal Cortes, and O. K. Bitzer Quintero. 2018. 'A comparative study of melatonin and immunomodulatory therapy with interferon beta and glatiramer acetate in a mouse model of multiple sclerosis', *Neurologia*.

Rawji, K. S., A. M. H. Young, T. Ghosh, N. J. Michaels, R. Mirzaei, J. Kappen, K. L. Kolehmainen, N. Alaeiilkhchi, B. Lozinski, M. K. Mishra, A. Pu, W. Tang, S. Zein, D. K. Kaushik, M. B. Keough, J. R. Plemel, F. Calvert, A. J. Knights, D. J. Gaffney, W. Tetzlaff, R. J. M. Franklin, and V. W. Yong. 2020. 'Niacin-mediated rejuvenation of macrophage/microglia enhances remyelination of the aging central nervous system', *Acta Neuropathol*, 139: 893-909.

Reiter, C. P., A. C. Christy, C. H. Olsen, and D. E. Bentzel. 2017. 'Effect of Home Cage Bedding in the Induction Chamber on Serum Cortisol and Corticosterone Levels in Response to Isoflurane-induced Anesthesia in C57BL/6J Mice', *J Am Assoc Lab Anim Sci*, 56: 118-21.

Religi, A., C. Backes, A. Chatelan, J. L. Bulliard, L. Vuilleumier, L. Moccozet, M. Bochud, and D. Vernez. 2019. 'Estimation of exposure durations for vitamin D production and sunburn risk in Switzerland', *J Expo Sci Environ Epidemiol*, 29: 742-52.

Reppert, S. M., D. R. Weaver, and C. Godson. 1996. 'Melatonin receptors step into the light: cloning and classification of subtypes', *Trends Pharmacol Sci*, 17: 100-2.

Risco, J., H. Maldonado, L. Luna, J. Osada, P. Ruiz, A. Juarez, and D. Vizcarra. 2011. 'Latitudinal prevalence gradient of multiple sclerosis in Latin America', *Mult Scler*, 17: 1055-9.

Rosenstein, R. E., D. A. Golombek, B. I. Kanterewicz, and D. P. Cardinali. 1991. 'Time-dependency for the bimodal effect of melatonin on calcium uptake in rat hypothalamus. Short communication', *J Neural Transm Gen Sect*, 85: 243-7.

Sabel, C. E., J. F. Pearson, D. F. Mason, E. Willoughby, D. A. Abernethy, and B. V. Taylor. 2021. 'The latitude gradient for multiple sclerosis prevalence is established in the early life course', *Brain*, 144: 2038-46.

Sae-Teaw, M., J. Johns, N. P. Johns, and S. Subongkot. 2013. 'Serum melatonin levels and antioxidant capacities after consumption of pineapple, orange, or banana by healthy male volunteers', *J Pineal Res*, 55: 58-64.

Saher, Gesine, Britta Brügger, Corinna Lappe-Siefke, Wiebke Möbius, Ryu-ichi Tozawa, Michael C Wehr, Felix Wieland, Shun Ishibashi, and Klaus-Armin Nave. 2005. 'High cholesterol level is essential for myelin membrane growth', *Nature neuroscience*, 8: 468-75.

Sandyk, R. 1992a. 'Successful treatment of multiple sclerosis with magnetic fields', *Int J Neurosci*, 66: 237-50.

———. 1993. 'Multiple sclerosis: the role of puberty and the pineal gland in its pathogenesis', *Int J Neurosci*, 68: 209-25.

———. 1997. 'Influence of the pineal gland on the expression of experimental allergic encephalomyelitis: possible relationship to the aquisition of multiple sclerosis', *Int J Neurosci*, 90: 129-33.

Sandyk, Reuven. 1992b. 'Clinical Case Report: SUCCESSFUL TREATMENT OF MULTIPLE SCLEROSIS WITH MAGNETIC FIELDS', *International Journal of Neuroscience*, 66: 237-50.

Saunders, P. R., J. Santos, N. P. Hanssen, D. Yates, J. A. Groot, and M. H. Perdue. 2002. 'Physical and psychological stress in rats enhances colonic epithelial permeability via peripheral CRH', *Dig Dis Sci*, 47: 208-15.

Schurr, A., and R. S. Payne. 2007. 'Lactate, not pyruvate, is neuronal aerobic glycolysis end product: an in vitro electrophysiological study', *Neuroscience*, 147: 613-9.

Schuster, C., F. Gauer, A. Malan, J. Recio, P. Pévet, and M. Masson-Pévet. 2001. 'The circadian clock, light/dark cycle and melatonin are differentially involved in the expression of daily and photoperiodic variations in mt(1) melatonin receptors in the Siberian and Syrian hamsters', *Neuroendocrinology*, 74: 55-68.

Schwartz, R. S., T. M. Koval, W. D. Edwards, A. R. Camrud, K. R. Bailey, K. Browne, R. E. Vlietstra, and D. R. Holmes, Jr. 1992. 'Effect of external beam irradiation on neointimal hyperplasia after experimental coronary artery injury', *J Am Coll Cardiol*, 19: 1106-13.

Sebastian, P., N. Cherbuin, L. F. Barcellos, S. Roalstad, C. Casper, J. Hart, G. S. Aaen, L. Krupp, L. Benson, M. Gorman, M. Candee, T. Chitnis, M. Goyal, B. Greenberg, S. Mar, M. Rodriguez, J. Rubin, T. Schreiner, A. Waldman, B. Weinstock-Guttman, J. Graves, E. Waubant, and R. Lucas. 2022. 'Association Between Time Spent Outdoors and Risk of Multiple Sclerosis', *Neurology*, 98: e267-e78.

Sedel, Frédéric, Delphine Bernard, Donald M Mock, and Ayman Tourbah. 2016. 'Targeting demyelination and virtual hypoxia with high-dose biotin as a treatment for progressive multiple sclerosis', *Neuropharmacology*, 110: 644-53.

Seguella, Luisa, Giovanni Sarnelli, and Giuseppe Esposito. 2020. 'Leaky gut, dysbiosis, and enteric glia activation: the trilogy behind the intestinal origin of Parkinson's disease', *Neural regeneration research*, 15: 1037-38.

Senanayake, Vijitha K., Wei Jin, Asuka Mochizuki, Bassirou Chitou, and Dayan B. Goodenowe. 2015. 'Metabolic dysfunctions in multiple sclerosis: implications as to causation, early detection, and treatment, a case control study', *BMC neurology*, 15: 154-54.

Seo, Hyobin, Chun-Hyung Park, Seokrip Choi, Woocheol Kim, Byung-Duk Jeon, and Seungpil Ryu. 2016. 'Effects of voluntary exercise on apoptosis and cortisol after chronic restraint stress in mice', *Journal of exercise nutrition & biochemistry*, 20: 16-23.

Sharman, E. H., S. C. Bondy, K. G. Sharman, D. Lahiri, C. W. Cotman, and V. M. Perreau. 2007. 'Effects of melatonin and age on gene expression in mouse CNS using microarray analysis', *Neurochem Int*, 50: 336-44.

Shelestak, J., N. Singhal, L. Frankle, R. Tomor, S. Sternbach, J. McDonough, E. Freeman, and R. Clements. 2020. 'Increased blood-brain barrier hyperpermeability coincides with mast cell activation early under cuprizone administration', *PLoS One*, 15: e0234001.

Sidhom, Y., I. Kacem, L. Bayoudh, M. Ben Djebara, Y. Hizem, S. Ben Abdelfettah, A. Gargouri, and R. Gouider. 2015. 'Season of birth and multiple sclerosis in Tunisia', *Mult Scler Relat Disord*, 4: 491-4.

Sierra-Ramírez, José Alfredo, Lourdes Saucedo-Bueno, Ana Lilia García-Hernández, Arnulfo Martínez-Dávalos, Camilo Rodríguez-López, Maria Elisa Drago-Serrano, and Marycarmen Godínez-Victoria. 2022. 'Moderate aerobic exercise on bone quality changes associated with aging and oxidative stress in BALB/c mice', *Journal of Biomechanics*, 135: 111035.

Simpson, S., Jr., W. Wang, P. Otahal, L. Blizzard, I. A. F. van der Mei, and B. V. Taylor. 2019. 'Latitude continues to be significantly associated with the prevalence of multiple sclerosis: an updated meta-analysis', *J Neurol Neurosurg Psychiatry*, 90: 1193-200.

Simpson, Steve, Leigh Blizzard, Petr Otahal, Ingrid Van der Mei, and Bruce Taylor. 2011. 'Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis', *Journal of Neurology, Neurosurgery & Psychiatry*, 82: 1132.

Singh, Harbindar Jeet, Hisham Ibrahim Saleh, Sergey Gupalo, and Effat Omar. 2013. 'Effect of melatonin supplementation on pregnancy outcome in Wistar-Kyoto and Sprague-Dawley rats', *Sheng li xue bao : [Acta physiologica Sinica]*, 65: 149-57.

Skobowiat, C., R. M. Sayre, J. C. Dowdy, and A. T. Slominski. 2013. 'Ultraviolet radiation regulates cortisol activity in a waveband-dependent manner in human skin ex vivo', *Br J Dermatol*, 168: 595-601.

Sloka, J. S., W. E. Pryse-Phillips, and M. Stefanelli. 2008. 'The relation of ultraviolet radiation and multiple sclerosis in Newfoundland', *Can J Neurol Sci*, 35: 69-74.

Snipes, G Jackson, and Ueli Suter. 1997. 'Cholesterol and myelin.' in, *Cholesterol* (Springer).

Söderholm, J. D., and M. H. Perdue. 2001. 'Stress and gastrointestinal tract. II. Stress and intestinal barrier function', *Am J Physiol Gastrointest Liver Physiol*, 280: G7-g13.

Solár, P., A. Zamani, L. Kubíčková, P. Dubový, and M. Joukal. 2020. 'Choroid plexus and the blood-cerebrospinal fluid barrier in disease', *Fluids Barriers CNS*, 17: 35.

Somlyo, A. P., and A. V. Somlyo. 1994. 'Signal transduction and regulation in smooth muscle', *Nature*, 372: 231-6.

Sotthibundhu, A., P. Phansuwan-Pujito, and P. Govitrapong. 2010. 'Melatonin increases proliferation of cultured neural stem cells obtained from adult mouse subventricular zone', *J Pineal Res*, 49: 291-300.

Sotthibundhu, Areechun, Kasima Ekthuwapranee, and Piyarat Govitrapong. 2016. 'Comparison of melatonin with growth factors in promoting precursor cells proliferation in adult mouse subventricular zone', *EXCLI journal*, 15: 829-41.

Spear, E. T., E. A. Holt, E. J. Joyce, M. M. Haag, S. M. Mawe, G. W. Hennig, B. Lavoie, A. M. Applebee, C. Teuscher, and G. M. Mawe. 2018. 'Altered gastrointestinal motility involving autoantibodies in the experimental autoimmune encephalomyelitis model of multiple sclerosis', *Neurogastroenterol Motil*, 30: e13349.

Stokkan, K. A., and R. J. Reiter. 1994. 'Melatonin rhythms in Arctic urban residents', *J Pineal Res*, 16: 33-6.

Sundberg, I., A. J. Rasmusson, M. Ramklint, D. Just, L. Ekselius, and J. L. Cunningham. 2020. 'Daytime melatonin levels in saliva are associated with inflammatory markers and anxiety disorders', *Psychoneuroendocrinology*, 112: 104514.

Susuki, Keiichiro. 2010. 'Myelin: a specialized membrane for cell communication', *Nature Education*, 3: 59.

Swank, G. M., and E. A. Deitch. 1996. 'Role of the gut in multiple organ failure: bacterial translocation and permeability changes', *World J Surg*, 20: 411-7.

Swirski, F. K., M. Nahrendorf, M. Etzrodt, M. Wildgruber, V. Cortez-Retamozo, P. Panizzi, J. L. Figueiredo, R. H. Kohler, A. Chudnovskiy, P. Waterman, E. Aikawa, T. R. Mempel, P. Libby, R. Weissleder, and M. J. Pittet. 2009. 'Identification of splenic reservoir monocytes and their deployment to inflammatory sites', *Science*, 325: 612-6.

Tamarindo, G. H., D. L. Ribeiro, M. G. Gobbo, L. H. A. Guerra, P. Rahal, S. R. Taboga, F. R. Gadelha, and R. M. Goes. 2019. 'Melatonin and Docosahexaenoic Acid Decrease Proliferation of PNT1A Prostate Benign Cells via Modulation of Mitochondrial Bioenergetics and ROS Production', *Oxid Med Cell Longev*, 2019: 5080798.

Tan, D. X., L. C. Manchester, L. Qin, and R. J. Reiter. 2016. 'Melatonin: A Mitochondrial Targeting Molecule Involving Mitochondrial Protection and Dynamics', *Int J Mol Sci*, 17.

Taylor, B. V., J. F. Pearson, G. Clarke, D. F. Mason, D. A. Abernethy, E. Willoughby, and C. Sabel. 2010. 'MS prevalence in New Zealand, an ethnically and latitudinally diverse country', *Mult Scler*, 16: 1422-31.

Tedroff, K., M. von Euler, and E. Dahlén. 2022. 'Melatonin usage in children and young adults, a registry-based cohort study', *Eur J Paediatr Neurol*, 39: 30-34.

Tengvall, K., J. Huang, C. Hellström, P. Kammer, M. Biström, B. Ayoglu, I. Lima Bomfim, P. Stridh, J. Butt, N. Brenner, A. Michel, K. Lundberg, L. Padyukov, I. E. Lundberg, E. Svenungsson, I. Ernberg, S. Olafsson, A. T. Dilthey, J. Hillert, L. Alfredsson, P. Sundström, P. Nilsson, T. Waterboer, T. Olsson, and I. Kockum. 2019. 'Molecular mimicry between Anoctamin 2 and Epstein-Barr virus nuclear antigen 1 associates with multiple sclerosis risk', *Proc Natl Acad Sci U S A*, 116: 16955-60.

Tepavčević, Vanja. 2021. 'Oligodendroglial Energy Metabolism and (re)Myelination', *Life (Basel, Switzerland)*, 11: 238.

Thomas, G., R. Tacke, C. C. Hedrick, and R. N. Hanna. 2015. 'Nonclassical patrolling monocyte function in the vasculature', *Arterioscler Thromb Vasc Biol*, 35: 1306-16.

Tillery, Erika E., Jennifer N. Clements, and Zach Howard. 2018. 'What's new in multiple sclerosis?', *The mental health clinician*, 7: 213-20.

Torkildsen, O., L. A. Brunborg, K. M. Myhr, and L. Bø. 2008. 'The cuprizone model for demyelination', *Acta Neurol Scand Suppl*, 188: 72-6.

Torkildsen, O., N. Grytten, J. Aarseth, K. M. Myhr, and M. T. Kampman. 2012. 'Month of birth as a risk factor for multiple sclerosis: an update', *Acta Neurol Scand Suppl*: 58-62.

Tosini, G., S. Owino, J. L. Guillaume, and R. Jockers. 2014. 'Understanding melatonin receptor pharmacology: latest insights from mouse models, and their relevance to human disease', *Bioessays*, 36: 778-87.

Trapp, Bruce, and Peter Stys. 2009. 'Trapp, B. D. & Stys, P. K. Virtual hypoxia and chronic necrosis of demyelinated axons in multiple sclerosis. Lancet Neurol. 8, 280-291', *Lancet neurology*, 8: 280-91.

Tremlett, H., F. Zhu, A. Ascherio, and K. L. Munger. 2018. 'Sun exposure over the life course and associations with multiple sclerosis', *Neurology*, 90: e1191-e99.

Turgut, M., Y. Uyanikgil, M. Baka, A. T. Tunc, A. Yavasoglu, M. E. Yurtseven, and S. Kaplan. 2005. 'Pinealectomy exaggerates and melatonin treatment suppresses neuroma formation of transected sciatic nerve in rats: gross morphological, histological and stereological analysis', *J Pineal Res*, 38: 284-91.

Vakilzadeh, G., F. Khodagholi, T. Ghadiri, A. Ghaemi, F. Noorbakhsh, M. Sharifzadeh, and A. Gorji. 2016. 'The Effect of Melatonin on Behavioral, Molecular, and Histopathological Changes in Cuprizone Model of Demyelination', *Mol Neurobiol*, 53: 4675-84.

Van den Bossche, J., L. A. O'Neill, and D. Menon. 2017. 'Macrophage Immunometabolism: Where Are We (Going)?', *Trends Immunol*, 38: 395-406.

Villapol, Sonia, Sébastien Fau, Sylvain Renolleau, Valérie Biran, Christiane Charriaut-Marlangue, and Olivier Baud. 2011. 'Melatonin Promotes Myelination by Decreasing White Matter Inflammation After Neonatal Stroke', *Pediatric Research*, 69: 51-55.

Voiculescu, S. E., N. Zygouropoulos, C. D. Zahiu, and A. M. Zagrean. 2014. 'Role of melatonin in embryo fetal development', *J Med Life*, 7: 488-92.

Vondrasová, D., I. Hájek, and H. Illnerová. 1997. 'Exposure to long summer days affects the human melatonin and cortisol rhythms', *Brain Res*, 759: 166-70.

Wacker, M., and M. F. Holick. 2013. 'Sunlight and Vitamin D: A global perspective for health', *Dermatoendocrinol*, 5: 51-108.

Walker, D. G., and L. F. Lue. 2015. 'Immune phenotypes of microglia in human neurodegenerative disease: challenges to detecting microglial polarization in human brains', *Alzheimers Res Ther*, 7: 56.

Wallin, Mitchell T., William J. Culpepper, Emma Nichols, Zulfiqar A. Bhutta, Tsegaye Tewelde Gebrehiwot, Simon I. Hay, Ibrahim A. Khalil, Kristopher J. Krohn, Xiaofeng Liang, Mohsen Naghavi, Ali H. Mokdad, Molly R. Nixon, Robert C. Reiner, Benn Sartorius, Mari Smith, Roman Topor-Madry, Andrea Werdecker, Theo Vos, Valery L. Feigin, and Christopher J. L. Murray. 2019. 'Global, regional, and national burden of multiple sclerosis 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016', *The Lancet Neurology*, 18: 269-85.

Wallner, Stefan, Margot Grandl, Tatiana Konovalova, Alexander Sigrüner, Thomas Kopf, Markus Peer, Evelyn Orsó, Gerhard Liebisch, and Gerd Schmitz. 2014. 'Monocyte to Macrophage Differentiation Goes along with Modulation of the Plasmalogen Pattern through Transcriptional Regulation', *PloS one*, 9: e94102.

Wehr, T. A., W. C. Duncan, Jr., L. Sher, D. Aeschbach, P. J. Schwartz, E. H. Turner, T. T. Postolache, and N. E. Rosenthal. 2001. 'A circadian signal of change of season in patients with seasonal affective disorder', *Arch Gen Psychiatry*, 58: 1108-14.

Welker, H. A., P. Semm, R. P. Willig, J. C. Commentz, W. Wiltschko, and L. Vollrath. 1983. 'Effects of an artificial magnetic field on serotonin N-acetyltransferase activity and melatonin content of the rat pineal gland', *Exp Brain Res*, 50: 426-32.

Wen, J., P. S. Ariyannur, R. Ribeiro, M. Tanaka, J. R. Moffett, B. F. Kirmani, A. M. Namboodiri, and Y. Zhang. 2016. 'Efficacy of N-Acetylserotonin and Melatonin in the EAE Model of Multiple Sclerosis', *J Neuroimmune Pharmacol*, 11: 763-73.

Willis, G. L., and S. M. Armstrong. 1999. 'A therapeutic role for melatonin antagonism in experimental models of Parkinson's disease', *Physiol Behav*, 66: 785-95.

Wolfson, C., and P. Talbot. 2002. 'Bacterial infection as a cause of multiple sclerosis', *Lancet*, 360: 352-3.

Wu, Bin, Caixing Sun, Fang Feng, Minghua Ge, and Liang Xia. 2015. 'Do relevant markers of cancer stem cells CD133 and Nestin indicate a poor prognosis in glioma patients? A systematic review and meta-analysis', *Journal of Experimental & Clinical Cancer Research*, 34: 44.

Wu, Cheng-Yang, Santhosh Satapati, Wenjun Gui, R. Max Wynn, Gaurav Sharma, Mingliang Lou, Xiangbing Qi, Shawn C. Burgess, Craig Malloy, Chalermchai Khemtong, A. Dean Sherry, David T. Chuang, and Matthew E. Merritt. 2018. 'A novel inhibitor of pyruvate dehydrogenase kinase stimulates myocardial carbohydrate oxidation in diet-induced obesity', *The Journal of biological chemistry*, 293: 9604-13.

Xu, Guangming, Yingmin Li, Chunling Ma, Chuan Wang, Zhaoling Sun, Yiwen Shen, Li Liu, Shujin Li, Xiaojing Zhang, and Bin Cong. 2019. 'Restraint Stress Induced Hyperpermeability and Damage of the Blood-Brain Barrier in the Amygdala of Adult Rats', *Frontiers in molecular neuroscience*, 12: 32-32.

Xu, X., X. Liu, S. Ma, Y. Xu, Y. Xu, X. Guo, and D. Li. 2018. 'Association of Melatonin Production with Seasonal Changes, Low Temperature, and Immuno-Responses in Hamsters', *Molecules*, 23.

Yacyshyn, B., J. Meddings, D. Sadowski, and M. B. Bowen-Yacyshyn. 1996. 'Multiple sclerosis patients have peripheral blood CD45RO+ B cells and increased intestinal permeability', *Dig Dis Sci*, 41: 2493-8.

Yamane, K., I. L. Indalao, J. Chida, Y. Yamamoto, M. Hanawa, and H. Kido. 2014. 'Diisopropylamine dichloroacetate, a novel pyruvate dehydrogenase kinase 4 inhibitor, as a potential therapeutic agent for metabolic disorders and multiorgan failure in severe influenza', *PLoS One*, 9: e98032.

Yang, T., R. Guo, and F. Zhang. 2019. 'Brain perivascular macrophages: Recent advances and implications in health and diseases', *CNS Neurosci Ther*, 25: 1318-28.

Yasaka, T., N. M. Mantich, L. A. Boxer, and R. L. Baehner. 1981. 'Functions of human monocyte and lymphocyte subsets obtained by countercurrent centrifugal elutriation: differing functional capacities of human monocyte subsets', *J Immunol*, 127: 1515-8.

Zhang, S., X. Zhu, X. Gui, C. Croteau, L. Song, J. Xu, A. Wang, P. Bannerman, and F. Guo. 2018. 'Sox2 Is Essential for Oligodendroglial Proliferation and Differentiation during Postnatal Brain Myelination and CNS Remyelination', *J Neurosci*, 38: 1802-20.

Zhang, Shuai, Matthew W. Hulver, Ryan P. McMillan, Mark A. Cline, and Elizabeth R. Gilbert. 2014. 'The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility', *Nutrition & Metabolism*, 11: 10.

Zhao, A., K. Zhao, Y. Xia, J. Lyu, Y. Chen, and S. Li. 2021. 'Melatonin inhibits embryonic rat H9c2 cells growth through induction of apoptosis and cell cycle arrest via PI3K-AKT signaling pathway', *Birth Defects Res*, 113: 1171-81.

Zhao, C., D. Ma, M. Zawadzka, S. P. Fancy, L. Elis-Williams, G. Bouvier, J. H. Stockley, G. M. de Castro, B. Wang, S. Jacobs, P. Casaccia, and R. J. Franklin. 2015. 'Sox2 Sustains Recruitment of

Oligodendrocyte Progenitor Cells following CNS Demyelination and Primes Them for Differentiation during Remyelination', *J Neurosci*, 35: 11482-99.

Zheng, G., G. Victor Fon, W. Meixner, A. Creekmore, Y. Zong, K. Dame M, J. Colacino, P. H. Dedhia, S. Hong, and J. W. Wiley. 2017. 'Chronic stress and intestinal barrier dysfunction: Glucocorticoid receptor and transcription repressor HES1 regulate tight junction protein Claudin-1 promoter', *Sci Rep*, 7: 4502.

Zheng, G., S. P. Wu, Y. Hu, D. E. Smith, J. W. Wiley, and S. Hong. 2013. 'Corticosterone mediates stress-related increased intestinal permeability in a region-specific manner', *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*, 25: e127-e39.

Zwirska-Korczala, K., J. Jochem, M. Adamczyk-Sowa, P. Sowa, R. Polaniak, E. Birkner, M. Latocha, K. Pilc, and R. Suchanek. 2005. 'Influence of melatonin on cell proliferation, antioxidative enzyme activities and lipid peroxidation in 3T3-L1 preadipocytes--an in vitro study', *J Physiol Pharmacol*, 56 Suppl 6: 91-9.